Analysis of the Loss in Heat and Acid resistance During Germination of Spores of *Bacillus* Species

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Abstract

A major event in the nutrient germination of spores of *Bacillus* species is release of spores’ large depot of dipicolinic acid (DPA). This event is preceded by both commitment, in which spores continue through germination even if germinants are removed, and loss of spore heat resistance. The latter event is puzzling, since spore heat resistance is due largely to core water content which does not change until DPA is released in germination. We now find that for spores of two *Bacillus* species the early loss in heat resistance during germination is most likely due to release of committed spores’ DPA at temperatures not lethal for dormant spores. Loss in spore acid resistance during germination also paralleled commitment, and was also associated with release of DPA from committed spores at acid concentrations not lethal for dormant spores. These observations plus previous findings that DPA release in germination is preceded by significant release of spore core cations suggest there is a significant change in spore inner membrane permeability at commitment. Presumably this altered membrane cannot retain DPA during heat or acid treatments innocuous for dormant spores, resulting in DPA-less spores that are rapidly killed.
Introduction

Spores of *Bacillus* species are metabolically dormant and extremely resistant to a variety of environmental stresses, most notably wet heat (1,2). For example, spores of *Bacillus subtilis* water can survive for many hr in water at 80°C, a temperature that rapidly kills growing cells of this species (3). There are a number of factors that contribute to spore resistance to wet heat; a major one is the low water content in the spores’ central core, which can be as low as 25% of wet weight for spores suspended in water (2,4). There are several reasons for the low spore core water content, including the: i) restriction of water uptake into the spore core by the large peptidoglycan (PG) cortex that surrounds the spore inner membrane (IM) and core; and ii) uptake late in spore formation of a huge amount (~ 20% of core dry weight) of pyridine-2,6-dicarboxylic acid (dipicolinic acid (DPA)) into the core, where the DPA is present as a 1:1 chelate with divalent cations, predominantly Ca\(^{2+}\) (CaDPA); this CaDPA displaces significant amounts of water from the maturing spore core. *B. subtilis* spores as well as *Clostridium perfringens* spores that lack CaDPA have higher core water content and are killed rapidly at temperatures that give minimal if any killing of CaDPA-replete spores (5,6).

Spores of *Bacillus* and *Clostridium* species are significant agents of food spoilage and food borne disease in large part because of their extreme resistance, and wet heat is probably the most frequently used agent to eliminate spores from foods. As a consequence, there is much interest in the mechanisms of spore resistance to wet heat, as well as methods to reduce or eliminate spores’ extreme resistance properties. While *Bacillus* spores can survive for long periods in their dormant, resistant state, if given the proper signal, generally one or more specific nutrients, spores will initiate the process of germination in which spores’ resistance and dormancy are lost (1,7). In particular, a major early germination event is the rapid release of
spores’ CaDPA that results in a significant rise in core water content. This is followed by hydrolysis of the PG cortex by either of two redundant cortex lytic enzymes (CLEs), CwlJ and SleB, allowing the core to swell and take up more water, raising the core water content to ~ 80% of wet weight and allowing enzymatic reactions in the core to begin. Cortex PG hydrolysis is not essential for CaDPA release during spore germination, but can considerably accelerate the rate of CaDPA release from individual spores of several Bacillus species (7-9).

While a dramatic event in Bacillus spore germination, CaDPA release is preceded by loss of resistance to heat and Hg\(^{2+}\), release of Na\(^+\), K\(^+\), H\(^+\) and Zn\(^{2+}\), and a significant change in the elastic light scattering intensity from individual spores (10-14). The phenomenon of commitment, whereby spores continue through germination even if a germinant is removed or dissociated from spores, also precedes CaDPA release (15-19). The occurrence of all these events well before CaDPA release during spore germination suggests that they are due to one fundamental change that commits a spore to germinate. For example, if there is an increase in the permeability of the spores’ IM associated with or the cause of commitment, this would explain the release of monovalent cations which would almost certainly be more soluble in spores than is the spores’ huge CaDPA depot, the great majority of which is most likely insoluble (20,21). This putative IM permeability change might also explain the increase in spores’ Hg\(^{2+}\) sensitivity well before CaDPA release.

