Heat Activation and Heat-induced Dormancy of *Bacillus stearothermophilus* Spores[1]

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Abstract

Finley, N. (University of Missouri, Columbia), and M. L. Fields. Heat activation and heat-induced dormancy of *Bacillus stearothermophilus* spores. Appl. Microbiol. **10**:231–236. 1962.—Heat-induced dormancy was observed when spores of two strains of *Bacillus stearothermophilus* were heated in distilled water at 80, 90, and 100 C. At temperatures above 100 C, true activation occurred; however, maximal activation was not achieved until temperatures of 110 to 115 C were employed. A heat treatment of 115 C for 3 min was required to induce maximal activation in one suspension of strain 1518 spores, whereas a heat treatment of 110 C for 7 to 10 min was adequate for the other suspension of strain 1518 spores. Spores from both strain M suspensions required heat treatments of 110 C for 9 to 15 min for maximal activation. The degree to which the spores could be activated was strain dependent and variable among spore suspensions of the same strain.

The germination and outgrowth of all spores, regardless of strain and suspensions source, were significantly reduced when the spores were heated in M/120 phosphate buffer at maximal or near maximal activating temperatures. It was suspected that phosphate lowered the heat resistance of the spores to the extent that the heat treatments were lethal to a portion of the populations.

The association of heat with spore germination has a unique place in the biology of the bacterial endospore. Curran and Evans (1944, 1945) were the first workers to demonstrate systematically that sublethal heat (62 to 95 C) could induce dormant spores to germinate. For most of their work, thermotolerant, mesophilic strains were used; however, selected thermophilic strains were also investigated, including strain 1518 of *Bacilius stearothermophilus*. This thermophile is of particular interest to the food processor and food microbiologist because the spore form is extremely heat resistant and the vegetative form, which is capable of growth at temperatures of 70 C or slightly higher, is responsible for flat sour spoilage of low acid, canned foods.

The first reference to activation of endospores at temperatures greater than 100 C was made by Brachfeld (1955). In his studies of *B. stearothermophilus* strain 1518, he found that spores suspended in distilled water and heated at 105 C for 5 min gave maximal plate counts. Brachfeld also introduced the concept of "heat-induced dormancy." He found that subjecting spores of strain 1518 to temperatures of 55 to 85 C resulted in significantly decreased plate counts. Titus (1957), working with a sugar isolate of *B. stearothermophilus* which he referred to as strain Z, reported that heating spores of this strain at 230 F (110 C) for 6 to 10 min resulted in maximal activation. These observations by both Brachfeld and Titus are of particular interest because they indicate that *B. stearothermophilus* spores require a greater amount of heat for activation than do spores of other *Bacillus* species.

In all of the studies mentioned above, the type of medium in which the spores were heated was noted to have some effect on activation. However, only Brachfeld (1955) investigated phosphate buffers, the heating meduim commonly used in thermal death time studies. He observed that M/20 phosphate buffer significantly decreased average plate counts, whereas M/80, M/200, M/800, and M/2,000 phosphate buffers had no effect on germination and outgrowth. Similarly, Williams and Hennessee (1956) noted that the apparent heat resistance of *B. stearothermophilus* spores was affected by the concentration of the phosphate buffer in which the spores were heated. With decreasing molal concentration of phosphate over the range of M/15 to M/120, resistance of the spores to lethal heat (120 C) increased. Most of the observed differences, however, were attributed to a carryover of phosphate into the plating medium rather than to combined phosphate-heating effects. Williams and Hennessee suggested that the increased resistance of the spores in M/120 phosphate buffer might be due to increased spore germination and outgrowth.

Since it was necessary to know the maximal activation temperatures and times for later studies involving activation of *B. stearothermophilus* spores in food extracts, and since different values have been reported in the literature for the maximal activation of *B. stearothermophilus* spores (Brachfeld, 1955; Titus, 1957), the work reported herein was undertaken. It was felt also that the effect of M/120 phosphate buffer at maximal activation temperatures should be included since it was to be used as a reference menstruum.

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MATERIALS AND METHODS

Cultures. Two strains of *B. stearothermophilus* were used in this study. One was the National Canners Association strain 1518 and the other was an isolate from cream-style corn. The latter will be referred to as strain M in this report. The identities of both strains conformed according to the description of the species provided by Gordon and Smith (1949) and Smith, Gordon, and Clark (1952).

