Heat Shock Affects Permeability and Resistance of *Bacillus stearothermophilus* Spores†

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Heat shock of dormant spores of *Bacillus stearothermophilus* ATCC 7953 at 100 or 80°C for short times, the so-called activation or breaking of dormancy, was investigated by separating the resulting spores by buoyant density centrifugation into a band at 1.240 g/ml that was distinct from another band at 1.340 g/ml, the same density as the original spores. The proportion of spores at 1.240 g/ml became larger when the original dormant spores were heated for a longer period of time, but integument-stripped dormant spores were quickly and completely converted to spores with a band at 1.240 g/ml. The spores with bands at both 1.240 and 1.340 g/ml were germinable faster than the original dormant spores and thus were considered to be activated. The spores with a band at 1.240 g/ml, which were considered to be fully activated, were apparently permeabilized, with a resulting complete depletion of dipicolinic acid, partial depletion of minerals, susceptibility to lysozyme action, permeation of the gradient medium, changed structural appearance in electron micrographs of thin-sectioned spores, and partly decreased heat resistance (D<sub>100</sub> = 453 min) compared with the original dormant spores (D<sub>100</sub> = 760 min). However, the fully activated spores with a band at 1.240 g/ml, although devoid of dipicolinic acid, still were much more resistant than germinated spores or vegetative cells (D<sub>100</sub> = 0.1 min). The spores with a band at 1.340 g/ml, which were considered to be partly activated, showed no evidence of permeabilization and were much more heat resistant (D<sub>100</sub> = 1,960 min) than the original dormant spores. This phenomenon of super-resistance may involve either in situ induction or selection of a preexisting subpopulation.

Activation, which is usually accomplished by sublethal heating (heat shock) of water-suspended spores, breaks spore dormancy and overcomes the inability or increases the ability of spores to germinate and grow under otherwise favorable conditions. Curran and Evans (8) first demonstrated systematically that heat shock can induce heat shock to germinate. Keynan and Evenchik (17) have defined terms, reviewed the relevant literature, and listed hypotheses to explain the phenomenon.

The spores of *Bacillus stearothermophilus* are said to be unusually dormant relative to those of other species. On conventional plate counting media without prior heat treatment, less than 10% of the total number of spores (by direct chamber count) germinate and form colonies, but about 50% do so after optimum heat activation (7). Virtually all do so after treatment with 0.5 N hydrochloric acid (4). Optimum activation is a function of temperature, time, and pH and is also dependent on the presence of buffer and nutrient broth in the suspension medium in order to protect against killing (5). In certain strains of *B. stearothermophilus*, dormancy may be induced, rather than alleviated, by prior heating at sublethal temperatures (10).

In the present study of heat shock, with *B. stearothermophilus* ATCC 7953 spores used as a model, the key finding was that activated spores, as well as dormant and germinated spores (27), could be separated from a heterogeneous population into two distinct fractions by the use of buoyant density centrifugation with a selected gradient medium. Characterization of these fractions showed that the fully activated spores became permeabilized at the outer membrane and, thus, lysozyme susceptible and that the partly activated spores became substantially more heat resistant than the original dormant spores.

**MATERIALS AND METHODS**

Dormant smooth colony spores of the standard sterilization test strain ATCC 7953 (NCA 1518, NCIB 8157) of *B. stearothermophilus* were produced at 60°C in the medium described by Forster (11) and handled as described previously (1, 25). Each lot of spores was used within 1 month after it was placed in refrigerated storage, and it was washed in distilled water before each use. The native lysozyme-resistant spores were stripped of their coats and outer pericortex membranes and thus were converted to lysozyme-susceptible spores by treatment with thioglycolic acid (25).

Heat shock was studied with spores that were suspended in distilled water at an optical density of about 1.7 measured at 600 nm with a spectrophotometer (DU-50; Beckman Instruments, Inc., Irvine, Calif.). Samples of 3 ml were distributed in glass ampoules (inner diameter, 6 mm), which were sealed and immersed in a mineral oil bath at 100 or 80°C, removed at successive times, and chilled in ice. For routine purposes, heat shock was accomplished at 100°C for 10 min. The exposure time included the time that was necessary to reach the desired temperature within the ampoule.

