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Mechanisms of Induction of Germination of *Bacillus subtilis* Spores by High Pressure

Madan Paidhungat,[1]† Barbara Setlow,[1] William B. Daniels,[2] Dallas Hoover,[3] Efstathia Papafragkou,[3] and Peter Setlow[1]*

Department of Biochemistry, University of Connecticut Health Center, Farmington, Connecticut 06032,[1] and Departments of Physics[2] and Animal and Food Science,[3] University of Delaware, Newark, Delaware 19717

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Spores of *Bacillus subtilis* lacking all germinant receptors germinate >500-fold slower than wild-type spores in nutrients and were not induced to germinate by a pressure of 100 MPa. However, a pressure of 550 MPa induced germination of spores lacking all germinant receptors as well as of receptorless spores lacking either of the two lytic enzymes essential for cortex hydrolysis during germination. Complete germination of spores either lacking both cortex-lytic enzymes or with a cortex not attacked by these enzymes was not induced by a pressure of 550 MPa, but treatment of these mutant spores with this pressure caused the release of dipicolinic acid. These data suggest the following conclusions: (i) a pressure of 100 MPa induces spore germination by activating the germinant receptors; and (ii) a pressure of 550 MPa opens channels for release of dipicolinic acid from the spore core, which leads to the later steps in spore germination.

Spores of various *Bacillus* species are metabolically dormant and can remain in this state for long periods of time (18, 24). Given the proper stimulus, however, these spores can initiate metabolism and macromolecular synthesis during spore germination and outgrowth (5, 18). The latter processes are normally triggered by germinants in the form of low-molecular-weight nutrients such as sugars and amino acids (5, 18). Spores have a number of receptors for germinants, with *Bacillus subtilis* spores having three receptors for known germinants and two with as yet undiscovered ligands (16, 18). Upon ligand binding, these receptors trigger the release of the spore core's large depot of pyridine-2,6-dicarboxylic acid (dipicolinic acid [DPA]) along with its associated divalent cations, predominantly Ca^{2+}, from the spore core (16, 18). As studied most intensively with *B. subtilis* spores, binding of germinants to their receptors and subsequent DPA release also triggers the activation of cortex-lytic enzymes (CLEs) that degrade the spore cortex, a large peptidoglycan structure that prevents the dormant spore core from expanding (5, 14, 18). There are two CLEs in *B. subtilis*, CwlJ and SleB, either of which is sufficient alone for nearly normal spore germination (9, 12); in contrast, *cwlJ sleB* mutants are severely compromised in the complete spore germination process (9, 23). The activation of CwlJ during spore germination is directly due to the Ca^{2+}-DPA released from the spore core, and exogenous Ca^{2+}-DPA will trigger spore germination by activating CwlJ (14, 18). In contrast, the precise mechanism for SleB activation is less clear. Although SleB activation is not directly due to DPA release, SleB does become active in DPA-less spores, possibly due to some change in the structure of or stress upon the spore cortex (14, 15).

Spores of a number of *Bacillus* species are also germinated by pressures of 100 to 600 MPa (1,000 to 6,000 atm) (6, 7, 11, 27, 28, 29). This process has drawn significant attention recently, because germinated spores are more sensitive to agents such as heat than are dormant spores (7, 11, 24). We have been studying the gene products required for spore germination (14, 15, 16) and in the process of these studies have generated strains lacking combinations of germinant receptors and CLEs. An analysis of the germination of spores of these various strains in response to high pressure is the subject of this communication.

