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Maturation of Released Spores Is Necessary for Acquisition of Full Spore Heat Resistance during *Bacillus subtilis* Sporulation▽

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The first ~10% of spores released from sporangia (early spores) during *Bacillus subtilis* sporulation were isolated, and their properties were compared to those of the total spores produced from the same culture. The early spores had significantly lower resistance to wet heat and hypochlorite than the total spores but identical resistance to dry heat and UV radiation. Early and total spores also had the same levels of core water, dipicolinic acid, and Ca and germinated similarly with several nutrient germinants. The wet heat resistance of the early spores could be increased to that of total spores if early spores were incubated in conditioned sporulation medium for ~24 h at 37°C (maturation), and some hypochlorite resistance was also restored. The maturation of early spores took place in pH 8 buffer with Ca^{2+} but was blocked by EDTA; maturation was also seen with early spores of strains lacking the CotE protein or the coat-associated transglutaminase, both of which are needed for normal coat structure. Nonetheless, it appears to be most likely that it is changes in coat structure that are responsible for the increased resistance to wet heat and hypochlorite upon early spore maturation.

Spores of *Bacillus* and *Clostridium* species are dormant and extremely resistant to a variety of agents, including high temperatures, radiation, and many chemicals (19, 31). This extreme spore resistance has major applied implications, because spores of *Bacillus* and *Clostridium* species are vectors for food spoilage and a number of diseases, some of which are food borne. As a consequence, there is much interest in the mechanisms of spore resistance as well as methods for spore inactivation.

The most commonly used method for spore inactivation is wet heat treatment, which is extremely effective, even though spores are generally resistant to temperatures ~40°C higher than their growing-cell counterparts (4, 19, 31). Factors involved in spores' extreme resistance to wet heat include (i) the protection of spore DNA by its saturation with the α/β-type small, acid-soluble spore proteins (SASPs); (ii) the thick proteinaceous spore coat layer; (iii) the thick layer of cortex peptidoglycan surrounding the spore core; (iv) the high level of pyridine-2,6-dicarboxylic acid (dipicolinic acid [DPA]) and its associated divalent cations, mostly Ca^{2+}, in the spore core; and, most importantly, (v) the low water content of the core of spores suspended in water (4, 5, 19, 31, 32). High spore wet heat resistance is acquired late in sporulation, largely in parallel with the uptake of DPA by the developing forespore and the final decrease in spore core water content. However, the precise time of acquisition of full spore heat resistance during sporulation is not completely clear because (i) precise measurements of wet heat resistance are usually carried out only on purified spores; (ii) analysis of acquisition of wet heat resistance can be carried out only with spore populations, and the lack of precise synchrony in sporulation of cell populations can complicate kinetic analysis of events in sporulation; and (iii) the specific conditions in sporulating cultures, including the composition of the medium and the environment in the mother cell surrounding the developing spore, may change during sporulation, and such changes could modify spore wet heat resistance compared to that of purified spores in water.

It is also clear that the wet heat resistance of individuals in spore populations can vary significantly (1, 3, 9, 13, 19, 27, 34, 36). Indeed, analyses of spore killing by wet heat as a function of time often suggest that there is a significant level of more wet-heat-resistant spores in populations (27). In addition, analysis of the behavior of individual spores exposed to potentially lethal temperatures in water has indicated that while individual spores release >95% of their DPA in only a 1- to 2-min period, this release follows a lag period which varies tremendously between individual spores (39). The latter difference between individuals in spore populations is not genetic, and fractionation of spore populations has resulted in the isolation of a significant fraction of spores that have elevated wet heat resistance and a lower core water content than the population as a whole (6).

The findings noted above indicate that genetically identical spore populations exhibit significant heterogeneity in their wet heat resistance. The causes of this heterogeneity are not clear, but there are a number of possible reasons. (i) One could be stochastic differences in expression of a key gene or genes whose products play significant roles in determining spores' core water content, such as levels of α/β-type SASPs or DPA or coat structure (4, 5, 8, 16, 24, 30, 32). (ii) A second one could be related to the asynchrony in sporulation noted above, in particular, the fact that the sporulation medium will change as

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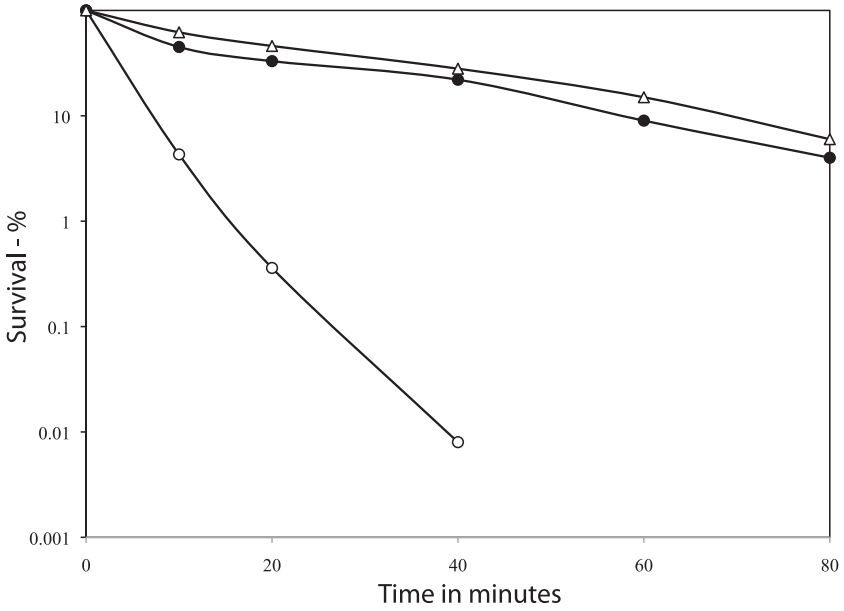