Heat resistance, which was measured during the initial decade or more of decline as described previously (1), was expressed as a D value (minutes required for a decimal reduction in CFU on exposure of 10<sup>7</sup> to 10<sup>8</sup> viable spores or cells per ml in distilled water at a given temperature, usually 100°C).

Germination without outgrowth was accomplished by heat
shocking the spores, cooling them, incubating them in the exudate solution at room temperature for about 12 h, and then centrifugally separating the fraction of germinated spores into the interface between solutions of 1.180 and 1.200 g of Nycodenz (Nyegaard and Co., Oslo, Norway) per ml. Germination was also accomplished by use of the medium described by Foerster (11), but outgrowth ensued. Germinability was measured by the reduction in optical density and the percentage conversion to phase-dark spores.

Vegetative cells were obtained in the logarithmic phase of growth by incubating heat-shocked spores in the medium described by Foerster (11) without agar on a shaker for 2.5 h, washing the cells in 0.1 M phosphate buffer and water, and then separating them between Nycodenz solutions as described above for germinated spores.

Wet density was determined by centrifugal buoyant density sedimentation at 5°C and 6,000 × g for 16 h (the long time was used to ensure equilibration, and the low force was used to prevent pressure-induced germination). Incremental discontinuous gradients of Nycodenz were used as described previously (21). The results are expressed as grams of wet protoplast per milliliter of wet protoplast for the lysozyme-susceptible spores or for the vegetative cells in which Nycodenz penetrated to the inner periplasmic membrane, and as grams of wet sporoplast per milliliter of wet sporoplast for the lysozyme-resistant spores in which Nycodenz penetrated to the outer pericell membrane.

Lysozyme susceptibility (sensitivity) was determined by suspending the spores in 0.1 M phosphate buffer at pH 7.0 to an optical density of about 0.5, adding 100 µg of egg white lysozyme per ml, and measuring the percentage conversion to phase-dark spores and the reduction in optical density.

The amount of dipicolinic acid (DPA) was determined by the method of Janssen et al. (16). Mineral contents were analyzed by inductively coupled plasma emission spectroscopy (model 955 Atomcomp; Jarrell-Ash Division, Fisher Scientific Co., Waltham, Mass.).

The methods for electron microscopy were essentially those described previously (3), but with the following changes. The spores were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2. They were then embedded in 1% Noble agar, which was diced and washed in the buffer. The agar-embedded spores were further fixed with 1% OsO₄ in the buffer, dehydrated through a graded ethanol series, cleared with propylene oxide, and embedded in resin of either PolyBed 812 (Polysciences Inc., Warrington, Pa.) or HXSA/VCD (Ladd Research Industries Inc., Burlington, Vt.). The embedded spores were cut into thin sections and then stained with uranyl acetate and lead citrate. Micrographs were taken with an electron microscope (model EM-300 or CM-10; Philips).

RESULTS

The activation of dormant spores by heat shock is a process that progresses with time and involves an abrupt change in individual spores rather than a gradual change in all spores, as indicated by increased colony counts with time on conventional plating media (7). The process is accompanied by the partial release of DPA and calcium (5, 17, 24), so that activated spores should be less dense than dormant spores. Therefore, the activated spores in a heterogeneous population after heat shock should be separable by means of buoyant density centrifugation.

Dormant native spores of B. stearothermophilus ATCC 7953 became separated into two distinct bands, without visible intermediate material, after various heat shock conditions and then centrifugation in Nycodenz gradients (Table 1). One band contained spores with a density of 1.340 g/ml (1.340-g/ml spores), which was the same value that was obtained for the original population of dormant spores and that was found previously for lysozyme-resistant dormant spores of this strain (21, 30). This value reflects the density of the sporoplast, i.e., the structures bounded by the outer pericell membrane. The other band contained spores with a density of 1.240 g/ml (1.240-g/ml spores), which reflects the density of the protoplast (see below). As time progressed, at either 80 or 100°C the proportion of the 1.240-g/ml spores increased and the optical density of the suspension decreased until a plateau was reached. The reduction in optical density in a fully converted population after heat shock was much less than that in a germinated population.

