Kinetics of Germination of Wet-Heat-Treated Individual Spores of *Bacillus* Species, Monitored by Raman Spectroscopy and Differential Interference Contrast Microscopy^V

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Raman spectroscopy and differential interference contrast (DIC) microscopy were used to monitor the kinetics of nutrient and nonnutrient germination of multiple individual untreated and wet-heat-treated spores of *Bacillus cereus* and *Bacillus megaterium*, as well as of several isogenic *Bacillus subtilis* strains. Major conclusions from this work were as follows. (i) More than 90% of these spores were nonculturable but retained their 1:1 chelate of Ca^{2+} and dipicolinic acid (CaDPA) when incubated in water at 80 to 95°C for 5 to 30 min. (ii) Wet-heat treatment significantly increased the time, T_{lag} , at which spores began release of the great majority of their CaDPA during the germination of *B. subtilis* spores with different nutrient germinants and also increased the variability of T_{lag} values. (iii) The time period, $\Delta T_{\text{release}}$, between T_{lag} and the time, T_{release} , at which a spore germinating with nutrients completed the release of the great majority of its CaDPA, was also increased in wet-heat-treated spores. (iv) Wet-heat-treated spores germinating with nutrients had higher values of I_{release} , the intensity of a spore's DIC image at T_{release} , than did untreated spores and had much longer time periods, ΔT_{lys} , for the reduction in I_{release} intensities to the basal value due to hydrolysis of the spore's peptidoglycan cortex, probably due at least in part to damage to the cortex-lytic enzyme CwlJ. (v) Increases in T_{lag} and $\Delta T_{\text{release}}$ were also observed when wet-heat-treated *B. subtilis* spores were germinated with the nonnutrient dodecylamine, while the change in I_{release} was less significant. (vi) The effects of wet-heat treatment on nutrient germination of *B. cereus* and *B. megaterium* spores were generally similar to those on *B. subtilis* spores. These results indicate that (i) some proteins important in spore germination are damaged by wet-heat treatment, (ii) the cortex-lytic enzyme CwlJ is one germination protein damaged by wet heat, and (iii) the CaDPA release process itself seems likely to be the target of wet-heat damage which has the greatest effect on spore germination.

Spores are resting states of some microorganisms and are formed under conditions unfavorable for the survival of growing cells. In bacteria of *Bacillus* species, spores are formed in sporulation and are metabolically dormant and extremely resistant to heat, desiccation, radiation, and many toxic chemicals (27). Spores of these and related species are common in foodstuffs, and the growing organisms of some of these species can cause food spoilage or food-borne disease (6, 27, 28). Consequently, there has been much work directed toward understanding the mechanism of spores' extreme resistance, in particular to wet heat, which is the treatment used most commonly to destroy spores in foodstuffs and medical devices. Overall, this work has led to a fairly complete understanding of the mechanism of spore resistance to wet heat, and factors responsible for spore wet-heat resistance include the (i) high level of dipicolinic acid (DPA) and its associated divalent cations (generally Ca^{2+} , giving CaDPA) in the spore's central core; (ii) spore core's low core water content—as little as 25% of the core wet weight; (iii) types and amounts of divalent cations associated with DPA; and (iv) protection of spore

DNA by its saturation with a group of α/β -type small, acid-soluble spore proteins (6, 27).

The factors responsible for causing spore wet-heat resistance have been reasonably well defined. However, there is much less definitive knowledge of how wet heat kills spores. Under normal conditions, neither DNA damage nor oxidative reactions are involved in wet-heat killing of spores (27). However, both protein denaturation and enzyme inactivation are associated with spore killing by wet heat (4, 5, 30, 36), although neither process has been definitively shown to cause wet-heat killing, and specific proteins/enzymes whose inactivation causes spore killing by wet heat have not been identified. Wet-heat treatment often causes the release of the spore core's CaDPA depot, presumably due to rupture of the spore's inner membrane permeability barrier. However, significant spore protein denaturation can take place during wet-heat treatment and prior to CaDPA release (27, 36). Equally importantly, high levels of wet-heat-killed spores of a number of *Bacillus* species can retain CaDPA if treatment conditions are not too extreme (4, 5). These wet-heat-killed spores retain CaDPA and can still germinate slowly, although they are blocked in spore outgrowth, perhaps due to the inactivation of one or more key metabolic enzymes. However, wet-heat-killed spores that have lost CaDPA do not germinate and have suffered massive protein denaturation (4, 5). It appears possible, then, that analysis of the germination defect(s) in wet-heat-killed spores may fa-

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cilitate the identification of one or more spore proteins that are inactivated by wet-heat treatment and whose inactivation may be associated with spore death.

The germination of spores of *Bacillus* species takes place in two stages, and a number of proteins, including nutrient germinant receptors (GRs), channel proteins for CaDPA release, and cortex-lytic enzymes (CLEs) are essential for completion of germination, while proteins essential for cell metabolism and macromolecular synthesis are crucial for spore outgrowth (19, 26). In stage I of germination, nutrient germinants bind to specific GRs located in the spore's inner membrane, and this triggers the release of small molecules from the spore core, most notably the CaDPA, and the small molecules released are replaced by water. The peptidoglycan cortex is hydrolyzed in stage II of germination, and in spores of *Bacillus* species, this cortex hydrolysis is catalyzed by either of two redundant CLEs, CwlJ and SleB. Cortex hydrolysis then allows core expansion and further water uptake, followed by spore outgrowth. The inactivation of any of these key germination proteins could affect the germination kinetics and perhaps the viability of spores after wet-heat treatment. Consequently, by comparing the germination of untreated and wet-heat-treated spores with a variety of germinants, it may be possible to identify proteins whose damage by wet heat decreases spore germination.

The kinetics of germination of spore populations is usually monitored by measuring the optical density at 600 nm of spore cultures, which falls ~60% upon completion of germination, or the release of spores' CaDPA depot, one of the early events in the germination process (26). However, the kinetics of germination of individual spores cannot be determined from these population measurements due to the large heterogeneity in the times at which individual spores in populations initiate germination events (3, 11, 21). Thus, a detailed understanding of spore germination requires analyses of individual spores' germination behavior. Raman spectroscopy is widely used in biochemical studies, as this technique has high sensitivity and responds rapidly to subtle changes in molecular structure (10). When Raman spectroscopy is combined with confocal microscopy and optical tweezers, the resultant laser tweezers Raman spectroscopy (LTRS) allows the nondestructive, noninvasive detection of biochemical processes at the single-cell level (3, 9, 31). Indeed, LTRS has been used to analyze both the CaDPA level and the kinetics of germination of individual spores of a number of *Bacillus* species (3, 9, 11). Dual-trap LTRS and elastic light scattering have also been used to investigate dynamic processes during the wet-heat treatment of individual spores of *Bacillus* species (36). Recently, differential interference contrast (DIC) microscopy has also been used to monitor the germination of hundreds of individual spores simultaneously (35, 37). In the present work, we have used DIC microscopy and LTRS to characterize the germination of untreated and wet-heat-treated individual spores of three *Bacillus* species and with a number of different germinants. The results of these analyses provide strong evidence that some key proteins involved in spore germination are inactivated during wet-heat treatment.