Preparation of spore suspensions. Four spore suspensions, two of each strain, were prepared for this study. Sporulation was carried out in Roux flasks containing approximately 200 ml of a modified nutrient agar composed as follows: nutrient agar, 1.5%; supplemental plain agar, 0.5%; glucose, 0.05%; $MnSO_4 \cdot H_2O$, 30 ppm. Titus (1957) and Ordal (1957) have shown that small quantities of manganese and a readily available carbon source greatly enhances sporulation. The supplemental agar was considered necessary for adequate moisture retention during the extended incubation period. Twenty-four-hour, nutrient broth cultures were used to inoculate the agar surfaces. Nine flasks were prepared at the same time for each of the two strains. One preparation of the two strains was incubated at 52 C for 6 days, whereas a second preparation of the two strains was incubated at 52 C for 11 days. The reason for the difference in incubation times was that a delayed sporulation occurred with both strains in the second set. Earlier experience indicated that the degree of cleanliness eventually attained in the resulting spore suspensions depended upon the percentage of spores initially present. Consequently, it was decided to allow the second preparations to incubate for a longer period of time to increase the ratio of spores to vegetative cells.

The first step in the cleaning procedure was to flood the flasks with sterile distilled water. The surface growths then were rubbed gently with a sterile glass rod and the resulting suspensions collected in sterile flasks. Approximately 250-ml aliquots were transferred to sterile centrifuge bottles and centrifuged for 15 min at 2,000 rev/min (RCF = 800). Approximately three-fourths of the supernatant was discarded and the remaining supernatant was used for resuspension of the compacted spores and vegetative cells. Suspensions of the same strain were combined and the resulting collections recentrifuged, decanted, and made up to volume (50 ml) with sterile distilled water. These were transferred to sterile flasks containing magnetic stirring bars.

An enzyme preparation, lysozyme,[2] was used to eliminate vegetative cells from the suspensions without resorting to a heat treatment. Titus (1957) demonstrated clearly that this digestive enzyme system is capable of lysing vegetative cells of *B. stearothermophilus* without adversely affecting the spores. A 0.5 mg per ml concentration of lysozyme was added to the suspensions and the mixtures incubated at 52 C for 2 hr. During this time they were agitated constantly with a magnetic stirrer. The adequacy of lysis was determined by preparing smears of the suspensions and examining them microscopically. The smears were stained with saturated, aqueous malachite green for 7 min over boiling water, washed with tap water, and counterstained with 0.25% aqueous safranin (spores stain green, whereas vegetative cells stain red). No intact vegetative cells were found, thus indicating that the 2-hr incubation time was adequate; however, considerable amounts of vegetative debris were observed.

To rid the suspensions of lysozyme and vegetative debris which tended to clump the spores, the suspensions were filtered first through a column (6 by ½ in.) of tightly packed glass wool by means of aspirator suction, and then washed ten times with sterile distilled water by means of centrifugation. The washing procedure was conducted as follows. Each filtered suspension was transferred to sterile, 50-ml centrifuge tubes fitted with rubber caps and centrifuged for 12 min at 3,000 rev/min (RCF = 2,000). The supernatants were decanted and discarded. Small quantities of sterile distilled water were added with intermittent shaking to resuspend the spores until the original 50-ml volume was attained. The procedure then was repeated. Even after ten washes, not all debris had been removed, but it was reduced to a point considered to be insignificant. Additional washes would have resulted only in the loss of additional spores.

Finally, the suspensions were transferred to sterile, Waring microblenders and blended for 1 hr in a 0 F storage room to insure complete disintegration of spore clumps. The cold environs quickly dissipated the heat generated by the blender, thus eliminating any possibility of unintentional spore activation. The spores were stored at 4 C until used in subsequent experiments. Fifty per cent or more of the initial spore populations probably were lost in carrying out the foregoing steps. However, adequate numbers were available for the study since only fractions of the four suspensions prepared were used eventually. The number of viable, non-heat-activated spores in the final suspensions, as determined by dilution plate counts with dextrose-tryptone agar,[3] ranged from 2×10^7 to 9×10^7 spores per ml.

