Heat-Induced Temperature Sensitivity of Outgrowing Bacillus cereus Spores†

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Inactivation of Bacillus cereus spores during cooling (10°C/h) from 90°C occurred in two phases. One phase occurred during cooling from 90 to 80°C; the second occurred during cooling from 46 to 38°C. In contrast, no inactivation occurred when spores were cooled from a maximum temperature of 80°C. Inactivation of spores at a constant temperature of 45°C was induced by initial heat treatments from 80 to 90°C. The higher temperatures accelerated the rate of inactivation. Germination of spores was required for 45°C inactivation to occur; however, faster germination was not the cause of accelerated inactivation of spores receiving higher initial heat treatments. Repair of possible injury was not observed in Tryptcase soy broth (BBL Microbiology Systems), peptone, beef extract, starch, or L-alanine at 30 or 35°C. Microscopic evaluation of spores outgrowing at 45°C revealed that when inactivation occurred, outgrowth halted at the swelling stage. Inhibition of protein synthesis by chloramphenicol at the optimum temperature also stopped outgrowth at swelling; thus protein synthesis may play a role in the 45°C inactivation mechanism.

Examination of Bacillus cereus spore survival during heating and cooling demonstrated that spore response could not always be predicted with data generated at constant temperatures (14). After rapid heating (ca. 900°C/h) to 90°C, spores were inactivated in two distinct temperature ranges during cooling at rates of 5 or 10°C/h. Thermal inactivation occurred during cooling from 90 to 80°C; the population remained stable during cooling from 80 to 50°C; and a second period of inactivation occurred during cooling from 50 to 35°C. Inactivation that occurred at the lower temperatures was not observed when spores were heated slowly (20 or 40°C/h) to 80°C before cooling (16a). This phenomenon was observed with three of four B. cereus foodborne illness-related strains studied.

The apparent low temperature inactivation of spores observed during cooling from 90°C must be viewed with more than academic curiosity. If the inactivation represents injury rather than death of spores, potentially viable spores may remain undetected with standard microbiological analysis. Repair of injured spores in food and subsequent growth may lead to potentially hazardous situations (3). Temperature sensitivity of B. cereus spores at 45 to 47°C has been reported (1, 5, 6). Strains with temperature sensitivity are generally isolated by mutagenic techniques such as treatment with nitrosoguanidine (1). Mutant spores with defective germination systems, RNA synthesis, protein synthesis, DNA synthesis (5), and cell development and division systems (6) during outgrowth have been observed. These strains exhibited normal vegetative growth at permissive (30 to 35°C) and nonpermissive (45 to 47°C) outgrowth temperatures.

Heating is known to induce a number of changes in spores through the process of activation (2). Faster germination rates, less exacting germination requirements, increased metabolic activity (17), and changes in spore proteins and enzymes (29) have been reported. Activation has also been shown to cause apparent increases in populations of some spore suspensions (12). Germination is a degradative process that has been extensively reviewed (11). During germination, spores lose heat resistance and refractivity. Proteins (24) and RNA are degraded to provide components for subsequent synthesis occurring during outgrowth (26). Outgrowth of spores is a highly ordered process (26, 30). Synthesis of RNA commences, followed shortly thereafter by protein synthesis and finally by DNA synthesis. Enzymes are synthesized in a specific order and only for limited periods of time (34).

The appearance of germinating and outgrowing spores under phase-contrast microscopy also follows an ordered progression. Spores turn from phase bright to dark during germination; then swell, elongate, and divide during outgrowth (30). Due to the systematic patterns in spore outgrowth, morphological observations may provide some insight into metabolic functions involved in inhibition.

In this paper, we intend to show that initial heat treatments can induce subsequent temperature sensitivity in B. cereus spores during outgrowth. This sensitivity is expressed both during cooling and at a constant temperature of 45°C. Attempts to repair damage and speculation on the metabolic function involved in heat-induced temperature sensitivity will be made.

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MATERIALS AND METHODS

Spore preparation. B. cereus F4810/72 spores were prepared on fortified nutrient agar and stored in distilled water at 4 ± 2°C as previously described (15).

Enumeration procedure. Samples were diluted in 0.1% peptone water and plated on plate count agar (Difco Laboratories) by the pour plate technique (8). Plates were incubated at 30°C for 24 ± 4 h before enumeration of colonies. Initial trials indicated that 24-h plate count agar counts of stressed spores were equal to those on mannitol-egg yolk-polymyxin
agar (19) and greater than those on Trypticase soy agar (BBL Microbiology Systems) at 24 or 48 h (data not shown).