MATERIALS AND METHODS

***Bacillus* strains used and spore preparation.** The *B. subtilis* strains used in this work are isogenic and were (i) PS832 (wild type), a prototrophic strain derived

from strain 168; (ii) FB10 (*gerB**), spores of which have a GR variant termed *GerB** that can respond to L-asparagine alone (17); (iii) PS3415 ($\uparrow$ *GerB**), spores of which have a 200-fold-elevated level of the *GerB** GR (2); (iv) FB111 (*cwlJ*), spores of which lack the CLE CwlJ (15); and (v) FB112 (*sleB*), spores of which lack the CLE SleB (15). *B. cereus* T (originally obtained from H. O. Halvorson) and *B. megaterium* QM B1551 (originally obtained from H. S. Levinson) were also used in this work.

B. subtilis spores were prepared on 2×SG medium agar plates at 37°C without antibiotics. *B. cereus* spores were prepared at 30°C in defined liquid medium (14), and *B. megaterium* spores were prepared at 30°C in supplemented nutrient broth (14). Liquid cultures were harvested after 36 to 48 h of incubation. After incubation for 2 to 3 days, plates with *B. subtilis* strains were incubated for several days at 23°C to allow completion of lysis of sporulating cells. Spores were then scraped from the plates and suspended in water. Harvested spores were purified by repeated centrifugation and washing with water, as well as several sonication treatments for the *B. megaterium* and *B. subtilis* spores, but not the *B. cereus* spores so as not to damage these spores' exosporium (14, 16). Purified spores were stored at 4°C in water protected from light and were free (98%) of growing and sporulating cells, germinated spores, and cell debris as observed by phase-contrast microscopy.

Spore wet-heat treatment and measurement of spore viability and CaDPA content. *B. subtilis* and *B. cereus* spores were incubated in water at $\sim 5 \times 10^8$ /ml at 90 to 95°C, and *B. megaterium* spores at $\sim 3 \times 10^8$ /ml in water were incubated at 80°C. The viability of wet-heat-treated spores was measured by serial dilution in water at various times during high-temperature incubation, spreading aliquots on L broth medium plates (18), incubating the plates for 24 to 36 h at 37°C, and finally counting colonies. Incubation for longer times gave no increase in the numbers of colonies.

Spores' CaDPA content was determined by Raman spectroscopy using LTRS as described previously (3, 9). Individual spores suspended in water were randomly trapped by a 780-nm laser beam, and Raman scattering excited by the same laser beam was acquired by a charge-coupled device (CCD). The spores' CaDPA content was determined from the intensity of the CaDPA-specific Raman band at $1,017\text{ cm}^{-1}$ (3, 9).

Spore germination. *B. subtilis* PS832 spores were germinated in (i) 25 mM Tris-HCl buffer (pH 8.3) with 10 mM L-alanine at 37°C; (ii) 50 mM KPO₄ buffer (pH 7.4) with 10 mM L-asparagine–10 mM D-glucose–10 mM D-fructose (AGFK) at 37°C; or (iii) 25 mM HEPES buffer (pH 7.4) with 0.8 mM dodecylamine at 45°C. FB111 and FB112 spores were germinated in 25 mM Tris-HCl buffer (pH 8.3) with 10 mM L-alanine at 37°C. PS3415 and FB10 spores were germinated in 25 mM Tris-HCl buffer (pH 8.3) with 0.5 mM L-asparagine at 37°C. *B. cereus* spores were germinated in 25 mM Tris-HCl buffer (pH 8.3) with 10 mM L-alanine at 37°C. *B. megaterium* spores were germinated in 25 mM KPO₄ buffer (pH 7.4) with 10 mM glucose at 37°C. Except for dodecylamine germination, spores were heat activated prior to germination by incubation in a water bath at 70°C for 30 min for *B. subtilis* and *B. cereus* spores and at 60°C for 15 min for *B. megaterium* spores, and spores were cooled on ice for at least 15 min prior to germination experiments.

Monitoring of spore germination. Analysis of the germination of single individual spores by LTRS was carried out as described previously (3, 9). Analysis of the germination of multiple individual spores by DIC microscopy was also done as described previously (35, 37). Briefly, heat-activated spores (1 μ l; $\sim 10^8$ spores/ml in water) were spread on the surface of a microscope coverslip that was then dried in a vacuum desiccator for 5 to 10 min. Coverslips were then mounted on and sealed to a microscope sample holder kept at a constant temperature. The DIC microscope was set such that the polarizer and analyzer were crossed, and thus the DIC bias phase was zero (37). After adding preheated germinant/buffer solution to spores on the coverslips, a digital CCD camera (16 bits, 1,600 by 1,200 pixels) was used to record the DIC images at a rate of 1 frame per 15 s for 60 to 90 min. These images were analyzed with a computation program in Matlab to locate each spore's position and to calculate the averaged pixel intensity of an area of 40 by 40 pixels that covered the whole individual spore on the DIC image. The DIC image intensity of each individual spore was plotted as a function of the incubation time (with a resolution of 15 s), the initial intensity at T_0 (the first DIC image recorded after the addition of the germinant) was normalized to 1, and the intensity at the end of measurements was normalized to zero. Invariably, the latter value had been constant for ≥ 10 min at the end of measurements. From the time-lapse DIC image intensity, we can determine the time of completion of the rapid $\sim 75\%$ fall of spore DIC image intensity, which is concomitant with the time of completion of spore CaDPA release (this time is defined as T_{release}), as confirmed by Raman spectroscopy with LTRS (37). CaDPA release kinetics during the germination of individual spores were described by the parameters T_{lag} and $\Delta T_{\text{release}}$, where T_{lag} is the time between the

mixing of spores with germinants and the initiation of most CaDPA release (see Results and Fig. 4), and $\Delta T_{\text{release}} = T_{\text{release}} - T_{\text{lag}}$. Two additional parameters, I_{lag} and I_{release} , used to describe the germination of individual spores were defined as the intensities of a spore's DIC image at T_{lag} and T_{release} , respectively. Given the significant alterations in DPA release kinetics seen during the germination of wet-heat-treated spores (see Results), we also defined the additional germination parameters, T_1 , I_1 , T_{lys} and ΔT_{lys} where T_1 is the time following T_{lag} when the most rapid rate of DPA release begins, I_1 is the DIC intensity at T_1 , T_{lys} is the time when spore cortex hydrolysis is completed as determined by the completion of the fall in the spore's DIC image intensity (11, 37), and $\Delta T_{\text{lys}} = T_{\text{lys}} - T_{\text{release}}$. Importantly, when comparing the kinetics of changes in Raman spectra and DIC image intensities, if the DIC intensity at T_{release} was normalized to zero, the curve of DIC image intensity was coincident with the curve for the intensity of the CaDPA-specific Raman band at $1,017\text{ cm}^{-1}$ (see Results). Consequently, the percentages of CaDPA in the spore core at T_{lag} and T_1 could be deduced from the curve of DIC intensity during spore germination.