FIG. 1. Heat resistance of early, early-matured, and total spores. *B. subtilis* strain PS533 was sporulated, and early, early-matured, and total spores were isolated and purified as described in Materials and Methods. The purified spores were incubated in 90°C water at an OD_{600} of 1, and viability was determined as a function of time as described in Materials and Methods. Symbols: ○, early spores; ●, early-matured spores; △, total spores.

sporulation proceeds, such that the medium in which the first spores are formed in sporulation will differ from that in which the last spores are formed (9, 33). If the latter is the case, then the spores formed first in sporulation might have different wet heat resistance than those formed either later or in the entire sporulation process, since the precise composition of the sporulation medium can have significant effects on spore wet heat resistance (4, 26, 27). In addition, if this prediction is borne out, it should be possible to analyze the properties of the spores formed early in sporulation to identify the factors that result in their altered wet heat resistance. Consequently, we isolated the ~10% of spores of *Bacillus subtilis* that are released first from their mother cells during sporulation and compared the wet heat resistance and other properties of these spores to those of the spores formed in the entire sporulation process. We found that the spores released first were significantly less resistant to wet heat than the total spores formed in a population. However, we further found that the low wet heat resistance of the spores released first in sporulation could be raised to that of the total spores if the initial spores formed were allowed to mature for ~20 h in buffer with divalent cations.

MATERIALS AND METHODS

***Bacillus* strains used and preparation and purification of early, early-matured, and total spores.** The *B. subtilis* wild-type strain used was PS533 (29), a derivative of the laboratory 168 strain, which carries plasmid pUB110, which encodes resistance to kanamycin (10 μg/ml). All other strains used were isogenic with strain PS533 but lacked plasmid pUB110 and were (i) PS3328 (*cotE*) (21), which has the majority of the gene encoding the spore coat morphogenetic protein CotE replaced with a tetracycline resistance cassette; (ii) PS4150 (*cotE gerE*) (5), which has the *cotE* mutation from PS3328 and also has the majority of the coding sequence of the *gerE* gene replaced by a spectinomycin resistance cassette; and (iii) KB101 (*tgl*) (25), which has a chloramphenicol resistance cassette replacing the majority of the coding sequence of the *tgl* gene encoding the spore-associated transglutaminase.

Sporulation was at 37°C in liquid 2× Schaeffer's-glucose (SG) medium (60 ml in a 500-ml flask or 120 ml in a 1-liter flask) without antibiotics, and total spores were harvested after ~48 h and were purified as described previously (20). For isolation of the spores formed initially in sporulation, cultures were harvested when ~10% of spores had been released from sporangia as determined by phase-contrast microscopy. The sample (30 to 60 ml) was centrifuged (10 min, 8,000 × *g*) and washed twice with cold deionized water, and the pellet fraction was suspended in a small volume of deionized water and was then layered on 50% Nycodenz medium (Sigma Chemical Co., St. Louis, MO) at 1 volume of spores per 10 volumes of Nycodenz. The tubes were centrifuged at 20°C for 45 min at 13,000 × *g*. Under these conditions, free spores pelleted, while spores in sporangia as well as unsporulated cells remained at the top of the Nycodenz layer. The upper layer was removed, and the pelleted spores were washed 3 to 4 times with cold water and stored at 4°C. This fraction, termed early spores, consisted of almost exclusively free, phase-bright spores and was free (>98%) of sporulating cells or germinated spores, as observed by phase-contrast microscopy. The yield of these early spores was routinely 5 to 10% of the spores obtained after 48 h (termed total spores). In some experiments, the medium in the supernatant from the initial centrifugation used to isolate the early spores was saved (conditioned medium), filter sterilized, and used in subsequent experiments.

Early spores were matured by incubation at an optical density at 600 nm (OD_{600}) of 1 to 3 in 1 to 2 ml of the filter-sterilized conditioned medium or in other solutions and did not germinate during these incubations, as determined by phase-contrast microscopy. After incubation at 37°C for 16 to 24 h, the spores were isolated by centrifugation, washed several times with water at 4°C, and stored in 4°C water at an OD_{600} of 0.5 to 1. These spores were termed early-matured spores.

Analysis of acquisition of spore wet heat resistance during sporulation. For precise analysis of changes in spore wet heat resistance throughout sporulation, strain PS533 was sporulated as described above in a 500-ml flask. At various times, 1-ml samples were harvested by centrifugation and washed twice with 1 ml water by centrifugation, and the final pellet was stored frozen for later analysis of DPA. A second 1-ml sample was briefly sonicated to disperse aggregated sporulating cells and diluted 20-fold in water at either 23°C (to determine total numbers of viable cells/spores) or 90°C to determine wet heat resistance. During incubation at 90°C, aliquots were removed and diluted in 23°C water, and 10-μl aliquots of further serial dilutions in water were spotted on L-broth agar plates

containing kanamycin. The plates were incubated for 24 to 36 h at 37°C, after which no further colonies appeared and colonies were counted.

The 1-ml samples saved for DPA analysis were boiled for 15 min in 1 ml of water and centrifuged, and the supernatant was saved. DPA was determined in the supernatant by its fluorescence with 50 μM $TbCl_3$ in 25 mM HEPES buffer (pH 7.4), as described previously (20, 37).