Table 1. Optical and buoyant densities of native and stripped B. stearothermophilus ATCC 7953 spores during heat shock

<table>
<thead>
<tr>
<th>Spore type</th>
<th>Temp (°C)</th>
<th>Time (min)</th>
<th>Relative optical density (%)</th>
<th>Buoyant wet density (g/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>80</td>
<td>0</td>
<td>100</td>
<td>1.340 (100)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>96.8</td>
<td>1.340 (80)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>120</td>
<td>96.0</td>
<td>1.340 (50)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>180</td>
<td>95.7</td>
<td>1.340 (30)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>360</td>
<td>94.7</td>
<td>1.340 (10)</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>1.340 (100)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>88.4</td>
<td>1.340 (50)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>87.3</td>
<td>1.340 (25)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
<td>87.2</td>
<td>1.340 (20)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>150</td>
<td>87.3</td>
<td>1.340 (10)</td>
</tr>
<tr>
<td>Stripped</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>1.385 (100)</td>
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<td></td>
<td></td>
<td>10</td>
<td>87.8</td>
<td>1.240 (100)</td>
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<td>84.7</td>
<td>1.240 (100)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>150</td>
<td>84.7</td>
<td>1.240 (100)</td>
</tr>
</tbody>
</table>

* (OD₆₀₀ at given time)/(OD₆₀₀ at time zero), where OD₆₀₀ is the optical density at 600 nm.

The values in parentheses indicate the estimated percentage distribution of the total amount in the gradient.
EFFECTS OF HEAT SHOCK ON *B. STEAROTHERMOPHILUS* SPORES

<table>
<thead>
<tr>
<th>Spore type</th>
<th>Content (µmol/mg [dry wt] of spores)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DPA</td>
</tr>
<tr>
<td>Dormant spore</td>
<td>83.1</td>
</tr>
<tr>
<td>Partly activated spore</td>
<td>67.6</td>
</tr>
<tr>
<td>Fully activated spore</td>
<td>0.05</td>
</tr>
</tbody>
</table>

*Note:* The values are given in terms of µmol/mg dry weight of spores.

In our experiments, the counts declined exponentially in the thermal death curves from the start of exposure at 100°C.

Dormant spores of strain ATCC 7953 germinating in the medium of Foerster (11) did so asynchronously and slowly (about 20% conversion to phase-dark spores in 30 min) but eventually exceeded 80% germination in 120 min, with outgrowth ensuing after germination so that some spores were grown out while others were not yet germinated. The 1.240-g/ml fraction of heat-shocked spores germinated more rapidly (about 80% in 30 min) than the dormant spores, and so it was considered to contain fully activated spores. The 1.340-g/ml fraction of heat-shocked spores also germinated more rapidly (about 85% in 30 min) than the dormant spores. In recognition of their more rapid germinability and their high density and other similarities to dormant spores (see below), spores in the 1.340-g/ml fraction were designated as partly activated.

The original dormant spores and the two fractions of activated spores were analyzed for their DPA and mineral contents (Table 2). The original high levels of DPA and minerals were mostly retained in the partly activated 1.340-g/ml spores. In the fully activated 1.240-g/ml spores, however, the DPA content was depleted entirely, while the minerals were depleted only partially. Results of previous reports indicated that dipicolinate, as well as minerals, is only partially lost after heat activation (5, 17, 24).

This leakage of cytoplasmic constituents after heat shock suggested that the fully activated 1.240-g/ml spores became permeabilized. This was confirmed by adding lysozyme and observing 87% conversion of the originally phase-bright, fully activated spores to phase-dark, germinated spores in 30 min, whereas the original dormant spores and the partly activated spores remained refractile (less than 5% conversion in 30 min). Lysozyme susceptibility was also indicated by a reduction in the optical density.

Because heat activation caused relaxation or disruption of the outer membrane that was sufficient to allow penetration of the lysozyme to its peptidoglycan substrate in the cortex, smaller molecules such as Nycodenz would also be able to penetrate the outer membrane and should then permeate through the cortex to the inner periplasm membrane. This was confirmed by stripping away the coat and outer membrane of the dormant spores with thioglycolic acid and determining the buoyant density with Nycodenz. The density of the dormant stripped spores (1.385 g/ml) was higher than that of the dormant native spores (1.340 g/ml), indicating that Nycodenz permeated through the exposed cortex to the inner membrane of the dormant stripped spores so that the resulting density value reflected that of the protoplast (Table 1). This density (1.385 g/ml) and the corresponding protoplast water content of dormant stripped spores (29.5%, on a wet weight basis) were about the same as those reported previously (1, 21). The density of the fully activated stripped spores (1.240 g/ml) was the same as that of the fully activated native spores (Table 1), reflecting the protoplast density in both cases and indicating that their integuments were equally permeable to Nycodenz.