Strains used and spore preparation. The *B. subtilis* strains used in this work were all derivatives of strain 168, with the wild-type strain being PS832. The strains used were as follows: FB72 (termed ger3) (Δ*gerA*::*spc* Δ*gerB*::*cat* Δ*gerK*::*erm* [16]), in which the genes encoding the three major germinant receptors are inactivated; FB85 (termed ger5) (Δ*gerA*::*spc* Δ*gerB*::*cat* Δ*gerK*::*erm* Δ*yndDEF*::*tet* Δ*yfkQRT*::*neo* [16]), in which all five genes encoding germinant receptors are inactivated; FB113 (Δ*cwlJ*::*tet* Δ*sleB*::*spcI* [16]), in which the genes encoding the two CLEs are inactivated; FB114 (Δ*gerA*::*spc* Δ*gerB*::*cat* Δ*gerK*::*erm* Δ*sleB*::*tet* [14]); FB115 (Δ*gerA*::*spc* Δ*gerB*::*cat* Δ*gerK*::*erm* Δ*cwlJ*::*tet* [14]); and PS2307 (Δ*cwlD*::*cat* [19]), which has a spore cortex that is not attacked by CLEs. Spores of all strains were prepared at 37°C on 2× SG medium agar plates without antibiotics and harvested, cleaned, and stored as described previously (13, 15). All spores used in this work were free (>98%) of growing and sporulating cells, germinated spores, and cell debris, as determined by examination with a phase-contrast microscope. Spores were treated with sodium dodecyl sulfate and urea to remove spore coats and then washed as described previously (1). Removal of spore coats has been shown to alter spore resistance to some but not all agents and can also modify spore germination (3, 14, 24, 18).

Pressure treatment and analysis of spore viability and ger-

* Corresponding author. Mailing address: Department of Biochemistry, University of Connecticut Health Center, Farmington, CT 06032. Phone: (860) 679-2607. Fax: (860) 679-3408. E-mail: setlow@sun.uchc.edu.

† Present address: Maxygen Incorporated, Redwood City, CA 94063.

mination. Spores were routinely pressure treated without prior heat activation, since heat activation is not needed to get high levels of germination of *B. subtilis* spores (6, 27, 28, 29). Small aliquots (1 to 1.5 ml) of spores at an optical density at 600 nm (OD_{600}) of 1 (~1.5×10^8 CFU/ml) in water (measured pH, ~7) or of 10 (1.5×10^9 CFU/ml) in 25 mM KPO_4 (pH 7.4) were heat sealed in sterile Tygon tubing sections (5/16 in. outside diameter and 3/16 in. inside diameter; catalog no. S-50-HL) and treated for 30 min in an apparatus built at the University of Delaware (2). This apparatus uses bis(2-ethylhexyl)sebacate (Alfa Aesar, Ward Hill, Mass.) as the compressing fluid, and the times to achieve the desired pressures were ≤2 min. The initial temperature was routinely 23°C, giving a maximum temperature in the compressing fluid due to adiabiatic heating of 55°C at 550 MPa (a rise of ~6°C/100 MPa; V. M. Balasubramaniam, personal communication), but this temperature decreased rapidly by conduction to the pressure vessel. In addition, the maximum temperature in the water in which the spores were suspended was only ~40°C (a rise of 3°C/100 MPa at most [25]). The pH in the spore suspensions will also change slightly upon pressure treatment (10), but pressure-induced germination and inactivation of spores is relatively insensitive to pH at values between of 5 and 10 (6, 21). After pressure treatment, samples were cooled on ice and diluted in water and aliquots of the various dilutions were plated in duplicate on Luria broth (LB) medium agar plates (16) and incubated for ~18 h at 37°C in order to determine spore viability. At the pressures and temperatures used, the level of spore killing was always ≤50% (see below). Consequently, the assessment of spore viability on LB plates was also a measure of the germination of the spores of ger3 and ger5 strains, since only 0.1 to 0.2% of ger3 and ger5 spores germinate with nutrients in 18 h at 37°C (16). In contrast, >90% of wild-type spores germinate and give rise to colonies on LB medium plates in 18 h at 37°C (16). After pressure treatment, in some cases the concentration of spores was adjusted to 0.1 M in Tris-HCl (pH 8.5) and the spores were incubated for 1 h at 37°C and examined with the phase-contrast microscope. Untreated spores do not germinate under these conditions (14, 16; data not shown), but the incubation allows spores in which germination has been triggered to complete the germination process and turn from bright to dark in the phase-contrast microscope. In a few experiments, the spores were centrifuged after pressure treatment and the supernatant fluid and pellet fractions were assayed for DPA as described previously (15, 20).