Germination and outgrowth measurements. Germination was evaluated by one of two procedures. Loss of heat resistance was employed to monitor germination rates. Samples (1 to 2 ml) were transferred to 13- by 100-mm tubes and heated at 70°C for 15 min before plating.

Microscopic examination was used in studies on germination and outgrowth. A drop of spore suspension was dried on a clean glass slide at ca. 65°C. Dried samples were subsequently rehydrated and examined at 1,000 × magnification with a phase-contrast microscope (Nikon). One hundred spores were scored as phase bright, phase dark, swollen, elongated, dividing (30), or ghost. A ghost had the appearance of a hollow shell. Judgment was required to distinguish one stage from another; therefore, intermediate stages (e.g., between swollen and elongated stages) were occasionally enumerated. Generally, the order in which sample slides were examined was randomized to remove bias, and each slide was examined twice.

Heat activation and inoculation procedures. Unless otherwise indicated, spores were heat activated at 70°C for 15 min in distilled water. Test media were held at ≤3°C during inoculation for <5 min to prevent germination.

Germination and survival during cooling. Heat-activated spores (10⁶/ml) in Trypticase soy broth (TSB; BBL) were heated rapidly to 90°C and cooled to 10°C at a rate of 10°C/h as previously described (16a). Viability and germination (loss of heat resistance) were monitored periodically during cooling. The same procedure was used for samples heated rapidly to 80°C before cooling.

Treatment of spores at constant temperatures. Heat-activated spores in TSB were heated at 90°C for 20 min and then transferred to water baths at 35, 45, or 55°C. Surface plating on mannitol-egg yolk-polymyxin was used for enumeration for samples immediately after heating and after 1 and 2 h of incubation. Two tubes for each treatment were evaluated at each sampling time.

Germination and survival in L-alanine and phosphate buffer at 45°C. Sodium phosphate buffer (36 mM, pH 7.0) with and without 1 mM L-alanine (L-Ala and buffer, respectively) were sterilized by autoclaving (121°C for 15 min). Tubes of buffer and L-Ala (0.9 ml) at 45°C were inoculated with heat-activated spores (0.1 ml) to achieve ca. 10⁶ spores per ml. Germination (loss of heat resistance) and survival were monitored periodically.

Variation of initial heat treatment in TSB. Heat-activated spores in TSB (ca. 10⁶ spores per ml) were heated at 70, 80, 82.5, 85, 87.5, or 90°C for 15 min. Heating at 85 to 90°C caused inactivation of a portion of the spore population. After all heat treatments (i.e., 70 to 90°C), tubes were held at 70°C for an additional 15 min. This incubation period stopped inactivation that occurred at 85 to 90°C and reduced the potential of sampling error caused by temperature/volume ratios. Tubes were then transferred to a 45°C water bath to initiate low temperature inactivation. Germination (loss of heat resistance) and viability were measured periodically. Each tube of TSB was used for one sample only. This entire segment of work was duplicated.

Inhibition of outgrowth by inhibitors of macromolecular synthesis. Minimum inhibitory concentrations of rifampin, chloramphenicol, and nalidixic acid for B. cereus were determined previously to be 0.5, 5, and 100 μg/ml (data not shown). Inhibitor solutions were filter sterilized, prepared weekly, and stored at 4 ± 2°C, with the exception of nalidixic acid, which was prepared daily. Individual tubes of TSB (10 ml) containing one of each of these inhibitors were tempered at 30 or 45°C before inoculation with heat-activated spores (ca. 10⁶/ml). Samples were periodically removed from each tube; the population was enumerated or germination and outgrowth were evaluated microscopically (or both). Before inoculation, spore suspensions contained >95% refractile spores.

Outgrowth of spores in TSB at 45°C after differing heat activation treatments. The effects of three different heat activation treatments on survival and simultaneous outgrowth were evaluated. The heat activation treatments evaluated were intended to elicit differing survival patterns at 45°C. In the first, spores were heat activated at 75°C for 15 min before inoculation of TSB (10 ml, ca. 10⁵ spores per ml) at 45°C. In the second, TSB (10 ml) at ≤3°C was inoculated (ca. 10⁶ spores per ml) and heated at 80°C for 15 min before transfer to 45°C. In the third, TSB (10 ml) at ≤3°C was inoculated with heat-activated spores (ca. 10⁶/ml), heated at 87.5°C for 15 min, held at 70°C for 15 min, and finally incubated at 45°C. Survival and outgrowth were evaluated periodically by enumeration and microscopic procedures, respectively.