Analytical procedures. The resistance of spores to lysozyme, wet heat, dry heat, shortwave UV radiation, and sodium hypochlorite was measured as described previously (6, 7, 20, 28, 38). Spore germination with various concentrations of either L-valine or a mixture of L-asparagine, D-glucose, D-fructose, and K^+ (AGFK; only the asparagine concentration was varied, while the concentration of each of the D-glucose, D-fructose, and K^+ components was held at 10 mM) was at 37°C, following heat activation of spores (OD_{600} = 1) in water for 30 min at 65°C and then cooling on ice. Germination was carried out in 25 mM HEPES buffer (made to pH 7.4 with KOH) plus 50 μM $TbCl_3$, and after germinant addition, germination was followed by measuring DPA release by the fluorescence of Tb-DPA in a multiwell plate reader as described previously (37). Levels of Ca in spore preparations were determined by inductively coupled plasma-mass spectrometry at the Dartmouth College Trace Elements Analysis Core in Hanover, NH (7). The Raman spectra of individual spores were determined by laser tweezers Raman spectroscopy (LTRS), and individual spores' DPA levels were determined from the intensity of the Raman band at 1,017 cm^{-1} (10). The core wet density of spores was measured by equilibrium density gradient centrifugation at 20°C on 57 to 76% Nycodenz gradients, and the spores' water content was determined from the density at which the spores banded in these gradients (23, 24).

RESULTS

Resistance properties of early spores and their maturation. To begin the analysis of the spores released first in sporulation, the first 10 to 15% of spores released during the sporulation of *B. subtilis* were purified, and their properties were compared with those of the total spores prepared in the same sporulation process. When the wet heat resistance of these spores was determined, the early spores were more sensitive to wet heat than were the total spores (Fig. 1). Examination of other resistance properties showed that the early spores were also more sensitive to sodium hypochlorite, although both early and total spores exhibited essentially identical resistance to dry heat and UV radiation (Fig. 2A to C).

Although early spores were significantly less wet heat resistant than total spores, it was possible that full spore wet heat resistance was not acquired before spores were first released from sporangia but required some period of maturation to develop fully. Indeed, when early spores were incubated for ~18 h in filtered medium from which these spores had been isolated, the wet heat resistance of these early-matured spores became essentially equal to that of the total spores (Fig. 1). The hypochlorite resistance of the early spores also increased after maturation, although the dry heat and UV resistance of the early-matured spores did not (Fig. 2A to C and data not shown). The maturation of the early spores in conditioned medium that led to full spore wet heat resistance took >6 h (Fig. 3).

While the initial attempts to mature the early spores used conditioned medium, it was of obvious interest to determine the specific requirements for this maturation process. The maturation process was not abolished if the conditioned medium was boiled but was abolished if EDTA was added to the conditioned medium (Fig. 4A). There was also little, if any, gain of wet heat resistance if the early spores were incubated in buffer alone (Fig. 4A and B). However, incubation in buffer plus

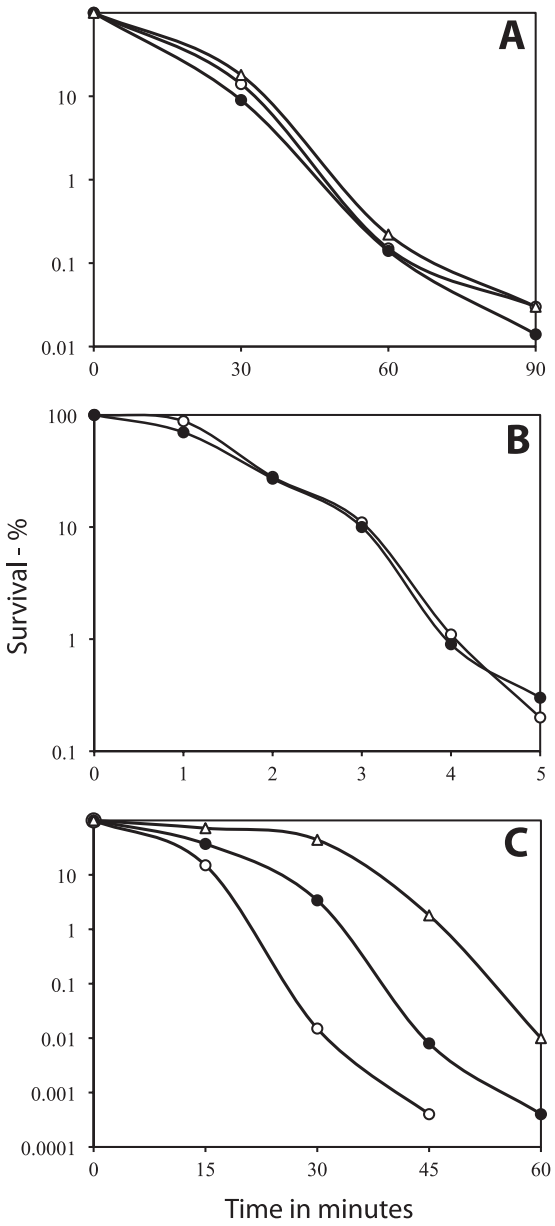

FIG. 2. Resistance to dry heat, short-wave UV radiation, and hypochlorite of early, early-matured, and total *B. subtilis* spores. Purified early, early-matured, and total *B. subtilis* PS533 spores were isolated, and their resistance to dry heat (120°C) (A), shortwave UV radiation at $3 \cdot 10^{-4}$ J/cm^2 · min (B), and sodium hypochlorite (0.25% in water at 23°C) (C) were determined as described in Materials and Methods. Symbols: ○, early spores; ●, early-matured spores; △, total spores.

$CaCl_2$ allowed full gain of wet heat resistance by the early spores, with $MgCl_2$ and $MnCl_2$ being less effective (Fig. 4B).