The gross appearance by electron microscopy of unstained, fully activated spores was different from that of dormant or partly activated spores, but not different from that of germinated spores. The fully activated and the germinated spores showed an electron-dense core and an electron-transparent periphery, whereas the dormant and the partly activated spores appeared to be entirely electron dense (data not shown).

The fine-structure appearance by electron microscopy with stained thin sections of fully activated spores, however, was identifiably different from that of dormant, partly activated, and germinated spores. In partly activated spores (Fig. 1A), the cortex and nucleoplasm were electron transparent, the ribosomes were indistinct, and the mesosomes were often seen (for a dormant spore with similar appearance, see Fig. 2B in reference 2). In fully activated spores (Fig. 1B), the cortex and nucleoplasm were electron opaque and the ribosomes were distinct. In germinated spores (Fig. 1C), the cortex was gone, the residual integument layers were often separated, and the nucleoplasm and cytoplasm resembled those in vegetative cells. The line seen just outside the protoplast (Fig. 1C) was believed to represent the residual outer membrane, but efforts to identify it specifically as such were unsuccessful. The size of the fully activated spores was larger than that of the partly activated and the dormant spores but smaller than that of the germinated spores. The average volume percentage of the sporoplast occupied by the protoplast was 42% for the fully activated spores and 29% for the partly activated spores, with the latter value being about the same as that reported previously for dormant spores (2).

The buoyant density of the protoplast in fully activated spores (1.240 g/ml), although less than that in partly activated spores (1.385 g/ml), was more than that in germinated spores and vegetative cells (1.192 g/ml) (Table 3). The value of 1.240 g/ml for germinated spores reported previously (21) was found, on review, to be misidentified and, instead, represents what are known to be fully activated spores. An experimental correlation enables conversion of the buoyant wet density to the protoplast water content in lysozyme-sensitive dormant spores (21), but the correlation is not applicable to lysozyme-sensitive activated spores, germinated spores, or vegetative cells.

The ability to separate the different types of spores into distinct buoyant density bands with Nycodenz gradients enabled a direct comparison of heat resistance in the different cell types (Table 3). The original population of dormant native spores had a mean D100 value of 760 min. This was somewhat higher than previously reported D100 values of 567 min (25) and 580 min (1), both of which were lowered to 238 min by stripping the integument from the spores. Full activation also lowered resistance, to a D100 value of 453 min. However, the fully activated spores still were several decades more resistant in D100 values than germinated spores.
The fully activated spores had a remarkably high $D_{100}$ value relative to their low protoplast density (Table 3) and a virtually complete absence of DPA (Table 2). This occurrence of DPA-less but heat-resistant spores confirms the similar occurrence in DPA-negative mutant spores (14; G. W. Dring and G. W. Gould, Spore Newsl. 7:130–131, 1981) and in demineralized-remineralized spores (R. E. Marquis, in T. J. Beveridge and R. J. Doyle (ed.), Bacterial Interactions with Mineral Ions, in press).

Heat shock caused the most remarkable change, however, in heat resistance of the partly activated spore population. This population had a mean $D_{100}$ value of 1,960 min, whereas the original population of dormant spores had a mean $D_{100}$ value of 760 min (Table 3). The actual $D_{100}$ value of partly activated spores could be even higher than 1,960 min, in that the population may have contained residual dormant spores and partly activated spores. Unfortunately, the two types of spores could not be separated because they had the same buoyant density.

**DISCUSSION**

The nature of the thermal activation process in spores has remained open to conjecture. Keynan and Evenchik (17) have listed six hypotheses to explain the phenomenon and have emphasized various lines of evidence indicating that activation enhances permeability, with the spore coat being the site of permeabilization. However, the outer pericortex membrane is now known to serve as the primary permeability barrier in the dormant spore, whereas the surrounding coat is porous (18). With this modification the permeabilization hypothesis explaining full activation was substantiated by the results of this study with *B. stearothermophilus* spores. Permeabilization of the outer membrane by heat shock was indicated by the susceptibility to lysozyme action and permeation of Nycodenz.