Inactivation and germination of spores of various strains. Treatment of wild-type spores with a pressure of 100 MPa at 23°C killed <25% of the spores (Table 1). This result was as expected, since even at a higher temperature, pressure this low produces little killing of germinated *B. subtilis* spores (27, 28). Pressure treatment at 550 MPa also produced <50% spore killing (Table 1), again as expected based on previous work (21, 27, 28). At a pressure of 550 MPa, removal of much of the spore coat protein had no significant effect on spore killing (Table 1).

Germination of wild-type spores of *B. subtilis* is induced by pressures of 100 to 600 MPa (6, 7, 11, 21, 27, 29). Indeed, phase-contrast microscopy of samples shortly after pressure treatment indicated that a pressure of 100 or 550 MPa induced ~30% of wild-type spores to germinate, although completion of the germination process required further incubation of the pressure-treated spores as described above (data not shown). However, for spores of most of the strains used, we did not directly assess spore germination induced by pressure but rather measured spore viability as an indirect indicator of the induction of spore germination as noted above. In contrast to wild-type spores, which produce ~1.5×10^8 colonies of spores/ml at an OD_{600} of 1 (~one colony/spore) on LB plates, ger3 and ger5 spores produce only 0.1 to 0.2% of this amount, since they do not respond to nutrient germinants (16) (Table 1). Consequently, any treatment that stimulates the germination of ger3 or ger5 spores results in an increase in colony formation by the spores of these strains (16). Since the viability of ger3 and ger5 spores was not increased significantly by a pressure of 100 MPa (Table 1), this indicates that very few of these spores were germinated by this treatment. However, the large increase in the viability of ger3 and ger5 spores treated with a pressure of 550 MPa indicated that germination of a significant percentage of the ger3 and ger5 spores was induced by this pressure (Table 1). This percentage may be as much as 30%, assuming that there is ~50% killing of the ger3 and ger5 spores, as is found with wild-type spores. Indeed, phase-contrast microscopy indicated that ~30% of ger3 and ger5 spores were induced to germinate by a pressure of 550 MPa at 23°C (data not shown). Spore coat removal again had no effect on the viability or the germination of ger3 or ger5 spores treated with a pressure of 550 MPa (Table 1).

In addition to germinant receptors, completion of nutrient-induced spore germination and resumption of vegetative growth also requires degradation of the spore cortex by either CwlJ or SleB (9, 12, 14, 23). In the absence of pressure treatment, ger3 *cwlJ* and ger3 *sleB* spores had the same low colony-forming ability as ger3 spores (Table 1), while *cwlJ sleB* spores had even lower colony-forming ability (Table 1), as seen previously (9, 23). Spores of a strain with a *cwlD* mutation, which

TABLE 1. Colony formation by spores of various strains with and without pressure treatment[a]

Strain	No. of colonies		
	No treatment	100 MPa	550 MPa
PS832 (wt)[b]	1.5×10^8	1.1×10^8	8.5×10^7
PS832 (wt) decoated[c]	1.6×10^8		8×10^7
FB72 (ger3)[b]	1.6×10^5	4.6×10^5	2.6×10^7
FB72 (ger3) decoated[c]	1.5×10^5		2.5×10^7
FB85b (ger5)[b]	3×10^5	2.7×10^5	2.7×10^7
FB85b (ger5) decoated[c]	1.5×10^5		2.2×10^7
FB114 (ger3 *sleB*)[b]	4.2×10^5		3.4×10^7
FB115 (ger3 *cwlJ*)[b]	1.3×10^5		2.7×10^7
FB113 (*cwlJ sleB*)[b]	10^4		10^4
PS2307 (*cwlD*)[b]	10^3		10^3

[a] Colony formation per milliliter by untreated or decoated spores of various strains after incubation for 18 h at an OD_{600} of 1 in water with or without pressure treatment for 30 min at a temperature of 23°C was determined as described in the text. Note that for ger3 and ger5 spores, colony formation is also an estimate of the induction of the germination of these spores by pressure treatment. wt, wild type.

[b] Values for these strains are averages of duplicate determinations in two different experiments; values in different experiments differed over a range ≤33%.