Other properties of early, early-matured, and total spores. Of the many factors that influence spore wet heat resistance, their core water content is probably the most important, with a lower core water content associated with higher spore resistance to wet heat (4). However, analysis of the water content of both early and total spores showed that, if anything, the early spores had a slightly lower core water content than the total spores (Table 1). Two other factors that can affect spore resis-

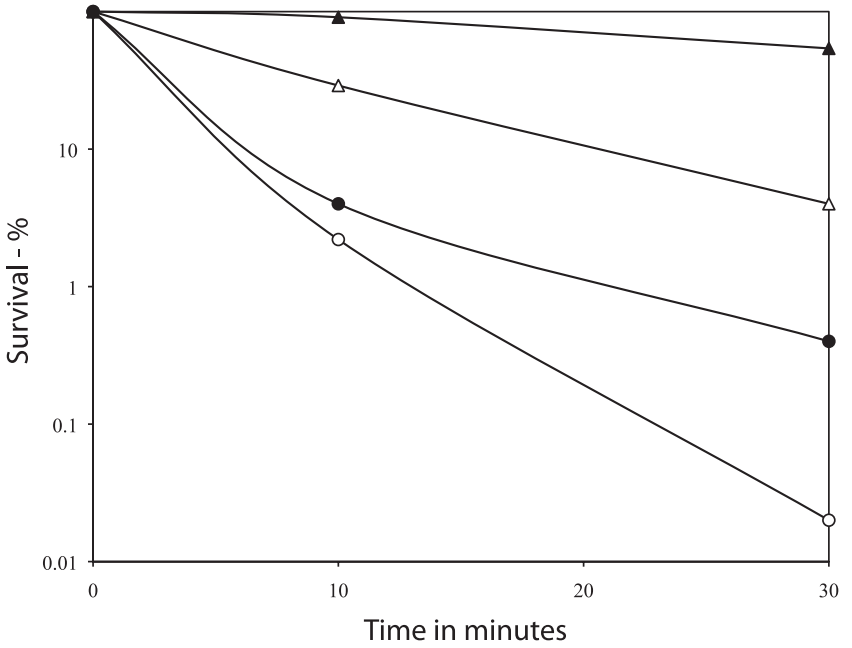

FIG. 3. Effects of maturation time on the increase in the wet heat resistance of early spores. Purified early spores of *B. subtilis* PS533 at an OD_{600} of 3 were incubated at 37°C in the conditioned medium from which they were isolated. At various times, samples (0.33 ml) were isolated and washed 3 times with 1 ml of 4°C water by centrifugation, the final pellet was suspended in 0.5 ml water, the OD_{600} was measured to quantify spore recovery, and the spores were stored at 4°C. The wet heat resistance of these spores was determined at 90°C with spores at an OD_{600} of 1 as described in Materials and Methods. The symbols used to designate the times of incubation of the early spores in the conditioned medium are as follows: ○, 0 h; ●, 3 h; △, 6 h; ▲, 24 h. The wet heat resistance of the spores after 24 h of incubation in the conditioned medium was almost identical to that of the total spores isolated after 48 h of sporulation, as noted in the legend to Fig. 1.

tance properties, including wet heat resistance, are the DPA level and the level of divalent cations, most of which are presumably chelated with the DPA (4). However, the levels of DPA in individual early, total, and early-matured spores were also very similar (Table 1). Levels of Ca, generally the most predominant cation chelated with DPA in spores (4), were also similar in early, total, and early-matured spores (Table 1). Indeed, Ca levels in these spores were quite similar to DPA levels on a mol/mol basis, as expected (Table 1 and data not shown).

Another important property of spores, in addition to their resistance, is their ability to resume metabolic activity in spore germination and outgrowth (30). To examine the germination properties of early and total spores, we determined the rates and extents of the germination of these spores with either L-valine, which triggers germination via the GerA germinant receptor, or a mixture of L-asparagine, D-glucose, D-fructose, and K^+ ions (AGFK), which triggers germination via the cooperative action of the GerB and GerK germinant receptors (Fig. 5A to D) (30). The total spores germinated well with high concentrations of either L-valine or AGFK, as expected, and the early spores also germinated well with these two types of germinants. In addition, examination of rates of germination with lower concentrations of L-valine or L-asparagine showed that both early and total spores germinated relatively similarly with various lower concentrations of these germinants as well (Fig. 5A to D).

Raman spectra of single early, early-matured, and total spores. To get further information on possible differences between early and total spores, Raman spectra of 40 individual early, early-matured, and total spores were obtained by LTRS and averaged (10) (Fig. 6). As expected, the spectra of these spores exhibited Ca-DPA-specific bands at 658, 824, 1,017, 1,395, 1,445, and 1,572 cm^{-1} (10, 11). However, there were some subtle differences between the spectra of early spores and those of early-matured and total spores. In particular, the ratios of the intensities of the Raman bands at 1,445 cm^{-1} and 1,395 cm^{-1} ($I_{1,445}/I_{1,395}$) were 1.15 ± 0.06 for early spores, 1.38 ± 0.10 for early-matured spores, and 1.47 ± 0.10 for total spores. The higher value of $I_{1,445}/I_{1,395}$ for early-matured and total spores could well reflect molecular differences between early spores and early-matured and total spores. The 1,395-cm^{-1} band has been assigned to the O—C—O symmetric stretch of Ca-DPA (10, 11), and the 1,445-cm^{-1} band has been attributed to both Ca-DPA and the C—H_2 deformation of proteins and lipids (17). It was previously observed that the Raman band in the 1,445- to 1,455-cm^{-1} region that is ascribed to the cortex, coat, and exosporium layers of individual *Bacillus cereus* spores is of higher intensity than the comparable band from the spore's core (11). Consequently, the higher value of the $I_{1,445}/I_{1,395}$ ratio in early-matured and total *B. subtilis* spores is consistent with some modification of the coat and/or cortex layers of early *B. subtilis* spores upon spore maturation. This is especially the case for the early-matured spores, which have the same Ca-DPA content as the early spores.