The degree of germination of multiple untreated spores of different strains was calculated by counting the germinated spores at various times and dividing by the number of dormant spores at the time of mixing of spores with germinants. For wet-heat-treated spores, only the spores retaining CaDPA were treated as dormant spores, since wet-heat-treated spores that have released their CaDPA do not germinate (4, 27). In these measurements, ≥ 300 individual spores were analyzed.

RESULTS

Spore killing and DPA content during wet-heat treatment.

As noted above, wet-heat treatment is routinely used for inactivation of spores of *Bacillus* species. Indeed, ~ 75 and $>90\%$ of wild-type *B. subtilis* spores were killed upon incubation for 5 min in water at 90 and 95°C, and longer incubation at these temperatures led to even more spore inactivation (Fig. 1a). However, even at these temperatures, most of the killed spores retained CaDPA, as spores incubated at 90°C for 15 to 30 min were inactivated 90 to 97% but 87 to 97% retained their CaDPA, with similar results for spores incubated at 95°C (Fig. 1a and b). Even incubation for 30 min at 95°C resulted in loss of CaDPA by only $\sim 60\%$ of the spores, although $\geq 99\%$ of these spores were killed (Fig. 1a and b). Thus, $\geq 80\%$ of the *B. subtilis* spores that retained CaDPA in populations that were wet heat treated under these conditions were dead, as seen previously (4, 5). Similar results were obtained with *B. cereus* and *B. megaterium* spores incubated at 90 or 80°C, respectively, for 15 min (data not shown), as found previously (5). These results indicate that while incubation in 80 to 95°C water for 15 to 30 min inactivates most spores, it probably does not severely damage the spore's inner membrane, the major barrier preventing release of the spore's small molecules. In previous work, the germination of wet-heat-killed spore populations that retained CaDPA was investigated and these spores did germinate, albeit slowly, with nutrient and nonnutrient germinants (4). However, the germination kinetics of individual wet-heat-inactivated spores that retain CaDPA has not been examined. Consequently, the Raman spectra and germination behavior of individual spores from populations that were wet heat inactivated but largely retained CaDPA have been studied.

In the Raman spectrum of an individual unheated wild-type *B. subtilis* spore (Fig. 1c, curve a), Raman bands at 662, 825, 1,017, 1,397, and 1,572 cm^{-1} are due to CaDPA (9, 13), the band at 1,004 cm^{-1} is due to phenylalanine in spore protein, and the bands at 1,650 and 1,665 cm^{-1} are associated with α -helical and irregular structures of the protein amide I (peptide bond C = O stretch), respectively (10). In examining the

FIG. 1. (a to c) Viability, CaDPA retention, and Raman spectra of wild-type *B. subtilis* spores with and without wet-heat treatment. (a and b) *B. subtilis* PS832 (wild-type) spores were incubated at either 90°C (○) or 95°C (▲) for various times, and spore viability (a) and the percentage of individual spores that retained CaDPA (b) were determined as described in Materials and Methods, by the examination of ~ 100 individual spores. (c) Raman spectra of individual *B. subtilis* PS832 spores without heat treatment (curve a), wet heat treated at 95°C for 30 min with retention of CaDPA (curve b), and wet heat treated at 95°C for 30 min with loss of CaDPA (curve c). The short dotted lines show the positions of the amide I Raman bands from protein in α -helical (1,650 cm^{-1}) or irregular (1,665 cm^{-1}) structures.

Raman spectra of spores that were wet heat treated at 95°C (Fig. 1c, curves b and c), one spore retained CaDPA (curve b) while another retained none (curve c). Further information from the Raman spectra of the heated spores concerns the state of the proteins in these spores. Thus, in curves a and b, the 1,645- to 1,670- cm^{-1} region of the Raman spectra of the unheated spore and the heat-treated spore which retained CaDPA looked almost identical, although there was slightly decreased intensity at 1,650 cm^{-1} and a moderate increase at 1,665 cm^{-1} in the wet-heat-treated spore that retained CaDPA (Fig. 1c; compare regions in the vertical dashed lines of curves a and b). The intensity of the Raman band at 1,650 cm^{-1} was even more reduced, and the band at 1,665 cm^{-1} was more prominent in the wet-heat-treated spore that had lost all CaDPA (Fig. 1c, compare curves b and c), indicating that much of the spore proteins' structure had changed from an α -helical to an irregular and presumably denatured state, concomitant with or soon after CaDPA release during wet-heat treatment, as seen previously (4, 5, 36).

FIG. 2. L-Alanine germination of untreated and wet-heat-treated wild-type *B. subtilis* spores. *B. subtilis* PS832 (wild-type) spores were left untreated (■), incubated at 90°C for 15 min (●) or 30 min (▲), or incubated at 95°C for 15 min (▼). Spore killing was determined as described in Materials and Methods and was as follows: 90°C for 15 min, 90%; 90°C for 30 min, 93%; 95°C for 15 min, 97%. After cooling, spores were heat activated at 70°C for 30 min and germinated with L-alanine and percentages of spore germination were determined by the intensity of DIC images of 300 to 400 individual spores that retained CaDPA as described in Materials and Methods.

A number of spore-specific proteins, including GRs, proteins comprising channels for CaDPA release, and CLEs, play key roles in spore germination (26). Consequently, the denaturation of any of these proteins should decrease spore germination and thus spore viability. However, previous work on the

germination of wet-heat-inactivated spore populations has given no significant information on protein inactivation that might affect the germination of wet-heat-treated spores (4, 5).

Kinetics of L-alanine germination of wet-heat-treated *B. subtilis* spores. Analysis of the L-alanine germination of multiple individual wild-type *B. subtilis* spores that retained CaDPA after a wet-heat treatment that gave ~90% killing showed that ~75% of these spores germinated with L-alanine, although more slowly than did untreated spores (Fig. 2). Increasing either the time or the temperature of the wet-heat treatment resulted in even slower germination of the treated spores. In contrast to the unheated spores, a significant percentage of the wet-heat-treated spores that retained CaDPA did not germinate in 60 min, and this percentage increased with more-severe wet-heat treatment.

While the analysis of the behavior of spore populations described above provided some information on the germination behavior of wet-heat-inactivated spores, the germination curves obtained were only an average of the behaviors of many different spores whose individual behavior could differ significantly. Therefore, we turned to examination of the L-alanine germination of individual untreated and wet-heat-treated wild-type *B. subtilis* spores (Fig. 3). Individual untreated spores germinated rapidly, most by ~20 min, and the intensities of DIC images of individual untreated spores were constant before the intensity decreased sharply as spores germinated, and the rapid CaDPA release for individual spores took place al-

FIG. 3. (a to d) L-Alanine germination of 10 individual untreated or wet-heat-treated wild-type *B. subtilis* spores. *B. subtilis* PS832 (wild-type) spores were either left unheated (a) or incubated at 90°C for 15 (b) or 30 (c) min or at 95°C for 15 min (d), spores were germinated with L-alanine, and the germination of the individual spores was followed by DIC microscopy as described in Materials and Methods. a.u., arbitrary units.