The loss in spore wet heat resistance well before CaDPA release in germination, however, has been much more difficult to understand, since spores’ core water content does not change during germination until CaDPA is released. However, if there is a major change in the spore’s IM upon commitment, perhaps the IM of committed spores is much less able to retain its integrity at a high temperature that has a minimal effect on dormant spores. As a consequence
CaDPA is released from committed spores at this high temperature, and when this happens the core water content rises and the committed and now DPA-less spores become heat sensitive and are rapidly killed (5,22). Indeed, oxidative damage to the dormant spore’s IM makes this membrane much less able to retain CaDPA at an elevated temperature that has minimal effects on dormant spores, and also increases the IM’s permeability (23-25). To test whether spore IM strength decreases markedly prior to CaDPA release in germination and to gain a better understanding of factors that influence spore resistance properties, we have examined the kinetics of: i) commitment; ii) loss of spore resistance to heat as well as acid; iii) loss of DPA at elevated temperatures or acid concentrations that do not result in DPA release from dormant spores; and iv) the normal release of CaDPA, all during spore germination by nutrients. This work was carried out with wild-type *Bacillus megaterium* and *B. subtilis* spores and with isogenic mutant spores that lack the CLE CwlJ, as CaDPA release during germination of individual *cwlJ* spores is much slower than with wild-type spores (8,9,14).
Materials and Methods

**Bacillus strains used and spore preparation and purification.** The *B. subtilis* strains used were: i) PS533 (26) that is isogenic with prototrophic 168 laboratory strain PS832, but also carries plasmid pUB110 encoding kanamycin resistance (10 μg/ml); ii) FB111 (27) that is isogenic with PS832 and has a deletion replacement mutation in the *cwlJ* gene in which most *cwlJ* coding sequence is replaced by a tetracycline resistance (10 μg/ml) cassette; iii) FB113 (27) that is isogenic with FB111 and has a deletion replacement mutation in the *sleB* gene in which most *sleB* coding sequence is replaced by a spectinomycin resistance (100 μg/ml) cassette; and iv) PS2307 (28) that is isogenic with PS832 and carries a deletion replacement mutation in the *cwlD* gene in which most *cwlD* coding sequence is replaced by a chloramphenicol resistance (5 μg/ml) cassette; CwlD encodes the enzyme essential for generating muramic-δ-lactam in spore cortex PG that is the recognition determinant for CLEs (7,29). The *B. megaterium* strains used were: i) QM B1551, a wild-type strain originally obtained from H.S. Levinson; and ii) PS4164 (8), a derivative of strain QM B1551 that carries a deletion replacement mutation in the *cwlJ* gene in which most *cwlJ* coding sequence is replaced by a spectinomycin resistance cassette.

Spores of *B. subtilis* strains were prepared at 37°C on 2x Schaeffer’s-glucose plates without antibiotics as described previously (5,30). After 2-3 d at 37°C, plates were held at 23°C for 2-3 d and then spores were scraped from plates, purified as previously described, and stored in water at 4°C protected from light. *B. megaterium* spores were prepared at 30°C in liquid supplemented nutrient broth medium with vigorous shaking to provide good aeration (8). After ~ 48 hr, cultures were harvested by centrifugation, washed extensively with water, purified as described previously (8) and stored as described above. All spores used in this work were free (>
98%) of growing or sporulating cells, germinated spores and cell debris as seen by phase contrast microscopy.

**Spore germination.** Spores were routinely germinated after a heat shock in water, 30 min at 75°C for *B. subtilis* spores and 15 min at 60°C for *B. megaterium* spores, followed by cooling on ice. Unless noted otherwise, *B. subtilis* spores were germinated at 37°C and an optical density at 600 nm (OD_{600}) of 0.5 in 25 mM K-Hepes buffer (pH 7.4) with 10 mM L-valine as the germinant, and *B. megaterium* spores were germinated at 23°C and an OD_{600} of 0.5 in 25 mM K-Hepes buffer (pH 7.4) with 1 mM glucose as the germinant. The low temperature and glucose concentration were used for *B. megaterium* spores to slow germination sufficiently to allow for easier analysis of germination events.