Effect of mutations affecting coat structure on wet heat resistance of early and total spores. The work noted above indicated that some spore properties, in particular, wet heat resistance, change significantly after spores are released from their sporangia and, further, that there may be changes in the

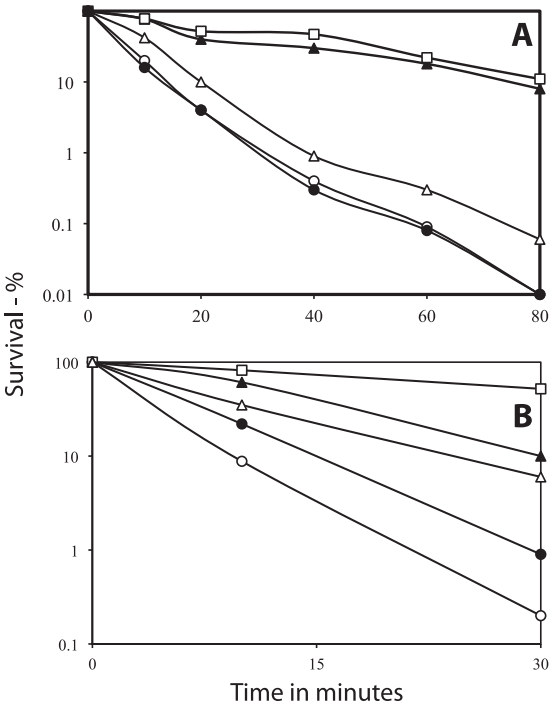

FIG. 4. Effects of various incubation conditions on the increase in the wet heat resistance of early spores during their maturation. Purified early spores of *B. subtilis* PS533 were isolated, and their wet heat resistance was determined with or without ~20 h of incubation at 37°C in various solutions as described in the legend to Fig. 3 and in Materials and Methods. The symbols denoting the various incubation conditions are as follows: (A) ○, no incubation; ●, incubation in conditioned medium plus 10 mM EDTA; △, incubation in 25 mM Tris-HCl (pH 8.0); ▲, incubation in conditioned medium that was boiled for 15 min and then cooled to 37°C; □, incubated in untreated conditioned medium; (B) ○, no incubation; ●, incubated in 25 mM Tris-HCl (pH 8.0); △, incubated in 25 mM Tris-HCl (pH 8.0) plus 2 mM $MgCl_2$; ▲, incubated in 25 mM Tris-HCl (pH 8.0) plus 2 mM $MnCl_2$; □, incubated in 25 mM Tris-HCl (pH 8.0) plus 2 mM $CaCl_2$.

spore coat/cortex layers during spore maturation. Indeed, it is known that at least some features of the spore coat can change significantly very late in sporulation and even after spore release from the sporangium, in particular, the degree of at least some coat protein cross-linking by a coat-associated transglutaminase and possibly other coat enzymes (8, 12, 14, 15, 18, 25, 35, 40). This finding as well as the role of at least some coat proteins or coat layers in spore resistance to wet heat (2, 5, 16, 35) suggested that it would be valuable to examine the role of some coat proteins in spore wet heat resistance as well as in the maturation of early spores. The coat proteins that were examined for these effects were (i) CotE, as *cotE* spores lack the outer coat as well as at least some inner coat proteins (8), and (ii) the coat-associated transglutaminase, Tgl, which is involved in the cross-linking of some coat proteins even after spores are released from sporangia (15, 25, 40). We also examined spores of a strain lacking both CotE and the sporulation-specific transcription factor GerE, as *cotE gerE* spores lack most of their coat layers and retain only a thin rind or shell of very insoluble protein on the outside of the spore (5). Analysis of the wet heat resistance of purified total *cotE*, *tgl*, and *cotE gerE* spores showed that, as found previously, *cotE* spores had wet heat resistance similar to that of wild-type spores and higher than that of *cotE gerE* spores (5) (Fig. 7A). The total *tgl* spores also had slightly lower wet heat resistance than total wild-type spores (Fig. 7A). Examination of the wet heat resistance of early *cotE*, *cotE gerE*, and *tgl* spores indicated that these early spores were also less wet heat resistant than the total spores, with the early *cotE* and *cotE gerE* spores being much more wet heat sensitive (Fig. 7B and data not shown). However, the total *cotE*, *cotE gerE*, and *tgl* spores had higher wet heat resistance than the early spores, suggesting, albeit not proving, that these early spores' wet heat resistance had increased significantly as sporulation proceeded (Fig. 7B).

Time of acquisition of full spore wet heat resistance during sporulation. The results given above suggested that full spore resistance to wet heat is acquired well after spores are initially released from their sporangia. This suggestion is counter to the generally held view that spore wet heat resistance is acquired in parallel with the spore's accumulation of DPA and associated divalent cations that is accompanied by the final lowering of the spore core's water content (25). However, the acquisition of wet heat resistance during sporulation has generally been measured by treating sporulating cells for ~30 min with temperatures of ~75°C. While incubation at this temperature will rapidly kill growing and sporulating cells, ~75°C is well below the temperature needed to give any significant killing of spores. Consequently, we isolated sporulating cells at various times during sporulation beginning when few if any spores had been released from sporangia; treated these samples at 90°C, a temperature that kills ~70 to 90% of total spores in 60 min (Fig. 1); and measured the survival in these samples (Fig. 8). The DPA level in the culture at the various times of harvest was also measured, and at the first sampling time it was ~50% of maximum and was close to maximal throughout the remainder of the incubation (Fig. 8). Most importantly, all spores in samples in the first three harvest times had lower-than-maximum wet heat resistance, even though maximum DPA levels were acquired by the third harvest time. Indeed, it was not until ~40 h later that maximum wet heat resistance was acquired, and this was well after the great majority of spores had been released from their sporangia (Fig. 8). These results, then, strongly indicate that spores' full wet heat resistance is not