TABLE 1. Mean values and standard deviations of parameters of the L-alanine germination of untreated and wet-heat-treated wild-type *B. subtilis* spores^a

Treatment	T_{lag} (min)	T_1 (min)	$T_{release}$ (min)	$\Delta T_{release}$ (min)	$\Delta(T_{release} - T_1)$ (min)	ΔT_{lys} (min)	I_{lag} (%)	$I_{release}$ (%)	Germination (%)
None	12.6 ± 10.3	13.8 ± 10.5	14.8 ± 10.5	2.2 ± 0.6	1.0 ± 0.2	5.2 ± 2.1	94 ± 3	23 ± 8	98.0
90°C, 15 min	13.3 ± 9.5	16.8 ± 10.0	18.0 ± 10.0	4.7 ± 2.2	1.3 ± 0.5	11.7 ± 3.9	83 ± 10	29 ± 9	78.6
90°C, 30 min	16.3 ± 11.5	22.2 ± 12.1	23.7 ± 12.1	7.4 ± 4.4	1.5 ± 0.6	12.3 ± 4.5	81 ± 12	23 ± 13	75.1
95°C, 15 min	28.8 ± 14.9	37.8 ± 19.8	38.7 ± 19.9	9.9 ± 10.2	0.9 ± 0.3	17.1 ± 7.6	72 ± 14	31 ± 23	38.4

^a Untreated or wet-heat-treated *B. subtilis* PS832 spores were germinated with L-alanine for 90 min as described in Materials and Methods, and values for various germination parameters were calculated from those obtained from 30 individual spores as shown in Fig. 3 and 4a. The percent germination of the various spore preparations was determined by analysis of the germination of 300 to 400 spores that retained CaDPA as described in Materials and Methods.

most in parallel (Fig. 3 and data not shown). However, the germination kinetics of wet-heat-treated spores were significantly different from those of untreated spores, and it was not just that almost all of the wet-heat-treated spores germinated more slowly overall. In particular, with the wet-heat-treated spores, there were often slow decreases in the intensities of spores' DIC images before the sharp decrease. In addition, the germination kinetics of wet-heat-treated spores were more heterogeneous, as wet-heat-treated spores showed increased standard deviations of the values of $\Delta T_{release}$, ΔT_{lys} , and $I_{release}$ (Table 1); a longer heat treatment time or elevated temperature increased this heterogeneity (Fig. 3b to d and 4a). To quantitatively describe the kinetics of spore germination with L-alanine, values of T_{lag} , $T_{release}$, and $\Delta T_{release}$ (Fig. 4a; see Materials and Methods) were measured for 30 to 40 randomly chosen untreated spores and spores given different wet-heat treatments (Table 1). Strikingly, the mean times for all of these germination parameters increased from 2.3- to 4.5-fold in the most severely wet-heat-treated spores. It was also noted that during the germination of an untreated spore, the decrease in normalized DIC intensity was almost identical to the CaDPA release determined by LTRS prior to the completion of CaDPA release at $T_{release}$ (Fig. 4b). Indeed, when the DIC image intensity at $T_{release}$ was normalized to zero, the curve of the DIC image intensity was coincident with the curve of the CaDPA level. A similar correspondence between decreases in DIC intensity and CaDPA levels was also observed for wet-heat-treated spores (Fig. 4c), indicating that DIC image intensity could be used to quantify the CaDPA release from individual wet-heat-treated spores.

It was clear that the CaDPA release kinetics during L-alanine germination of most wet-heat-treated spores were quite different from those of the untreated spores in a number of ways (Fig. 3 and 4 and data not shown), as with the wet-heat-treated spores: (i) even prior to T_{lag} , there was often a slow fall in DIC image intensity and the CaDPA level and the amount of this fall was greatest in spores that germinated the most slowly, as was evident when the DIC image intensities at T_{lag} , I_{lag} values, were examined; and (ii) events following T_{lag} were often biphasic, with an initial slow fall in DIC image intensity, and thus the CaDPA level, prior to more rapid decreases (Fig. 3 and 4 and data not shown). In order to quantitate the latter phenomena, we used an additional kinetic parameter, T_1 , to denote the time of the start of the most rapid phase of most CaDPA release, with T_{lag} remaining as the time of the initiation of CaDPA release, determined by the slope of the first phase of CaDPA release (Fig. 4a and c), although some indi-

vidual germinating wet-heat-treated spores exhibited a decrease in ~20% of their CaDPA level before T_{lag} (Fig. 3b to d). The DIC image intensities at T_{lag} , T_1 , and $T_{release}$ were also measured (Table 1), and these values were paralleled by the spore's CaDPA level (data not shown). The period of slow fall in DIC image intensity and CaDPA level between T_{lag} and T_1 could also be discerned during the germination of unheated spores, although the rates of decrease between T_{lag} and T_1 were significantly faster with the unheated spores. Determination of all of the parameters of the L-alanine germination of multiple untreated and wet-heat-treated spores (Table 1; data not shown) led to a number of notable findings. (i) Average values of T_{lag} increased >2-fold with wet-heat-treated spores, as did the fall in DIC image intensity and CaDPA release prior to T_{lag} . (ii) Average values of $\Delta T_{release}$ increased >4-fold in the most severely wet-heat-treated spores, and this is likely an underestimate of the decrease in the actual rates of CaDPA release, since in the more severely wet-heat-treated spores, a notable amount of the fall in DIC image intensity and CaDPA level took place prior to T_{lag} . (iii) Most of the increase in $\Delta T_{release}$ values with wet-heat-treated spores was in the time between T_{lag} and T_1 , with this period increasing from ~1 min in untreated spores to ~9 min in the most severely wet-heat-treated spores, while $\Delta(T_{release} - T_1)$ values were relatively similar for both unheated and wet-heat-treated spores—again, however, this similarity is somewhat overstated, since the differences between I_1 and $I_{release}$ values decreased with the severity of spore wet-heat treatment. (iv) The ΔT_{lys} value, which is most likely the time needed for spore cortex hydrolysis following complete CaDPA release, increased >3-fold in the most severely wet-heat-treated spores. (v) The standard deviations of the values of T_{lag} , $\Delta T_{release}$, ΔT_{lys} , and $I_{release}$ were also increased in wet-heat-treated spores, indicating an increased variability/heterogeneity in germination among individual wet-heat-treated spores.

AGFK and L-asparagine germination of wet-heat-treated *B. subtilis* spores. In addition to L-alanine, *B. subtilis* spores are also germinated by a mixture of L-asparagine, glucose, fructose, and K⁺ (AGFK) that requires the participation of two different GRs, GerB and GerK, while L-alanine germination requires only the GerA GR (19). As found with L-alanine germination, wet-heat treatment also significantly slowed wild-type *B. subtilis* spore germination with AGFK (Fig. 5a). Comparing the average AGFK germination parameters of multiple untreated and wet-heat-treated wild-type *B. subtilis* spores (Table 2) revealed the same general findings as found with L-alanine germination.