Unless noted otherwise, spore germination was measured by monitoring DPA release by the fluorescence of released DPA with TbCl\textsubscript{3} (50 μM) in germination incubations as described above, and in a multi-well fluorescence plate reader as described previously (15). In most experiments Tb-DPA fluorescence was read directly, but in some experiments spores were germinated at an OD_{600} of 5 and aliquots were diluted 10-fold to final concentrations of 50 μM TbCl\textsubscript{3} and 25 mM K-Hepes buffer (pH 7.4) for fluorescence measurements.

**Measurement of commitment, heat resistance, acid resistance and heat- or acid-induced DPA release.** For measurement of commitment, at various times 200 μl aliquots of spores germinating at an OD_{600} of 0.5 as described above were made 10 mM in D-alanine (*B. subtilis* spores) or 170 mM in acetic acid (*B. megaterium* spores) to halt further commitment to germinate while allowing committed spores to continue germination events, as shown previously and in the current work (15; and data not shown). The percentage of spores that had committed to germinate at time t in germination was defined as the percentage of total DPA in the spore
population that was released when D-alanine or acetic acid was added at time t and incubations were continued until DPA release had stopped (see Fig. 1A). Germination at time t was defined as the percentage of total DPA released at time t (15). Note that control experiments have shown that when these concentrations of D-alanine or acetic acid are added to spores together with nutrient germinants, there is no subsequent DPA release (15; and data not shown).

For measurement of the loss in wet heat resistance during spore germination, spores were germinated at an OD$_{600}$ of 0.5 in 25 mM K-Hepes buffer (pH 7.4) plus 50 μM TbCl$_3$, and the release of CaDPA was monitored fluorometrically as described above. At various times, aliquots of the germinating culture were diluted 1/100 in sterile water at either 80°C (B. subtilis) or 75°C (B. megaterium), incubated for 30 min (B. subtilis) or 20 min (B. megaterium) and then cooled and diluted further. For analysis of the loss in spore acid resistance, at various times aliquots of the germinating culture were diluted 1/10 in 220 mM HCl at 23°C, incubated for 30 min, diluted 1/100 in L-broth and then further in water. Aliquots (10 μl) of various dilutions were then spotted on Luria (L) broth plates (5) with appropriate antibiotics and the plates were incubated at 30 or 37°C until no more colonies appeared (generally ≤ 36 hr). Control analyses showed that there was no killing of the starting dormant spores by these heat or acid treatments, that samples heat treated prior to germinant addition or immediately upon germinant addition gave the same number of colonies, and there was ≤ 20% killing of the starting dormant spores by the heat or acid treatments when these were carried out for 1 hr (data not shown).

For analysis of DPA release from germinating spores given heat or acid treatments that are not lethal for dormant spores, spores were germinated at an OD$_{600}$ of 5 in 25 mM K-Hepes buffer (pH 7.4) with either 10 mM L-valine or 1 mM D-glucose as described above. For measurement of DPA release without a heat or acid treatment, at various times 30 μl aliquots of
germinating cultures were added to 270 μl of ice cold 25 mM K-Hepes buffer (pH 7.4) - 55 μM TbCl₃ (termed dilution mix) that was immediately frozen in dry ice-ethanol and kept frozen until later analysis. For measurement of heat-induced DPA release from germinating spores, 30 μl of the germinating culture was added to 270 μl of dilution mix at 75°C (B. subtilis) or 70°C (B. megaterium), held for 30 min (B. subtilis) or 20 min (B. megaterium) at these temperatures and then frozen in dry ice-ethanol. The fluorescence of frozen samples was read immediately after each sample was thawed. For measurement of acid-induced DPA release from germinating spores, 45 μl of germinating culture was mixed with 5 μl of 2 M HCl, the mixture incubated for 30 min at 23°C, 22 μl mixed with 168 μl of 30 mM K-Hepes (pH 7.4) plus 24 mM freshly prepared NaOH, 10 μl of 1 mM TbCl₃ was added and the Tb-DPA fluorescence was read immediately. Control experiments showed that there was no significant DPA release from dormant spores of these species incubated for 30 min at the temperatures or acid concentrations used in these experiments (data not shown).
Commitment and loss of heat resistance and acid resistance in B. subtilis spore germination. Previous work has found that one of the earliest events in spore germination that can be easily measured is commitment, which precedes CaDPA release (15-19). For wild-type B. subtilis spores commitment precedes DPA release by 8-10 min (Fig. 1A), although this difference is smaller with spores of other species, including Bacillus cereus and B. megaterium (15; and see below). Since we were interested in analyzing other germination events, in particular loss of resistance properties, that appear to take place prior to DPA release, it would be helpful if times of spore commitment and DPA release were as far apart as possible. One way to greatly increase the time between these two events is by deleting the CLE CwlJ, as cwlJ spores of several Bacillus species exhibit decreased rates of DPA release in germination, both as individual spores and as populations (8,9). More importantly, times for commitment of cwlJ B. subtilis spores are similar to those of wild-type spores, as shown previously and in current work (15) (Fig. 1A). Thus we used wild-type and cwlJ spores in germination experiments examining events prior to DPA release.