TABLE 1. DPA levels and spore core wet density in early, early-matured, and total spores[a]

Spores analyzed	DPA level (amol/spore)	Ca level (mg/g dry wt)	Spore wet density (g/ml)
Early	260 ± 24	35[b]	1.351
Early-matured	254 ± 27[c]	41[d]	ND[e]
Total	216 ± 31	34[b]	1.348

[a] The DPA levels in 40 individual early, early-matured, or total spores of *B. subtilis* PS533 were determined by LTRS as described in Materials and Methods, and values were averaged and standard deviations were calculated. The spore core wet density of early and total spores and Ca levels in early, early-matured, and total spores were determined as described in Materials and Methods.

[b] These values were reduced ≤10% by washing the spores with 10 mM EDTA.

[c] Early spores were matured in conditioned medium for ~24 h at 37°C.

[d] Early spores were matured for ~24 h at 37°C in 25 mM Tris-HCl buffer (pH 8.0) plus 2 mM $CaCl_2$, and these spores were not washed with EDTA.

[e] ND, not determined.

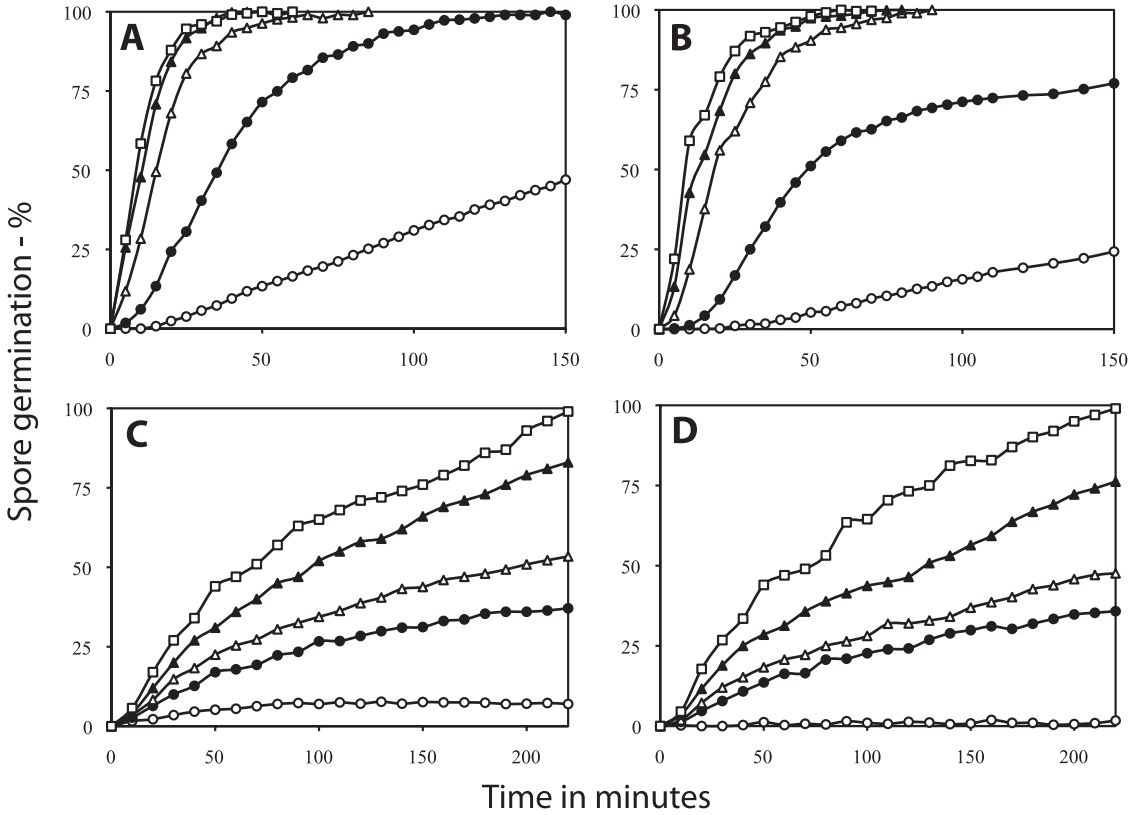

FIG. 5. Germination of early and total spores with valine and AGFK. Purified early (A and C) and total (B and D) spores of strain PS533 were germinated with various concentrations of either L-valine (A and B) or L-asparagine (C and D) plus 10 mM (each) D-glucose, D-fructose, and K^+, and spore germination was followed as described in Materials and Methods. The symbols denoting the concentrations of L-valine are as follows: □, 10 mM; ▲, 5 mM; △, 2 mM; ●, 0.7 mM; ○, 0.3 mM. The symbols denoting the L-asparagine concentrations used are as follows: □, 10 mM; ▲, 3 mM; △, 1 mM; ●, 0.5 mM; ○, no L-asparagine added.

acquired until well after spores have acquired maximum DPA levels and have been released from their sporangia.

It was also notable in this experiment that while spore wet heat resistance clearly increased well after the maximal DPA level was accumulated, the wet heat resistance of the spores at the early harvest times appeared to be significantly higher than that seen for purified early spores (compare Fig. 1 and 8). We have not studied the reasons for this difference, but two possible explanations are that (i) wet heat resistance during sporulation was measured in 50-fold-diluted sporulation medium, and this could certainly affect spore wet heat resistance (27), and (ii) at the first three harvest times, many of the spores were still in the mother cell even after the brief sonication treatment to disrupt clumps of cells, and the mother cell environment could also provide increased wet heat resistance to unreleased spores. In contrast, at the last three times of harvest, essentially all the spores in the culture were present as free spores after the brief sonication treatment (data not shown).