Evaluation of potential injury. Heat-activated spores in L-Ala (ca. 10⁶ spores per ml) were incubated for 2 h at 45°C to induce damage. The suspension was then vortexed, and 0.1 ml was delivered into 9.9 ml of potential repair medium previously tempered at 30 or 35°C. The dilution factor was sufficient to stop L-Ala germination after transfer (data not shown). Components initially screened as potential repair agents at 35°C included 0.1% peptone, 0.1% beef extract, 0.1% soluble starch, 20% sucrose, 0.25% glucose, 0.25% dipotassium phosphate, 0.1% calcium lactate, 0.5% sodium pyruvate, and 0.2% magnesium sulfate; 1 mM L-Ala was used as a control. Further studies were conducted at 30°C in duplicate with peptone, beef extract, starch (single trial), L-Ala, and TSB with or without inhibitors of macromolecular synthesis to insure that observed increases in numbers were not the result of multiplication of cells. Penicillin G (500 U/ml; Pfizer) was used in addition to the inhibitors mentioned above.

Statistical analysis. The linear portions of 45°C survivor curves in TSB after various initial heat treatments were analyzed by least-squares regression analysis. Data from 15 to 260 min were included in regression analysis. Log transformations of the percentage of survivors were used to obtain the best estimates and 95% confidence intervals of slopes (inactivation rate constants) and intercepts. These kinetic constants were subjected to analysis of variance by using IVAN (33) and regression analysis of slope (log₁₀ scale) or intercept versus temperature by using MULTREG (32). Differences among the means of factors which had significant F-tests were tested by using the method of least significant differences (28).

RESULTS AND DISCUSSION

Germination and survival of spores during cooling. Spores heated rapidly to 90°C and cooled at a rate of 10°C/h exhibited two phases of inactivation as described previously (16a). No germination was apparent until the second phase of inactivation commenced at 44 to 46°C (Fig. 1). Germination was more rapid and more extensive than inactivation during cooling.

Spores heated only to 80°C, however, maintained a constant population throughout the cooling process (Fig. 2). These data suggest that the lack of low-temperature inactivation during cooling from 80°C observed by Johnson et al.
(16a) was not the result of inactivation of sensitive spores during slow heating (20 or 40°C/h) to 80°C. Instead, inactivation of spores at temperatures between 46 and 38°C was stimulated by the higher heat treatment.

**Effect of constant temperature incubation in TSB on spore survival.** Spores heated in TSB at 90°C for 20 min received approximately the same lethal heat treatment as spores being cooled from 90 to 80°C at 10°C/h (15). The response of heated spores to subsequent incubation at 35, 45, or 55°C is presented in Fig. 3. Significant inactivation was observed only with 45°C incubation. Maximal rates of inactivation occurred at 45 to 47°C (data not shown). Previous work has shown that germination of *B. cereus* spores does not occur at 55°C, slow germination occurs at 45°C, and optimum germination occurs at 35°C (16). Incubation at 45°C was selected for subsequent evaluation of the low-temperature spore inactivation. 

Previously reported D_{95°C}-values for *B. cereus* strain F4810/72 range from 5.6 (15) to 9.5 min (20), with a z-value of 8.9°C (15). From these data, one would not anticipate any measurable spore inactivation at 45°C (D_{45°C}, ca. 10^6 min). Our results, however, show that spore inactivation can occur at 45°C with D-values of 96 to 120 min. Therefore, inactivation of this strain at 45°C seems to occur by a mechanism other than that involved in "normal" thermal inactivation of bacterial spores.

**Effect of germination on inactivation.** Survival and germination of *B. cereus* spores in L-Ala and in buffer are presented in Fig. 4. Limited germination and no inactivation were apparent in buffer, whereas L-Ala supported significant initial germination and inactivation in 2 h. These data show that germination is required for 45°C inactivation to proceed. These data are also in agreement with the lack of spore inactivation at 55°C, a temperature above the germination temperature range (16).

**Effect of initial heat treatment on spore survival and germination at 45°C.** Figure 5 illustrates the 45°C survival of *B. cereus* spores previously subjected to heat treatments at 70 to 90°C in TSB. The rate of inactivation of *B. cereus* spores in TSB at 45°C increased when the temperature of the initial heat treatment was increased. There was no appreciable lag before the onset of inactivation (P = 0.979). No inactivation occurred when spores were heated at 70°C. After initial heat treatments ranging from 70 to 90°C, the inactivation rate constant at 45°C can be determined by the following equation: \( \log_2(-\text{slope}) = 0.1135(T - 12.24) \), where \( T \) is the temperature (degrees Celsius) of the initial heat treatment (\( r^2 = 0.904 \)).