In addition to commitment, another event prior to DPA release in B. megaterium spore germination is loss of heat resistance, and loss of spore heat resistance is closely associated with commitment (10,18). Analysis of wild-type and cwlJ B. subtilis spores also showed that loss of spore wet heat resistance was closely associated with commitment, and with cwlJ spores, loss of wet heat resistance preceded DPA release by 20-50 min (Fig. 1B,C).

In addition to their resistance to wet heat, dormant spores are also very resistant to acid, as even 1 M HCl kills B. subtilis spores only slowly at 23°C (31). Since release of monovalent ions, including H⁺, is reported to precede CaDPA release during germination of B. megaterium spores...
(16,18), it seemed possible that spores might also become sensitive to external H⁺ early in germination. Consequently we examined the loss of acid resistance during germination of wild-type and cwlJ B. subtilis spores (Fig. 2). Strikingly, loss of these spores’ resistance to an HCl concentration that gave no killing of dormant spores took place very early in spore germination, certainly well before DPA release by wild-type spores and much earlier than DPA release from cwlJ spores, just as seen for loss of DPA following heat treatment of germinating spores.

**Release of DPA during B. subtilis spore germination by heat or acid treatment.** The loss of spore wet heat resistance well before DPA release during spore germination has been puzzling, since spores that retain CaDPA would be expected to retain their low core water content and thus retain full wet heat resistance (11,13). However, a possible reason for the early loss in spores’ wet heat resistance in germination is that DPA is actually released during the heat treatment to measure spore wet heat resistance. This release would generate a DPA-less spore that would then be rapidly killed at the temperatures used for assessing spore wet heat resistance (5,22).

To determine whether the possibility noted above is in fact the case, wild-type and cwlJ B. subtilis spores were germinated and aliquots were analyzed directly at various times for DPA release. DPA release was also measured when samples taken at the same times were incubated at a temperature below levels that are rapidly lethal for dormant spores but that have been shown to release DPA from spores that have been damaged in various ways (23-25). The results of this experiment were striking, as amounts of DPA released from samples taken at various times and measured directly were much lower than when these same samples were given a heat treatment that is not lethal for dormant spores (Fig. 3A). The times between the curves for total DPA released normally in germination as well as after the heat treatment were also similar to the differences seen between commitment and DPA release, in particular for cwlJ spores (compare...
The differences between curves for normal DPA release as well as after the heat treatment during germination of \textit{cwlJ} spores were also almost identical to those seen with germinating FB113 spores which lack both redundant CLEs either of which can hydrolyze cortex PG sufficiently to complete spore germination (data not shown) (7,27). A similar difference between curves for normal DPA release and DPA release after heat treatment was also seen with spores of strain PS2307 that lack the protein CwlD that forms the muramic-δ-lactam in cortex PG that is the recognition determinant for spore enzymes that specifically cleave cortex PG (data not shown) (7,32,33). Note that the germination conditions in the experiments to measure DPA release with and without a heat treatment were different than those used when commitment and loss of heat resistance during germination were measured, in particular that the spore concentration in the experiments measuring DPA release after a heat treatment was 10-fold higher than when commitment and heat or acid resistance were measured.  