DISCUSSION

The results reported in this work indicated that spores of *B. subtilis* do not acquire their full wet heat resistance until they are released from the sporangium and well after DPA levels are maximal in spores and core water content is minimal. As a consequence, spores harvested just after their release from the sporangium have significantly lower wet heat resistance than spores harvested ~24 h later. There appear to be two main questions that arise from this observation: (i) what is the reason for the lower wet heat resistance of early spores, and (ii) what are the changes that take place whereby early spores mature and acquire full wet heat resistance. With regard to the first question, the current work has ruled out a number of possible explanations for the lower wet heat resistance of early spores, as levels of DPA, water content, and Ca^{2+}, all factors that influence the wet heat resistance of mature dormant spores (4), were essentially identical in both early and total spores. In addition, the levels of UV and dry heat resistance of early and total spores were also identical, and resistance to these agents, in particular, UV, is dependent on the spores' levels of α/β-type SASPs (4, 19, 31, 32), suggesting that the levels of these DNA-binding proteins are also similar in early and total spores. Indeed, α/β-type SASP synthesis in the developing spore ends well before full DPA accumulation (32). With these factors eliminated as contributing to the lower wet heat resistance of early spores, two possible causes remain. One is the amount and/or the cross-linking status of the spores' peptidoglycan cortex, another spore layer that has been suggested to play a role in spore wet heat resistance (23, 24). This is certainly a possibility, although there is no evidence that the cortex structure is modified significantly after spores are re-

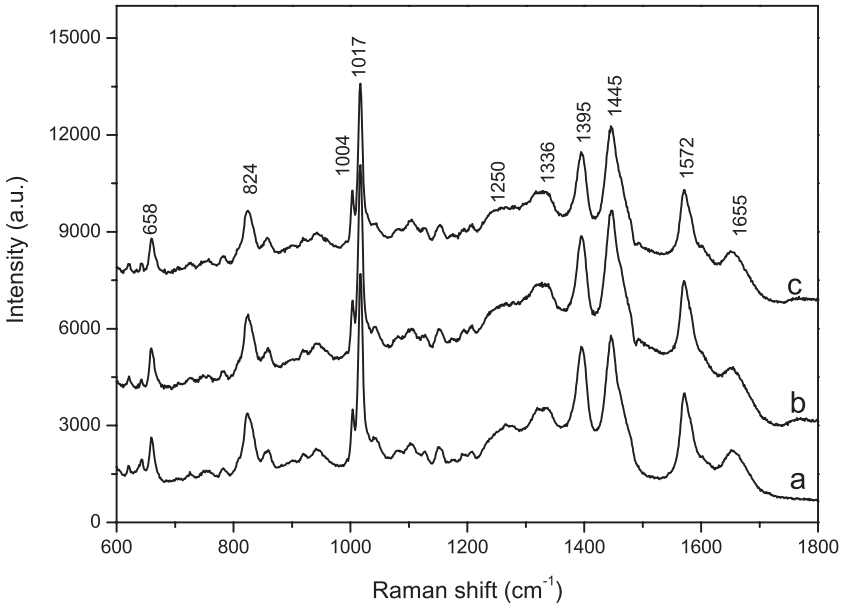

FIG. 6. Average Raman spectra of 40 individual early spores (curve a), early-matured spores (curve b), and total spores (curve c) of *B. subtilis* strain PS533. The various types of spores were isolated, and their Raman spectra were measured using LTRS with a laser power of 10 mW and an integration time of 30 s, as described in Materials and Methods. Early spores were matured in conditioned medium. The spectra from 40 individual spores of each type were averaged, and the ratios of the intensities of Raman bands at 1,395 and 1,445 cm^{-1} and the standard deviations were calculated.

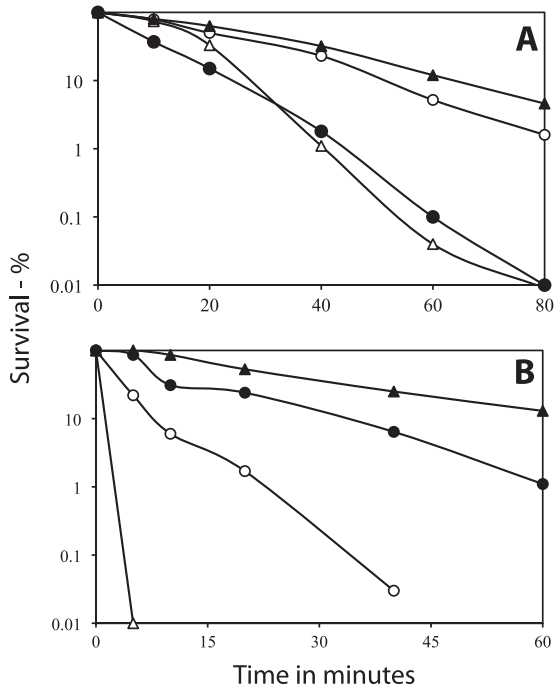

FIG. 7. Wet heat resistance of early and total spores of strains with spore coat defects. Purified early and total spores of strains with coat defects were isolated, spores were incubated at 90°C in water, and spore survival was measured as described in Materials and Methods. (A) Total spores of strains PS533 (wild-type) (○), PS3328 (*cotE*) (▲), PS4150 (*cotE gerE*) (△), and KB101 (*tgl*) (●) were examined; (B) early spores (○ and △) and total spores (● and ▲) of strains PS3328 (*cotE*) (△ and ▲) and KB101 (*tgl*) (○, ●) were examined. Early *cotE gerE* spores had wet heat resistance similar to that of early *cotE* spores (data not shown).