Figure 6 illustrates the effect of initial heat treatments on germination of spores at 45°C, calculated as the percentage of the viable population that was heat resistant at each sampling time. There is little difference in maximal germination among various initial heat treatments, with the exception of 90°C. This higher heat treatment may have caused a slightly lower level of germination. Severe heat treatments
HEAT-INDUCED OUTGROWTH SENSITIVITY IN B. CEREUS

FIG. 5. Survival of B. cereus F4810/72 spores in TSB at 45°C after initial 15-min heat treatment at 70°C, 80°C, 82.5°C, 85°C, 87.5°C, or 90°C and a 15-min holding period at 70°C. Solid lines represent data used in regression analysis.

FIG. 6. Percentage of germinated B. cereus F4810/72 spores during 45°C incubation in TSB previously heated for 15 min at 70°C, 80°C, 82.5°C, 85°C, 87.5°C, or 90°C, with holding at 70°C for an additional 15 min (see the text for details).

FIG. 7. Effect of 30°C incubation in potential repair media on B. cereus spores after inactivation at 45°C in L-Ala. Potential repair media included TSB (A), 0.1% beef extract (B), 0.1% peptone (C), L-Ala (D), and 0.1% soluble starch (E) with and without inhibitors of macromolecular synthesis. Symbols: L-Ala at 45°C ( ), no inhibitor ( ), nalidixic acid ( ), chloramphenicol ( ), rifampin ( ), and penicillin ( ).

(e.g., 90°C and perhaps 87.5°C) may have reduced the initial germination rate. These data demonstrate that the increased rate of 45°C inactivation observed with increased initial heat treatments was not due to the stimulation of more rapid germination.

Response of spores in potential repair media. Figure 7 presents results representative of trials on the potential repair of spore damage induced by incubation in L-Ala at 45°C. Greater than 80% of the initial population was inactivated during 2 h of incubation at 45°C in L-Ala before the inoculation of recovery media with or without inhibitors of macromolecular synthesis. Little difference was noted among the responses for L-Ala with or without inhibitors (Fig. 7D). The same was true for starch (Fig. 7E). The increase of the population in TSB, beef extract, and peptone was attributed to cell multiplication rather than spore repair since no increase in population was observed in the presence of any inhibitor. In these growth media, chloramphenicol, an inhibitor of protein synthesis, appeared to be less inhibitory than rifampin, nalidixic acid, and penicillin. Results for pyruvate, calcium lactate, magnesium sulfate, sucrose, glucose, and potassium phosphate at 35°C were similar to those of starch (data not shown).

Rappaport and Goepfert (22) reported that injury of vegetative cells of B. cereus occurred at 47°C. Repair of this damage was possible in 0.1% peptone and required RNA synthesis. If this same type of damage occurred in our work, the spores apparently lacked this repair machinery. A previous report on the effect of cooling B. cereus spores in TSB and in a rice-beef extract medium from 90°C suggested that 45°C inactivation occurred in both media (16a). The apparent viable population increased during cooling from 35 to 10°C at a rate of 5°C/h in rice-beef extract; however, the population remained constant through this temperature range in TSB. There appears to be a component in rice that either protects spores from lethal damage or promotes growth at lower temperatures of the cooling process, suggesting that apparent 45°C inactivation may indeed be repairable.

Effect of inhibition of macromolecular synthesis during germination and outgrowth of spores. The effect of inhibitors of protein, RNA, and DNA syntheses on outgrowth of B. cereus spores in TSB at optimum temperature (30°C) and at 45°C are presented in Fig. 8. At 30°C without inhibitors, B. cereus spores progressed rapidly from phase bright to phase dark, a majority of the spores were at the swollen stage by 1
h, and elongation and division were apparent by 2.5 h. Inhibition of RNA synthesis by rifampin had no effect on germination; however, swelling was limited and delayed. Furthermore, inactivation of <50% of the population occurred by 2.5 h. Swelling occurred normally in the presence of chloramphenicol, an inhibitor of the protein synthesis, and outgrowth did not proceed beyond this stage. The use of nalidixic acid, generally an inhibitor of DNA synthesis, delayed swelling, whereas elongated cells were observed after 2.5 h. By 4 h, many of these cells were extremely long and twisted, but no dividing cells were evident. Nalidixic acid did not inactivate outgrowing spores within 4 h at 30°C.