The finding that spores released DPA upon a normally sublethal heat treatment essentially in parallel with commitment and that the committed spores appeared to be acid-sensitive suggested that the committed spores might also release DPA during an acid treatment not lethal for dormant spores. This was again the case, as with both wild-type and \textit{cwlJ} \textit{B. subtilis} spores DPA release with an additional acid treatment during spore germination was significantly earlier than normal DPA release (Fig. 3B). The differences between the acid-induced DPA release and normal DPA release curves were also similar to those for commitment and normal DPA release (compare Fig. 1A, 3B). The large difference between curves for normal DPA release during germination of \textit{cwlJ} \textit{B. subtilis} spores and DPA release after an acid treatment of these germinating spores was also seen during germination of PS2307 spores that lack muramic-δ-lactam in cortex PG (data not shown).
Commitment and DPA release following heat or acid treatment during *B. megaterium* spore germination. The results with *B. subtilis* spores showing that commitment, loss of spore heat resistance, acid resistance and DPA release upon a heat or acid treatment all took place with relatively similar kinetics were striking. Since these results suggested that there is a causal relationship between the latter events, it seemed worthwhile to learn if the same temporal relationship is seen during germination of spores of another *Bacillus* species. As noted above, loss of *B. megaterium* spore heat resistance has been shown to take place largely in parallel with commitment (10,13), so the spores of this species seemed ideal to examine.

*B. megaterium* spores germinate much faster than *B. subtilis* spores, but commitment still precedes DPA release from germinating wild-type spores by 2-5 min (Fig. 4). However, with *cwlJ* *B. megaterium* spores the time between commitment and DPA release was significantly larger (Fig. 4), as expected. Loss of *B. megaterium* spores’ resistance to acid and wet heat during germination also preceded normal DPA loss significantly, especially so for *cwlJ* spores (Fig. 5; and data not shown), as found previously for wet heat resistance (10,13). As found with *B. subtilis* spores, normal DPA release from germinating *B. megaterium* spores not given a heat or acid treatment was also significantly slower than if the germinating spores were given heat or acid treatments that are not lethal for dormant spores (Fig. 6; and data not shown). The separations between the curves for normal DPA release and heat-induced DPA release from germinating wild-type and *cwlJ* spores were also comparable to the separations between curves for commitment and normal DPA release during spore germination (compare Fig. 4, 6), and this was also the case when normal DPA release and acid-induced release of DPA from germinating wild-type and *cwlJ* *B. megaterium* spores were compared (data not shown).
Discussion

The work in this communication leads to a number of conclusions. First, lost of heat resistance precedes CaDPA release during \textit{B. megaterium} and \textit{B. subtilis} spore germination, and essentially in parallel with commitment. This was reported previously for spores of \textit{B. megaterium} (10,18), but has not been previously observed for \textit{B. subtilis} spores. Second, spore acid resistance is also lost essentially in parallel with commitment for spores of both \textit{B. megaterium} and \textit{B. subtilis}, and this has not been previously reported for spores of any \textit{Bacillus} species.

The third, and probably the major conclusion from this work is that there appears to be a simple explanation for the puzzling observations made years ago (10,13) that loss of heat resistance precedes CaDPA release during spore germination. Thus the current work indicates that the ability to retain DPA by germinating spores given a heat treatment that neither kills nor releases DPA from dormant spores is lost well before CaDPA release during germination and largely in parallel with commitment. Consequently, it is the DPA-less spores generated by the heat treatment to measure the loss in spore heat resistance during germination that are actually being killed. The fourth conclusion is that the explanation given above for wet heat resistance may also explain the loss in spore acid resistance well before loss of CaDPA during spore germination, as acid treatments that neither kill nor release DPA from dormant spores also released DPA from germinating spores well before normal CaDPA release. In addition, at least DPA-less \textit{C. perfringens} spores have greatly decreased resistance to HCl (6).