[c] Values for these strains are averages of duplicate determinations in one experiment, with duplicate determinations differing by ≤25%.

have a cortex that cannot be degraded by CLEs, had an even lower colony-forming ability than *cwlJ sleB* spores (Table 1), again as found previously (19, 22, 23). Germination of the ger3 *cwlJ* and ger3 *sleB* spores was induced at a significant level by a pressure of 550 MPa, but the apparent viability of *cwlJ sleB* and *cwlD* spores was not increased by treatment at this high pressure (Table 1). Note, however, that previous work has shown that spores of the latter two mutant strains can be recovered if the cortex is degraded with lysozyme (9, 19, 23).

DPA release from spores in response to a pressure of 550 MPa. The data given above indicate that completion of spore germination induced by a pressure of 550 MPa does not require the germinant receptors but does require cortex degradation by either CwlJ or SleB. In nutrient-induced spore germination, the activation of these CLEs is dependent on the binding of nutrients to germinant receptors (14, 18). Since the latter event cannot take place in ger3 or ger5 spores, a pressure of 550 MPa must activate CLEs either directly or by triggering a step in the normal spore germination pathway between the binding of a germinant to its receptor and the activation of CLEs. One such step in the nutrient-induced spore germination pathway is the release of DPA from the spore core, as this event activates CwlJ directly and SleB indirectly (14, 15, 18).

In order to examine whether a pressure of 550 MPa induces spore germination by causing release of DPA from the spore core, we used spores from strains FB113 (*cwlJ sleB*) and PS2307 (*cwlD*), whose cortexes do not degrade upon the triggering of spore germination (9, 12, 19, 23). The spores, at an OD_{600} of 10 in 25 mM KPO_4 (pH 7.5), were incubated at 32 or 40°C with 550 MPa of pressure for 30 min to maximize spore germination (27, 29) or at 75°C for 30 min with no pressure treatment and were then centrifuged. The levels of DPA in the supernatant and pellet fractions were then analyzed. Treatment with 550 MPa of pressure at 32 or 40°C caused the release of 66 or 93%, respectively, of the DPA of the *cwlJ sleB* spores and at 32°C caused the release of 77% of the DPA of the *cwlD* spores. (Values are averages of duplicate determinations from two experiments, with <15% variation among individual determinations.) Presumably, divalent cations are released along with the DPA, but we have not yet shown this directly. In contrast to the results with high-pressure treatment, the *cwlD* and *cwlJ sleB* spores incubated at ambient pressure for 30 min at 75°C released only ~3% of their DPA. A temperature of 75°C is well above the maximum possible sample temperature resulting from adiabiatic heating during pressure treatment at 40°C and 550 MPa. Note also that for wild-type spores that were incubated at an initial temperature of 32°C with 550 MPa of pressure for 30 min in 25 mM KPO_4 (pH 7.5), the level of killing was at least 30% (data not shown). Since spores of strains FB113 and PS2307 cannot degrade their cortexes (9, 12, 19, 23), the percentage of germination of the pressure-treated spores could not be determined by examination using the phase-contrast microscope. However, the levels of germination of wild-type spores treated with 550 MPa of pressure at 32 and 40°C were ~70 and ~90%, respectively, as determined by examination using a phase-contrast microscope (data not shown). Other workers have also found very high levels of spore germination at these temperatures and pressures (27, 29).

Conclusions, questions, and discussion. The work presented in this communication allows a number of conclusions. First, although the coats are important in spore resistance to some agents (3, 24), the coats are not important in the resistance of spores to high pressure. Second, although one or more spore coat proteins are involved somehow in spore germination (3, 18), the spore coats are not important in pressure-induced spore germination. Third, induction of spore germination at a pressure of 100 MPa requires at least one of the spore's germinant receptors. Previous work has shown that the rate of induction of germination of spores lacking two germinant receptors (GerA and GerB) by a pressure of 100 MPa is significantly reduced relative to that of wild-type spores (29). However, ~90% of *gerA gerB* spores can eventually be induced to germinate by this pressure (29). The residual pressure-induced germination of the double mutant spores was likely due to the remaining germinant receptors, predominantly the GerK receptor, since the germination of ger3 spores induced by 100 MPa of pressure was greatly reduced and ger5 spores produced no germination with this pressure (Table 1). Fourth, induction of spore germination by a pressure of 550 MPa requires no germinant receptors. Previous work had indicated that the germination of *gerA gerB* spores induced by a pressure of 600 MPa was essentially identical to that of wild-type spores (29). In the present work, we now show that the level of germination of spores lacking all germinant receptors with 550 MPa of pressure is identical to that of wild-type spores. Fifth, completion of spore germination induced by a pressure of 550 MPa requires spore cortex degradation by either of the spore's two CLEs. Thus, spore germination induced by this high pressure utilizes at least this part of the normal spore germination pathway. Sixth, treatment of *cwlD* and *cwlJ sleB* spores with 550 MPa of pressure causes release of DPA, and it is likely through this event that the pressure treatment induces spore germination. This further indicates that a pressure of 550 MPa does not trigger spore germination by direct activation of CLEs, since cortex degradation takes place after DPA release during nutrient-induced spore germination (18, 23).