leased from the sporangium. The second possibility is the spore coat, which has also been shown to contribute to spore wet heat resistance, as at least some coat-defective spores, including *cotE gerE* spores (5), have decreased wet heat resistance. In addition, a number of modifications in spore coat structure have been shown to take place following spores' release from the sporangium, including increased cross-linking of at least several coat proteins by Tgl (14, 15, 25, 40). This Tgl-dependent cross-linking alone was not essential for the maturation of early spores, but there are other events that take place in free spores, including proteolytic modification of at least one coat protein and almost certainly protein cross-linking due to the actions of additional enzymes (8, 14, 15, 35, 40). Modification of the coat certainly takes place after spores are released from the sporangia, and given that the intact mature coat is essential for full spore wet heat resistance, it seems reasonable to propose that it is molecular changes in the spore coat that result in increased spore wet heat resistance. Further pieces of evidence that it is the coat whose modification results in spore maturation following spore release from the sporangium are (i) the changes in spores' Raman spectrum of bands due at least in part to protein and likely spore coat protein upon early spore maturation and (ii) the fact that the hypochlorite resistance of early spores was lower than that of total spores, since the spore coat is the major factor in spore resistance to hypochlorite (38). Hypochlorite resistance also increased during spore maturation, although not fully to the resistance of the total spores. Unfortunately, how the spore coat provides hypochlorite resistance is not clear, nor is how the spore coat contributes to spore wet heat resistance.

In contrast to the results noted above suggesting that changes in the spore coat cause the increased spore wet heat

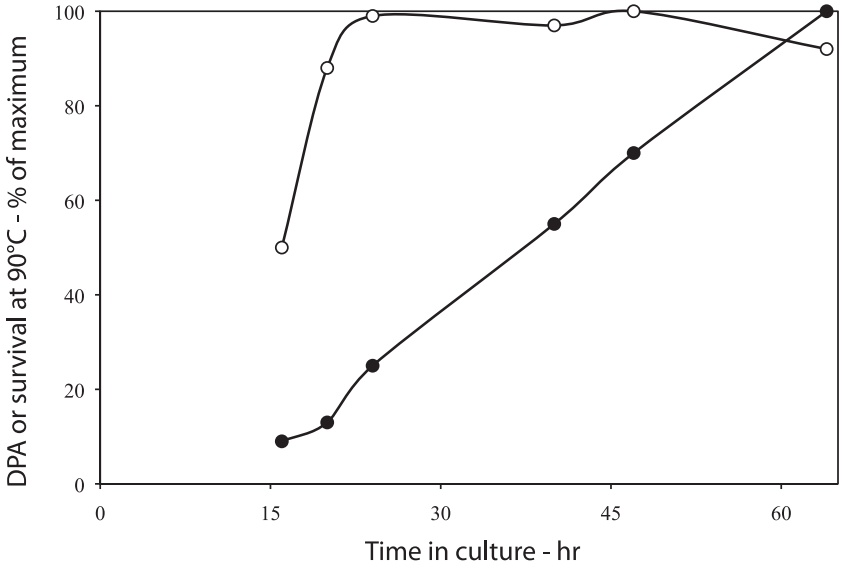

FIG. 8. Acquisition of full spore wet heat resistance during sporulation. Strain PS533 was sporulated, and DPA levels and wet heat resistance at 90°C were determined at various times in sporulation as described in Materials and Methods. DPA levels are expressed as the percentage of the maximum amount accumulated, and wet heat resistance is expressed as the percentage of maximum survival after 60 min at 90°C, with the maximum survival seen set at 100%, although the actual survival at 64 h was 23%. Time zero is the time when the culture was inoculated. The approximate percentages of spores that had been released from sporangia at various times were <1% at 16 h, 10% at 20 h, 30% at 24 h, and >70% at 40 h, as determined by examination by phase-contrast microscopy. Symbols: ●, wet heat resistance; ○, DPA level.

resistance upon early spore maturation, it is notable that *cotE* and *cotE gerE* early spores also appeared to exhibit a large increase in their wet heat resistance as sporulation proceeded. Since *cotE gerE* spores in particular lack most coat protein (5), this suggests that it may not be modification of the coat that results in increased spore wet heat resistance as sporulation proceeds but rather some modification of the spore cortex. However, this is by no means certain, since even *cotE gerE* spores retain a significant amount of coat protein, and much of the coat protein of *cotE gerE* spores seems likely to be highly cross-linked (5), although the timing and complete identity of these cross-links have not been determined.

The second major question is precisely what reactions are involved in the maturation of early spores that lead to increased wet heat resistance? Since well-washed early spores acquired full wet heat resistance when incubated in buffer plus Ca^{2+}, it is clear that factors needed for the maturation of early spores are associated with the spores themselves and are not some component from the mother cell that is released into the sporulation medium. As noted above, one type of reaction that may contribute to spore maturation is modification of spore coat structure, and at least some of the modifications in coat structure are catalyzed by spore-associated enzymes such as the YabG protease and Tgl. However, at least Tgl-catalyzed reactions are not essential for spore maturation, since (i) early *tgl* spores did acquire increased wet heat resistance when matured, and (ii) purified Tgl does not require Ca^{2+} for its activity (22). It appears to be likely that YabG activity also does not require divalent cations (35). Thus, either some additional coat maturation process is needed in order to acquire full spore wet heat resistance or further modification of spore cortex structure by a Ca^{2+}-dependent enzyme is needed for acquisition of full spore wet heat resistance following spore release from sporangia.

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