FIG. 4. (a to c) Typical kinetics of L-alanine germination of untreated and wet-heat-treated individual wild-type *B. subtilis* spores. (a) Normalized intensity of DIC images of individual *B. subtilis* PS832 (wild-type) spores that were germinated with L-alanine, with germination followed by DIC microscopy as described in Materials and Methods. Symbols: $\square$, untreated spore; $\triangle$ and $\circ$, individual spores wet heat treated at 90°C for 30 min that retained CaDPA. (b and c) Normalized intensities of DIC images and height of the Raman band at 1,017 cm^{-1} of an untreated spore (b) or a wet-heat-treated (15 min; 90°C) spore that retained CaDPA (c), both germinating with L-alanine and with spore germination monitored as described in Materials and Methods. Symbols: $\circ$, germination kinetics monitored by Raman spectroscopy; $\square$, germination kinetics monitored by DIC microscopy; $\diamond$, normalized DIC image intensities with the image intensity at $T_{release}$ set to zero. a.u., arbitrary units.

Results generally similar to those obtained with L-alanine and AGFK germination were also obtained when the L-asparagine germination of untreated and wet-heat-treated spores carrying a modified GerB GR, termed GerB*, that can trigger spore germination in response to L-asparagine alone was examined (Fig. 5d; Table 3). The L-asparagine germination of untreated and wet-heat-treated spores carrying ~ 200 -fold-elevated levels of the GerB* GR was also similar to the L-asparagine germination of unheated and wet-heat-treated spores with normal GerB* GR levels (Fig. 5d; Table 3). Note, however, that with 0.5 mM L-asparagine as the germinant, the spores with wild-type GerB* levels did not germinate as rapidly as spores with the high GerB* levels, as expected (2).

L-Alanine germination of wet-heat-treated *cwlJ* and *sleB* mutant *B. subtilis* spores. In addition to requiring GRs to trigger it, spore germination also requires the degradation of the spore's peptidoglycan cortex. *B. subtilis* spores have two CLEs, CwlJ and SleB, either of which is sufficient for the completion of spore germination (26). Analysis of the L-alanine germination of untreated and wet-heat-treated *B. subtilis* spores lacking SleB (FB112 spores) showed that the effects of wet-heat treatment on these spores' germination were very similar to those seen with wild-type spores (compare Fig. 5a and c; Tables 1 and 4). The one notable difference was that it required a higher temperature to significantly reduce the germination of the wild-type spores, 90°C versus 85°C, suggesting that the remaining CLE in the FB112 spores, CwlJ, was somewhat more wet heat labile than SleB.

In contrast to the germination of the spores containing only CwlJ (FB112 spores), that of spores containing only SleB (FB111 spores) was as resistant to wet-heat treatment as was that of wild-type spores, although there were also other differences between the germination of wild-type and that of *cwlJ* mutant spores (Fig. 5b and c; Table 4). In particular, the $\Delta T_{release}$ value, even for untreated FB111 spores, was much higher than for untreated wild-type or FB112 spores, as found previously when spore germination was monitored only by Raman spectroscopy (12). In addition, the initially high $\Delta T_{release}$ value of the FB111 spores was increased only slightly by the severest wet-heat treatment, in contrast to the ~ 3 -fold increase seen with the FB112 spores. It was also notable that the T_{lag} value of untreated FB111 spores was significantly lower than that of untreated wild-type or FB112 spores and that the FB111 spores' T_{lag} value was not decreased appreciably by wet-heat treatment (Tables 1 and 4).

Dodecylamine germination of wet-heat-treated *B. subtilis* spores. In addition to nutrients, spores of *Bacillus* species are also germinated by a number of nonnutrient agents, one of which is the cationic surfactant dodecylamine (22, 26). Germination of *B. subtilis* spores by dodecylamine does not involve the spores' GRs, and dodecylamine also does not directly activate CLEs (22, 26). Rather, dodecylamine directly or indirectly opens channels in the spore's inner membrane that allow CaDPA release (22, 29). This suggested that analysis of the kinetics of the dodecylamine germination of wet-heat-treated spores might provide new insight into the precise damage to the spore germination apparatus caused by wet-heat treatment, since any damage to GRs would not affect spores' germination with dodecylamine. The T_{lag} values for dodecylamine germination of untreated *B. subtilis* spores were >4 -fold higher

FIG. 5. (a to d) Germination of untreated and wet-heat-treated spores of various *B. subtilis* strains. PS832 (wild-type) spores were germinated with AGFK (a), FB111 (*cwlJ*) spores (b) and FB112 (*sleB*) spores (c) were germinated with L-alanine, PS3415 ($\uparrow$ GerB*) and FB10 (*gerB**) spores were germinated with L-asparagine (d), and the germination of ~ 300 individual spores was followed by DIC microscopy as described in Materials and Methods. Symbols in panels a and b: $\blacksquare$, unheated spores; $\bullet$, spores incubated at 90°C for 15 min; $\blacktriangle$, spores incubated at 90°C for 30 min. Symbols in panel c: $\square$, unheated spores; $\bullet$, spores incubated at 90°C for 15 min; $\blacktriangle$, spores incubated at 90°C for 30 min. Symbols in panel d: $\square$, unheated PS3415 spores; $\bullet$, PS3415 spores incubated at 90°C for 15 min; $\blacktriangle$, PS3415 spores incubated at 90°C for 30 min; ∇ , unheated FB10 spores; $\blacktriangledown$, FB10 spores incubated at 90°C for 30 min.

than for nutrient germination (Table 1 to 3 and 5), although as seen with nutrient germination, *B. subtilis* spores germinated more slowly and to a lesser extent with dodecylamine following wet-heat treatment (Fig. 6a to d). However, the multiphasic kinetics of CaDPA release seen during nutrient germination of wet-heat-treated spores (Fig. 3b to d) were much less evident during the dodecylamine germination of these spores (Fig. 6b to d). The changes in some parameters of dodecylamine germination upon wet-heat treatment were similar to those seen with nutrient germination, while changes in some other param-

eters were different (Table 5). Changes in germination parameters following wet-heat treatment that were similar for both dodecylamine and nutrient germinations were that (i) the values of T_{lag} , $\Delta T_{\text{release}}$, and $\Delta(T_1 - T_{\text{lag}})$ for dodecylamine germination were all increased by wet-heat treatment, while there was much less of an effect on $\Delta(T_{\text{release}} - T_1)$, and (ii) the I_{lag} and I_1 values for dodecylamine germination also decreased upon wet-heat treatment, the I_1 value in particular by a large amount (Tables 1 to 3 and 5). The change in a germination parameter that was different in wet-heat-treated spores germi-

TABLE 2. Mean values and standard deviations of parameters of the AGFK germination of untreated and wet-heat-treated wild-type *B. subtilis* spores^a

Treatment	T_{lag} (min)	$\Delta T_{\text{release}}$ (min)	$\Delta(T_{\text{release}} - T_1)$ (min)	ΔT_{lys} (min)	I_{lag} (%)	Germination (%)
None	7.6 $\pm$ 4.9	2.2 $\pm$ 0.9	0.9 $\pm$ 0.2	3.9 $\pm$ 1.6	96 $\pm$ 2	98.2
90°C, 15 min	12.3 $\pm$ 7.5	5.1 $\pm$ 3.4	1.1 $\pm$ 0.4	9.7 $\pm$ 5.3	83 $\pm$ 11	76.0
90°C, 30 min	16.5 $\pm$ 10.1	5.0 $\pm$ 2.3	1.1 $\pm$ 0.3	12.8 $\pm$ 9.9	78 $\pm$ 10	76.4

^a Untreated or wet-heat-treated *B. subtilis* PS832 spores were germinated for 60 min with AGFK as described in Materials and Methods, and values for various germination parameters were calculated from those obtained from 30 individual spores as shown in Fig. 4a. The percent germination of the various spore preparations was determined by analysis of the germination of ~ 300 spores that retained CaDPA as described in Materials and Methods.