The final conclusion is based on both our new observations and previously published work including the following. 1) Loss of spore heat resistance largely parallels commitment during germination, and the kinetics of commitment are approximately paralleled by: i) loss of spore acid resistance; ii) sensitization of spores to release of DPA during heat or acid treatments that
have no effects on dormant spores; and iii) none of the latter changes require cleavage of cortex PG. 2) Previous work has shown that release of much monovalent cations, almost certainly from the spore core, precedes CaDPA release during germination of spores of several *Bacillus* species (16,18). There is also release of much spore Zn$^{2+}$ prior to CaDPA release in *B. megaterium* spore germination (17), but it is not clear that all this Zn$^{2+}$ is released from the spore core. 3) There is a significant increase in individual spores’ elastic light scattering intensity well prior to CaDPA release during germination (19). Although the reason for this latter change is not known, it does indicate that there is some physical change in spores prior to CaDPA release, and this has been suggested to involve a change in spore size or refractive index and could also be due to changes in the IM. In thinking about the spores’ IM it is important to note that lipids in the dormant spore IM are generally immobile, and that the IM has an extremely low passive permeability not only to charged small molecules, but also to neutral small molecules including water (16,34-36). These novel properties of the spore IM are lost at least when spores complete germination (16,34), although the precise kinetics of these changes have not been examined.

Given all these observations, we propose that the key change that ties together all events prior to rapid CaDPA release in spore germination is a change in the permeability of the spores’ IM, much as was suggested ~35 years ago (37). Precisely how this change is brought about and what this change entails are not clear, but for nutrient germination this could involve the spores’ nutrient germinant receptors (GRs) that are located in the IM (7), since the time to establish commitment in nutrient germination is dependent on spores’ GR levels (15). However, it seems unlikely that it is the GRs themselves that allow passage of small molecules including CaDPA across the IM. Rather it is more likely that the movement of spore core small molecules across the IM is via a channel formed by the multiple SpoVA proteins (6,7). These proteins appear to be
distributed throughout the IM and are essential for CaDPA movement both into the developing spore during sporulation and out of the spore during spore germination. It is possible that small molecules other than CaDPA could also exit spores via SpoVA protein channels, although it is clear that these channels have some selectivity (7,38,39). In our model, when the increase in the IM’s permeability associated with commitment takes place we propose that this is caused by at least the partial opening of the SpoVA channels such that the very soluble monovalent cations along with as yet unknown anions can rapidly leave the spore core, while the largely insoluble CaDPA takes much longer to be released (40). In addition, the partial opening of SpoVA channels presumably results in a reduced ability of the IM to retain CaDPA upon a normally innocuous heat or acid treatment, much as is the case with oxidatively damaged dormant spores in which there is also a large increase in IM permeability (23-25). The early changes in apparent IM permeability and strength during spore germination could also be due to overall changes in the state of the spores’ IM, not just in the SpoVA channels. However, this seems less likely, since there is no observable change in the state of spore IM bulk lipids during spore germination until after CaDPA has been released and the spore cortex has been degraded (34).

While the model given above is just that, a model, it is consistent with the changes that take place prior to rapid CaDPA release during spore germination. There are also several additional points to consider concerning this model as follows. 1) With spores of many B. subtilis strains very slow CaDPA release is seen from individual germinating spores prior to very rapid release of CaDPA in 2-3 min (8,41). Presumably, this slow CaDPA release reflects the slow solubilization of CaDPA and its release through at least some opened SpoVA channels, while CaDPA release is then accelerated tremendously in individual spores by a mechanism that is not clear. 2) During spore germination, normal CaDPA release (8,9,14) but not DPA release
upon a heat or acid treatment, is much slower in the absence of the CLE CwlJ. The complete
explanation for this phenomenon is not known. However, CaDPA released from an individual
spore is known to activate this spore’s CLE CwlJ, perhaps directly, and this will most likely
increase the rate of cortex PG hydrolysis that will in turn further increase the rate of CaDPA
release (8,9,27). 3) B. subtilis spores treated with a high pressure (HP) of 150 megaPascals that
triggers germination via GRs also shows commitment similar to that seen with nutrient
germinants (42), and while it is not known if these HP-committed spores become moderately
heat sensitive, this certainly seems possible.