When cells were incubated at 45°C without inhibitors, the change from phase bright to dark was slower than that observed at the optimum temperature (Fig. 8). Swelling and elongation were also delayed. No division was apparent in 4 h, and ca. 40% of the initial spore population was inactivated by this time. In the presence of rifampin, swelling was halted almost completely, and the population was reduced by 80% within 2.5 h. Chloramphenicol again halted outgrowth at the swelling stage, and inactivation of the population lagged behind the rate observed with inhibition of RNA synthesis. The response to nalidixic acid was similar to that for rifampin in both stage of outgrowth affected and inactivation of the population.

These results agree with observations reported by others. Garrick-Silversmith and Torriani (7) reported that phase darkening of spores occurred before macromolecular synthesis. The synthesis of RNA has been shown to commence immediately after germination and peak during swelling (31). Swelling of spores is due primarily to the uptake of water and some nutrients (30); therefore, inhibition of RNA synthesis should result in primarily phase dark spores. Inhibition of protein synthesis during outgrowth prevented cell wall synthesis (31); thus elongation would not occur. Temperature-sensitive mutants lacking protein synthesis were halted at the stage of swelling during outgrowth (6).

Outgrowth proceeds in the absence of DNA replication (10). Ginsberg and Keynan (9) reported that mutants defective in DNA synthesis during outgrowth at 44°C formed long, curved cells with no division septa. The same morphology was observed with outgrowing parental strains incubated in “rich” media containing nalidixic acid. Hecker (13) reported that nalidixic acid may inhibit RNA synthesis in addition to DNA synthesis. In our studies, the synthesis of RNA, rather than DNA, may have been inhibited by nalidixic acid at nonpermissive temperatures, halting outgrowth at the phase dark or partially swollen stage.

It appears that morphological characteristics of spores during outgrowth may be used as indicators of systems that are sensitive to inactivation treatments—a population of primarily phase dark spores resulting from inhibition of RNA synthesis, a majority of swollen spores indicating defective protein synthesis, and elongated cells suggesting interference with DNA replication.

Stage of outgrowth sensitive to 45°C inactivation. Three heat activation treatments were employed to elicit differing levels of spore inactivation at 45°C. A 75°C heat treatment before inoculation of TSB at 45°C did not cause 45°C inactivation of B. cereus spores (Fig. 9A). Spores progressed normally through germination and outgrowth, and elongated spores were evident before 3 h. Heating of spores in TSB at 80°C resulted in a 50% reduction in the population at 45°C, and outgrowth appeared to be halted at swelling (Fig. 9B). Heating at 87.5°C before incubation at 45°C resulted in 45°C inactivation of 90% of the population, and outgrowth was halted at swelling (Fig. 9C). The cessation of outgrowth at the stage of swelling suggests that protein synthesis may be involved in inactivation of B. cereus F4810/72 spores at 45°C.

The role of protein synthesis in 45°C inactivation of germinated B. cereus spores is speculative. Three possible
functions should be considered: (i) incubation at 45°C may inhibit the synthesis of proteins that are required for survival of germinated spores at elevated temperature; (ii) heat activation before incubation at 45°C may inhibit the synthesis of proteins that are detrimental to the outgrowing spore; or (iii) protein synthesis is not altered, but the effects of an unrelated mechanism are expressed concurrently with protein synthesis. The second possibility is not consistent with data on the effect of chloramphenicol on spores incubated at 45°C (Fig. 8). Inhibition of protein synthesis at 45°C resulted in a faster rate of inactivation than that occurring in TSB without inhibitor. Protein synthesis, therefore, appears to supply some protection of spores at 45°C.

The spore proteins degraded during germination (25) appear to be similar to "heat shock" proteins (23). Both are associated with DNA (21, 27) and increase the melting point of DNA (25). Heat shock proteins are thought to protect DNA of bacteria, yeasts, and other species subjected to temperature up-shifts (4) and are produced by sporulating yeasts not previously exposed to heat (18). Bacterial spore proteins could serve the same function in B. cereus. Accelerated degradation of these proteins by activated proteases (29) would explain accelerated inactivation of germinated spores. This hypothesis is conjecture, and much work is required to verify these comments. Work in this area could also add information on the mechanism of heat resistance of bacterial spores.

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