TABLE 3. Mean values and standard deviations of parameters of the L-asparagine germination of untreated and wet-heat-treated *B. subtilis* spores with various levels of the GerB* GR^a

Strain and treatment	T_{lag} (min)	$\Delta T_{release}$ (min)	$\Delta(T_{release} - T_1)$ (min)	ΔT_{lys} (min)	I_{lag} (%)	$I_{release}$ (%)	Germination (%)
PS3415 ($\uparrow$ GerB*)							
None	5.5 $\pm$ 4.1	2.1 $\pm$ 0.8	0.9 $\pm$ 0.2	5.0 $\pm$ 1.4	92 $\pm$ 5	20 $\pm$ 8	98.3
90°C, 15 min	14.3 $\pm$ 14.5	5.9 $\pm$ 3.2	1.4 $\pm$ 0.6	17.8 $\pm$ 5.0	86 $\pm$ 9	37 $\pm$ 13	85.4
90°C, 30 min	27.0 $\pm$ 9.8	7.5 $\pm$ 7.0	1.0 $\pm$ 0.5	20.1 $\pm$ 5.5	72 $\pm$ 13	33 $\pm$ 9	21.9
FB10 (<i>gerB</i> *)							
None	30.1 $\pm$ 18.3	5.5 $\pm$ 3.2	1.1 $\pm$ 0.5	14.2 $\pm$ 6.8	92 $\pm$ 3	37 $\pm$ 8	51.7
90°C, 30 min	34.8 $\pm$ 26.0	10.4 $\pm$ 5.8	1.4 $\pm$ 0.3	19.3 $\pm$ 6.8	85 $\pm$ 13	43 $\pm$ 8	34.1

^a Untreated or wet-heat-treated *B. subtilis* PS3415 ($\uparrow$ GerB*) and FB10 (*gerB**) spores were germinated for 90 min with L-asparagine as described in Materials and Methods, and values for various germination parameters were calculated from those obtained from 30 individual spores as shown in Fig. 4a. The percent germination of the various spore preparations was determined by analysis of the germination of ~300 spores that retained CaDPA as described in Materials and Methods.

nating with dodecylamine was that, unlike with L-alanine, AGFK, or L-asparagine germination, the $I_{release}$ values in dodecylamine germination of wet-heat-treated spores were much closer to zero, such that the cortex hydrolysis period following CaDPA release was not as noticeable as in L-alanine germination of untreated or wet-heat-treated spores.

Nutrient germination of wet-heat-treated *B. megaterium* and *B. cereus* spores. In addition to *B. subtilis* spores, germination of *B. cereus* and *B. megaterium* spores is also decreased by wet-heat treatment, and these spores are also likely killed by protein denaturation, with a large percentage of the wet-heat-killed spores retaining CaDPA (4, 5). Therefore, it was of obvious interest to examine the kinetics of nutrient germination of individual wet-heat-treated *B. cereus* and *B. megaterium* spores (Fig. 7; Table 6). Perhaps not surprisingly, the effects of wet-heat treatment on the nutrient germination of spores of these two species were generally very similar to those seen with *B. subtilis* spores (Tables 1, 2, 4, and 6). Thus, wet-heat-treated *B. megaterium* and *B. cereus* spores also germinated more slowly than untreated spores, and except for the shorter T_{lag} value for wet-heat-treated *B. cereus* spores, all other nutrient germination parameters exhibited responses to wet-heat treatment similar to those of *B. subtilis* spores. These results thus suggested that wet heat affects spore germination by a similar mechanism in all three *Bacillus* species.

DISCUSSION

The work described in this communication shows clearly that wet-heat treatment greatly slows the germination of spores of *Bacillus* species, even though these spores may be largely dead in plate assays of viability. These nonculturable spores that germinate retain their CaDPA but respond only slowly to a variety of germinants in releasing their CaDPA. Since a significant amount of protein denaturation precedes CaDPA release during spore wet-heat treatment (36; P. Zhang, L. Kong, G. Wang, P. Setlow, and Y. Q. Li, unpublished results), it seems likely that the proteins denatured in this process would include one or more of the proteins that are essential for spore germination. There are three steps in the germination of spores of *Bacillus* species that require known proteins (26): (i) recognition of nutrient germinants by GRs, (ii) release of CaDPA perhaps through channels composed at least in part of SpoVA proteins, and (iii) hydrolysis of the spore's peptidoglycan cortex by the CLEs CwlJ and/or SleB. Loss of the activity of any proteins that are essential for these events would block germination, and it is possible that one or more unknown proteins play some role in signal transduction during the germination process, as the GerD protein might do (20). An obvious question then is which of these possible proteins is inactivated by wet heat leading to longer times at which individual spores initiate germination events.

Certainly, inactivation of GRs would block spore germina-

TABLE 4. Mean values and standard deviations of parameters of the L-alanine germination of untreated and wet-heat-treated *cwlJ* and *sleB* mutant *B. subtilis* spores^a

Strain and treatment	T_{lag} (min)	$\Delta T_{release}$ (min)	$\Delta(T_{release} - T_1)$ (min)	ΔT_{lys} (min)	I_{lag} (%)	$I_{release}$ (%)	Germination (%)
FB111 (<i>cwlJ</i>)							
None	20.4 $\pm$ 10.5	14.9 $\pm$ 5.6	1.0 $\pm$ 0.2	3.5 $\pm$ 1.1	76 $\pm$ 10	18 $\pm$ 5	99.4
90°C, 15 min	52.3 $\pm$ 19.9	15.5 $\pm$ 6.8	1.1 $\pm$ 0.4	14.6 $\pm$ 4.2	77 $\pm$ 8	22 $\pm$ 13	62.6
90°C, 30 min	55.2 $\pm$ 25.1	18.1 $\pm$ 7.9	1.1 $\pm$ 0.3	14.8 $\pm$ 6.9	79 $\pm$ 7	23 $\pm$ 3	34.1
FB112 (<i>sleB</i>)							
None	16.3 $\pm$ 9.5	2.7 $\pm$ 1.2	0.9 $\pm$ 0.1	3.2 $\pm$ 1.8	95 $\pm$ 3	13 $\pm$ 5	99.9
85°C, 15 min	19.5 $\pm$ 12.4	6.4 $\pm$ 3.3	1.0 $\pm$ 0.2	7.0 $\pm$ 3.3	94 $\pm$ 3	18 $\pm$ 5	67.3
85°C, 30 min	32.4 $\pm$ 20.5	7.4 $\pm$ 5.0	1.1 $\pm$ 0.2	9.0 $\pm$ 4.3	87 $\pm$ 7	19 $\pm$ 6	24.3

^a Untreated or wet-heat-treated *B. subtilis* FB111 (*cwlJ*) and FB112 (*sleB*) spores were germinated for 120 min with L-alanine as described in Materials and Methods, and values for various germination parameters were calculated from those obtained from 30 individual spores as shown in Fig. 4a. The percent germination of the various spore preparations was determined by analysis of the germination of ~300 spores that retained CaDPA as described in Materials and Methods.