Finally, if the model noted above for the early events in spore germination is indeed
correct, then it will be crucial to understand what is happening at a physical level that results in
the IM changes that lead to events prior to CaDPA release in germination. An ultimate complete
understanding of this phenomenon might also suggest ways to trigger this same change
artificially, and thus artificially trigger spore germination to promote relatively easy spore
killing.

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(10), triggered the thinking that led to this work.
References


Figures

Fig. 1A-C.
Fig. 1A-C. Kinetics of germination, commitment and loss of heat resistance during *B. subtilis* spore germination. 

A) Spores of *B. subtilis* strains PS533 (wild-type) (○, ●) and FB111 (cwlJ) (△, ▲) were germinated at an OD<sub>600</sub> of 0.5, and commitment (●, ▲) and germination (○, △) were determined as described in Methods. 

B,C) PS533 (wild-type) (B) and FB111 (cwlJ) (C) spores were germinated and commitment (●), germination (○) and loss of heat resistance (△) were measured as described in Methods. The values shown in the figure are averages of results from two (A) or three (B,C) replicate experiments, and there was ≤ 10% variation between replicates in values for commitment and germination, and ≤ 20% variation between replicates in values for heat resistant spores.
Fig. 2. Kinetics of DPA release and loss of acid resistance during *B. subtilis* spore germination.

Spores of *B. subtilis* strains PS533 (wild-type) (○, ●) and FB111 (cwlJ) (△, ▲) were germinated at an OD$_{600}$ of 0.5, and germination (○, △) and loss of acid resistance (●, ▲) were determined as described in Methods. The values shown in the figure are averages of results from three replicate experiments, and there was ≤ 10% variation between replicates in values for germination, and ≤ 20% variation between replicate values for acid resistant spores.
Fig. 3A,B. Kinetics of DPA release during germination of *B. subtilis* spores with and without a heat (A) or acid (B) treatment. Spores of *B. subtilis* strains PS533 (wild-type) (○, ●) and FB111 (*cwlJ*) (△, ▲) were germinated at an OD$_{600}$ of 5, and DPA release was measured at
various times either without (○, △) or with (●, ▲) (A) a heat treatment at 75°C for 30 min or
(B) an acid-treatment for 30 min at 23°C as described in Methods. Values shown are averages of
results from two independent replicate experiments, and values in replicates differed by ≤ 15%.
Fig. 4. Kinetics of *B. megaterium* spore germination and commitment. Spores of *B. megaterium* strains QM B1551 (wild-type) (○, ●) and PS4164 (cwI) (△, ▲) were germinated at an OD$_{600}$ of 0.5, and germination (○, △) and commitment (●, ▲) were determined as described in Methods. Values shown in this figure are averages of results from two independent experiments, and replicate values differed by ≤ 15.
Fig. 5. Kinetics of DPA release and loss of acid resistance during B. megaterium spore germination. Spores of B. megaterium strains QM B1551 (wild-type) (○, ●) and PS4164 (cwLJ) (△, ▲) were germinated at an OD$_{600}$ of 0.5, and germination (○, △) and loss of acid resistance (●, ▲) were determined as described in Methods. The values shown in the figure are averages of results from three replicate experiments, and there was < 10% variation in values for DPA release in replicates, and < 20% variation between replicates in values for acid resistant spores. Note that the wild-type spores used in this experiment are a different preparation than was used in Fig. 4.
Fig. 6. Kinetics of DPA release during germination of B. megaterium spores with or without a heat treatment. Spores of B. megaterium strains QM B1551 (wild-type) (○, ●) and PS4164 (cwlJ) (△, ▲) were germinated at an OD$_{600}$ of 5, and DPA release was measured at various times without (○, △) or with (●, ▲) a heat treatment as described in Methods. The values shown are averages of results from three independent experiments, and replicate values for germination differed by ≤ 15%, with ≤ 20% variation between replicates for the loss in spore heat resistance. Note that the wild-type spores used are from the same preparation used in Fig. 4.