A number of questions also arise from some of the conclusions listed above. One concerns the identity of the mechanism whereby a pressure of 100 MPa activates the spore's germinant receptors. The germinant receptors are located in the spore's inner membrane (8, 17), and a pressure of 100 MPa might activate these receptors either by altering membrane properties directly, perhaps by causing a decrease of membrane fluidity, or by causing a structural change in the receptor itself. Since we know neither the structure nor the mechanisms of activation or action of the germinant receptors, the nature of the mechanism involved in the effect of pressure on the germinant receptors is not clear.

A second question is how an even higher pressure (550 MPa) causes release of spore DPA. This release might be due to some effect of the pressure on the solubility of DPA in the spore core, although this seems unlikely, since the level of concentration of DPA in the spore core (1 to 2 M, if all of the DPA were soluble [4]) is far above that of DPA soluble at neutral pH (≤100 mM). Alternatively, high pressure may cause changes in the spore's inner membrane, allowing DPA to leak out. However, since such a high pressure should decrease membrane fluidity, this would be expected to retard DPA loss.

A third explanation is that a pressure of 550 MPa either directly or indirectly activates the spore's normal channels for DPA, much as a pressure of 100 MPa activates the germinant receptors. Recently the products of the *spoVA* operon have been implicated in DPA transport into developing spores (26), although neither the structure nor the mechanism of action or activation of spore DPA channels is known. However, it may be possible in the future to discriminate between the last two explanations for the high-pressure induction of DPA release by analyzing pressure-treated spores for release of other major small molecules that are not excreted during spore germination.

A third question concerns the precise state of spores immediately after they have been induced to germinate by a pressure of 550 MPa. As noted above, spores treated at this pressure have released their DPA. However, as seen with the wild-type ger3 and ger5 spores, completion of the germination of pressure-treated spores requires further incubation, suggesting that the pressure treatment has triggered germination solely by causing DPA release and that subsequent events are not affected by pressure. The process of spore germination has recently been divided into Stages I and II (23). Stage I is characterized by activation of the germinant receptors and release of DPA, while completion of Stage II requires cortex hydrolysis and the associated swelling and water uptake by the spore core. Enzyme action in the spore core, including the degradation of the spore's large cache of DNA binding proteins (small, acid-soluble spore proteins) and initiation of ATP production, only begins during Stage II of germination. Interestingly, previous work (27) has shown that immediately after wild-type spores are treated at a pressure of 600 MPa, the spores have lost their DPA but not small, acid-soluble spore proteins and do not generate ATP. Thus, these high-pressure-treated wild-type spores appear to be in Stage I of spore germination. Such Stage I germinated spores would not be as stable as dormant spores, since SleB slowly becomes active and catalyzes cortex degradation leading to complete spore germination (14, 15). However, DPA-less spores containing SleB that have not progressed beyond Stage I of germination can be isolated and maintained in this state for many days, in particular at low temperatures (15). Consequently, we propose that spores treated with ≥550 MPa of pressure are in Stage I of germination but will slowly complete the germination process, in particular during the extended incubation at the temperatures used to assess spore viability on plates. The resistance properties of spores immediately after treatment with 600 MPa of pressure are also consistent with these spores being in Stage I of germination (23, 27), but this remains a topic for further work.

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