TABLE 5. Mean values and standard deviations of parameters of the dodecylamine germination of untreated and wet-heat-treated wild-type *B. subtilis* spores^a

Treatment	T_{lag} (min)	$\Delta T_{\text{release}}$ (min)	$\Delta(T_{\text{release}} - T_1)$ (min)	I_{lag} (%)	I_{release} (%)	Germination (%)
None	31.1 ± 13.2	2.3 ± 0.9	0.8 ± 0.2	92 ± 3	12 ± 3	99.2
90°C, 15 min	54.8 ± 26.9	4.4 ± 2.3	1.6 ± 0.9	87 ± 6	8 ± 4	52.9
90°C, 30 min	53.9 ± 31.5	7.0 ± 2.5	1.0 ± 0.4	86 ± 5	8 ± 6	35.1

^a Untreated or wet-heat-treated *B. subtilis* PS832 spores were germinated with dodecylamine for 90 min as described in Materials and Methods, and values for various germination parameters were calculated from those obtained from 40 individual spores as shown in Fig. 6. The percent germination of the various spore preparations was determined by analysis of the germination of ~300 spores that retained CaDPA as described in Materials and Methods.

tion with nutrients, since nutrient germinants would not be recognized. In addition, at least some segments of the spore’s GR proteins will be outside the protective environment of the spore core, since some GR proteins are integral proteins in the spore’s inner membrane (26). However, there are a number of observations indicating that GR inactivation is not a reason for the longer times at which individual wet-heat-treated spores

initiated germination events as follows. (i) *B. subtilis* spore germination through activation of a number of different GRs was decreased relatively similarly by wet-heat treatment. While it is possible that different GRs would exhibit similar percentages of inactivation by wet heat, the proteins in different GRs, while clearly related, exhibit only 30 to 40% sequence identity and thus might be expected to have different percentages of

FIG. 6. (a to d) Dodecylamine germination of unheated and wet-heat-treated wild-type *B. subtilis* spores. Unheated or wet-heat-treated *B. subtilis* PS832 (wild-type) spores were germinated with dodecylamine, and spore germination was followed by DIC microscopy as described in Materials and Methods. In panel a, the germination of ~300 spores was followed whether or not they retained CaDPA, and in panels b to d, the germination of 10 randomly chosen spores that were left untreated (b), treated at 90°C for 15 min (c), or treated at 90°C for 30 min with retention of CaDPA (d) were followed. Symbols in panel a: ■, unheated spores; ●, spores incubated at 90°C for 15 min; ▲, spores incubated at 90°C for 30 min. a.u., arbitrary units.

FIG. 7. (a and b) Nutrient germination of wet-heat-treated *B. megaterium* and *B. cereus* spores. Heat-activated *B. megaterium* spores were germinated with glucose (a), and heat-activated *B. cereus* spores were germinated with L-alanine (b), and the germination of ~300 unheated or wet-heat-treated spores that retained CaDPA was followed by DIC microscopy as described in Materials and Methods. Symbols in panel a: ■, unheated spores; ●, spores incubated at 80°C for 15 min. Symbols in panel b: ■, unheated spores; ●, spores incubated at 90°C for 15 min. The viability of the *B. megaterium* and *B. cereus* spores was reduced >90% by the wet-heat treatment used, but ≥90% of these wet-heat-treated spores retained CaDPA.

wet-heat inactivation. Consequently, germination with different nutrient germinants would respond differently to wet-heat treatment. (ii) Overexpression of the GerB* receptor 200-fold accompanied by more rapid germination with L-asparagine did not reduce the decreases in spore germination caused by wet-heat treatment. If the GerB* GR was the target of the wet-heat treatment, spores with higher levels of GerB* would have been expected to require more-severe wet-heat treatment to reduce their germination to the same extent as spores with normal GerB* levels. (iii) Spore germination with dodecylamine was also decreased considerably by wet-heat treatment. Since dodecylamine germination does not involve the GRs (22), this strongly indicates that wet-heat treatment decreases spore germination by acting on a target other than GRs.

The second step at which wet-heat treatment could slow spore germination is cortex lysis by CLEs. Indeed, lack of cortex lysis by loss or inactivation of CLEs does block the completion of the germination of spores of *Bacillus anthracis*, *B. megaterium*, and *B. subtilis* (7, 8, 25, 26). In addition, values of $\Delta T_{\text{release}}$ are slowed 3- to 14-fold during nutrient germination of individual spores of *cwlJ sleB* mutant *B. megaterium* and *B. subtilis* strains, respectively (21, 25). Thus, wet-heat inactivation of CLEs can slow but does not abolish CaDPA release in spore germination, even although cortex lysis cannot take place, and loss of both CwlJ and SleB does not increase values of T_{lag} (21, 23–25). Thus, effects on CLEs, in particular CwlJ,

can be only a partial explanation for the effects of wet heat on spore germination, since *cwlJ sleB* mutant *B. megaterium* and *B. subtilis* spores still release CaDPA during nutrient germination, and this seems almost certain for *B. anthracis* spores that lack CLEs as well (7, 8, 25, 26). However, CLE inactivation, in particular inactivation of CwlJ by wet-heat treatment, does appear to be one reason for the slower germination of wet-heat-treated spores seen in this work as follows. (i) Times for most CaDPA release expressed as $\Delta T_{\text{release}}$ values increased significantly in wet-heat-treated spores, consistent with increases in $\Delta T_{\text{release}}$ values seen with untreated spores when CwlJ but not SleB was absent (21, 25). (ii) The time required for cortex hydrolysis, ΔT_{lys} , also increased significantly during nutrient germination of wet-heat-treated spores. (iii) Spores that contained only CwlJ were more sensitive to wet-heat treatment than wild-type spores or those that contained only SleB, as wild-type or *cwlJ* mutant spores required treatment at a higher temperature to obtain the same reduction in spore germination as spores that contained only CwlJ. These results indicated that in spores, CwlJ is significantly more heat labile than SleB, as was shown previously (1), and CwlJ in spores has also been shown to be significantly more sensitive than SleB to a number of oxidizing agents (33, 34). As with some segments of the GR proteins, CwlJ and SleB are also outside the spore core, in regions in or adjacent to the spore cortex (26). Consequently, these proteins would not be protected against wet