If conversion to lysozyme susceptibility by sublethal heating is found to occur with other spore species that are also important in food preservation, such pretreatment might provide a practical way to germinate spores and thus reduce the heat requirement in food processing. To our knowledge, the lysozyme susceptibility of activated spores has not been reported previously. The potential use of lysozyme as an antibacterial agent against vegetative cells in food processing has been suggested by Hughey and Johnson (15). They have shown that the cells of some pathogenic or spoilage organisms, such as *Clostridium sporogenes* 3679, are susceptible to lysozyme. Others, such as *Clostridium botulinum* 113B and 110E, are resistant to lysozyme alone but become susceptible with the addition of EDTA. On testing, however, we found that heat-activated (80°C for 10 min) spores of none of these three *Clostridium* strains (nor spores of *Bacillus subtilis* 168 or 4670) were susceptible to lysozyme or lysozyme-EDTA. The susceptibility of spores to lysozyme after heat activation and the potential applicability to food processing thus seem to be limited.

The fully activated spores of *B. stearothermophilus* were shown to have a buoyant density that was intermediate between the buoyant densities of dormant and germinated spores. This finding may explain in whole or in part the frequent observation of multiple bands in gradient density centrifugation of spore suspensions (6, 9, 20, 27, 29, 30), in which there may exist a subpopulation of fully activated spores. Subpopulations of smooth and rough colony types of dormant spores also have different buoyant densities (2) and so could also cause multiple bands.

**FIG. 1.** Electron micrographs of representative stained thin-sectioned spores of *B. stearothermophilus* ATCC 7953 after heat shock and centrifugation in a Nycodenz gradient. (A) Partially activated spore in a band at a density of 1.340 g/ml. (B) Fully activated spore in a band at a density of 1.240 g/ml. (C) Germinated spore in a band at a density of 1.192 g/ml. Bar, 0.2 μm.
The partly activated spores were shown to have a buoyant density that was the same as that of the original dormant spores and were alike in other characteristics of dormancy except faster germinability, hence their qualified designation as a type of activated spore in the definition used by Keynan and Evenchik (17). Partly activated spores apparently represent an intermediate type of spore in the transition from a fully dormant to a fully activated spore. Consequently, the usual perceptions of dormancy and activation may have to be modified.

The partly activated spores were remarkable also in that they were much more heat resistant than the original dormant spores, even though both spore types had the same sporoplast density. It can be hypothesized that heat shock induced a higher level of resistance in the partly activated spores. This increased resistance might be explained if heat shock caused expansion of the cortical peptidoglycan (26) against the intact coat, with a resulting lower water content in the protoplast (and higher water content in the cortex). This possibility is supported by the observation that heat shock did not induce super-resistance in coat-stripped spores (Table 1). Also relevant are findings that the elevation of temperature during sporulation causes increased resistance in the resulting spores of B. stearothermophilus ATCC 7953 (1) and that the increased dormancy of this strain can sometimes be induced by sublethal heat treatment (10).

The partly activated spores might be heated to a lethal temperature, with the amount of increase depending on the rate of heating, with the amount of increase depending on the rate of heating up to killing conditions. The increase in heat resistance of vegetative cells may be associated with the synthesis of heat shock proteins (28), but this mechanism seems unlikely in dormant spores unless heat shock releases their anabolic inactivity.

An alternative hypothesis envisages the selection by heat shock of a preexisting super-resistant subpopulation in an originally heterogeneous population of dormant spores. Such super-resistant, partly activated spores would not be separated in the centrifugation procedure because there is an upper limit of density in B. stearothermophilus spores of about 1.390 g/ml for the protoplast of lysozyme-sensitive spores (1), which is equivalent to about 1.340 g/ml for the sporoplast of lysozyme-resistant spores (21). The greater resistance in the subpopulation might be explained by a higher level of mineralization, which is an additive factor in thermoresistance at the limit of highest density and lowest protoplast water content (1). The hypothesis of a heat-selected, super-resistant subpopulation seems consistent with the phenomenon of a superdormant subpopulation in dormant spore populations, in which the germination rate decreases markedly and becomes a plateau after the first 10- or 100-fold decrease in the level of surviving ungerminated spores (13). However, germinability is said to be distributed heterogeneously, but heat resistance is not (12).

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LITERATURE CITED

2520 BEAMAN ET AL.