TABLE 6. Mean values and standard deviations of parameters of the nutrient germination of untreated and wet-heat-treated *B. cereus* and *B. megaterium* spores^a

Species and treatment	T_{lag} (min)	$\Delta T_{\text{release}}$ (min)	$\Delta(T_{\text{release}} - T_1)$ (min)	ΔT_{lys} (min)	I_{lag} (%)	I_{release} (%)	Germination (%)
<i>B. megaterium</i>							
None	2.1 ± 2.5	1.6 ± 0.5	1.1 ± 0.3	1.3 ± 0.6	97 ± 5	14 ± 5	99.0
80°C, 15 min	3.0 ± 2.9	2.8 ± 1.1	1.4 ± 0.4	5.9 ± 2.2	93 ± 6	32 ± 12	66.5
<i>B. cereus</i>							
None	12.2 ± 5.6	1.6 ± 0.8	0.8 ± 0.1	3.4 ± 1.6	95 ± 4	17 ± 5	98.4
90°C, 15 min	9.4 ± 9.0	9.8 ± 5.4	1.3 ± 0.3	8.9 ± 4.4	85 ± 10	20 ± 9	41.6

^a Untreated or wet-heat-treated *B. megaterium* and *B. cereus* spores were germinated for 90 min with glucose or L-alanine as described in Materials and Methods, and values for various germination parameters were calculated from those obtained from 20–30 individual spores as shown in Fig. 4a. The percent germination of the various spore preparations was determined by analysis of the germination of ~300 spores that retained CaDPA as described in Materials and Methods.

heat by the special properties of the spore core, in particular its low water content. Thus, it is not surprising that one of these CLEs would be more wet heat sensitive than proteins in the spore core and that the two CLEs would also exhibit different wet-heat resistance. However, while effects on CLEs may explain some of the decrease in $\Delta T_{\text{release}}$ values for CaDPA release during spore germination caused by wet heat, effects on CLEs alone will not abolish CaDPA release, only slow it!

The final possible event that could be blocked by wet-heat treatment is CaDPA release itself. There appear to be two possible ways that CaDPA release during spore germination could be blocked: (i) blockage of signal transduction between GRs and the CaDPA release mechanism, the latter perhaps involving the GerD protein or an unknown protein and (ii) blockage of CaDPA release directly by inactivating one or more proteins that comprise the CaDPA release mechanism, possibly a SpoVA protein (26). The first possibility seems unlikely, since spores lacking at least GerD germinate normally with dodecylamine (20) and germination with this agent is decreased by wet-heat treatment. However, there certainly could be some unknown protein involved in the mechanism signaling CaDPA release that is the target of wet-heat treatment. Another possibility is the CaDPA release process that may involve one or more of the SpoVA proteins (26). Like the GRs, the six proteins encoded by the *spoVA* operon are likely in or associated with the spore's inner membrane, as would seem to be important for proteins involved in CaDPA release during spore germination. It therefore seems reasonable to suggest that it is one or more of these proteins or the CaDPA release process itself that is the target of the wet-heat treatment that abolishes the germination of spores of *Bacillus* species that retain CaDPA.

The fact that wet-heat treatment has generally similar effects on CaDPA release in both nutrient and dodecylamine germination further suggests that dodecylamine triggers spore germination by activating the same CaDPA release mechanism that is used in nutrient germination and not by some nonspecific effect on the spores' inner membrane causing production of pores in this membrane that allow CaDPA release. Indeed, it was suggested previously by analysis of the germination of spore populations with dodecylamine that dodecylamine triggers spore germination by acting on some protein channel (22, 29).

The damage to the spore's germination apparatus generated by spore wet-heat treatment clearly precedes the CaDPA release caused by this treatment, as well as the extensive protein denaturation that accompanies CaDPA release. However, spores that release CaDPA during wet-heat treatment are already dead, likely because of protein denaturation that precedes CaDPA release. Some of the proteins denatured prior to CaDPA release are clearly involved in some fashion in spore germination, including at least the CLE CwlJ and perhaps one or more proteins involved more directly in the CaDPA release process. Inactivation of spores during wet-heat treatment is not caused by inactivation of CLEs, since lysozyme treatment of wet-heat-inactivated spores that retain CaDPA does not recover these spores (4, 5). However, it is possible that wet-heat damage to the spore's CaDPA release mechanism might be the way wet heat kills spores, in effect by making them incapable of germinating properly.

In addition to the major question of how wet-heat treatment slows and even abolishes the germination of spores of *Bacillus* species, there are a number of related questions. One such question is why inactivation or loss of CwlJ decreases the $\Delta T_{\text{release}}$ value in spore germination. This has been seen previously by examining CaDPA release from individual *cwlJ* spores of *B. megaterium* and *B. subtilis* and has been suggested to be due to a stimulation of CaDPA release by cortex hydrolysis catalyzed by CwlJ (21, 25), since CwlJ catalysis is activated by CaDPA released during germination, as well as by high concentrations of exogenous CaDPA (26). It is perhaps notable that the effect of CwlJ loss or inactivation by wet heat seems to be primarily on the initial slower phases of CaDPA release, with much less of an effect on the rate of release between T_1 and T_{release} . This is consistent with the initial slower phase or phases of CaDPA release activating CwlJ, with cortex hydrolysis by this enzyme in turn somehow leading to more-rapid CaDPA release. However, in the absence of CwlJ or with low CwlJ levels due to wet-heat treatment, the slower CaDPA release goes on much longer until some other change, perhaps activation of SleB by the decreased osmotic pressure in the spore core, finally leads to cortex hydrolysis and rapid CaDPA release. Indeed, *cwlJ sleB* mutant *B. megaterium* and *B. subtilis* spores exhibit only a monotonic release of CaDPA during germination (21, 25). This general scenario would also explain why there is biphasic release of CaDPA during spore germination. However, it is not clear why the CaDPA release kinetics during germination might be triphasic. It is also not clear why there would be much more CaDPA release prior to T_1 from wet-heat-treated spores germinating with nutrients than during dodecylamine germination.

The final notable question is why wet-heat treatment generally results in significant increases in T_{lag} values during both nutrient and nonnutrient germinations. Again, we have no answer for this question, as we know very little about events that take place during T_{lag} . The exception to this is commitment, whereby spores that have been incubated with a germinant but have not yet germinated, as evidenced by the absence of CaDPA release, will subsequently germinate even if the germinant is removed or its action is completely inhibited (32). Normally, commitment in nutrient germination shows a lag time similar to that of germination itself but always takes place a few min earlier than T_{lag} . Perhaps the time between commitment and germination is greatly increased by wet-heat treatment and this could be tested in spore populations, although perhaps not in individual spores. Unfortunately, the events that cause commitment are not known, nor are any other events that take place during T_{lag} , so this period in spore germination remains largely uncharted territory.

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