Maximal heat activation in distilled water. Diluted suspensions calculated to yield nonheated spore counts of 500 to 750 per ml were prepared by transferring aliquots of the original suspensions to 500-ml prescription bottles containing 300 ml of sterile distilled water and a small quantity of glass beads. After vigorously shaking for 1 min, 1-ml aliquots of the diluted suspensions were transferred to screw-capped test tubes (16 by 150 mm) containing 9 ml of distilled water. The test tube caps were equipped with rubber liners to insure an airtight seal. This 1:10 dilution reduced the number of unheated viable spores per ml to a

[2] Nutritional Biochemicals Corp., Cleveland, Ohio.

[3] Difco Laboratories, Inc., Detroit, Mich.

countable range (50 to 70), yet allowed for a threefold heat activation increase without subsequent dilutions.

Several series of tube dilutions were prepared from each diluted suspension in the previously described manner. These were subjected to temperatures ranging from 80 to 115 C. Spores from the first suspensions of both strains 1518 and M were heated at 80, 90, 100, 105, 110, and 115 C, whereas temperatures of 105, 110, and 115 C were used for the second suspensions. Thirteen tube dilutions constituted a series; one tube dilution served as an unheated control and the others were subjected to heat for varying lengths of time up to 15 min. The observed heating times did not include the initial "come-up-times"; these latter were 80 C, 2½ min; 90 C, 3 min; 100 C, 4 min; 105 C, 8 min; 110 C, 9 min; and 115 C, 12 min. The first tube was removed from the heating bath after the distilled water in which the spores were suspended reached the designated temperature. This was considered "0" time. Thereafter, the tubes were removed at 1-min intervals for the next 10 min, the last tube being removed after heating for 15 min. As each tube was taken from the heating bath, it was placed immediately in cold tap water to bring the temperature of the spores back to room temperature as quickly as possible.

Heat treatments at or below 100 C were carried out in a 100 C water bath; those above 100 C were carried out in an oil bath. The oil bath assembly consisted of another 100 C water bath equipped with 2 auxiliary, 500-w, knife-blade heaters and a supplemental thermostatic control. The water and mineral oil heating media were constantly circulated with air-driven agitators. A 150 C mercury thermometer sealed in an identical test tube (16 by 150 mm) containing 10 ml of distilled water was used to record the internal temperatures of the tubed suspensions.

Enumeration of the viable spores was performed by plating 1-ml quantities of the suspending menstrua with Dextrose Tryptone agar.[3] Triplicate plates were poured for each treatment and all plates were incubated at 52 C for 48 hr.

Effect of M/120 phosphate at maximal and submaximal activation temperatures. Spores from both suspensions of each of the two strains were heated in distilled water and M/120 Sorensen's phosphate buffer (pH 7.1) in repeated experiments. Procedures paralleled those just described for the determination of maximal heat activation in distilled water except that only duplicate tube dilutions were prepared with each heating menstruum and heating conditions were constant for each strain. Spores of strain 1518 were heated at 110 C for 7 min, whereas strain M spores were heated at 110 C for 9 min.

Results

Maximal heat activation in distilled water. The collected count data have been converted to percentages based on the unheated controls and expressed graphically to illustrate the responses of the two spore strains to the various heat treatments. In most instances the time-temperature responses resulted in curvilinear rather than straight-line relationships. To demonstrate these relationships more accurately, the graph lines as shown in Fig. 1, 2, 3, and 4 were computed statistically from first, second, and third degree equations. The points plotted about each line are the actual percentage data.

The heat activation curves for the first-suspension spores of strain 1518 are illustrated in Fig. 1. Heat treatments of 80, 90, and 100 C apparently deactivated rather than activated the spores. On the basis of unheated, viable counts (100% level), the 80 C heat treatment reduced counts approximately 50%. The 100 C heat treatment was intermediate since it reduced counts approximately 58%. Greatest reduction was found in the 90 C heat treatment where a reduction of approximately 65% was observed. The length of heating time at these temperatures had little or no effect. Had the heating time been extended beyond 15 min, it seems likely that activation or possibly less deactivation might have occurred. The 105 C curve indicates that the spores were activated slightly after 3 min, and the percentage of activation slowly increased with time up to 6 min. Here it remained fairly stationary over the next 4 min. Between the 10- and 15-min interval further activation occurred. After heating for 15 min at 105 C, the viable spore count increased 42% over the unheated control. Heating at higher temperatures indicated that the spores of this suspension were susceptible to still greater activation. At 110 C, the heated spore counts increased approximately 85% over the unheated spore counts after the spores had been heated for 7 to 10 min.

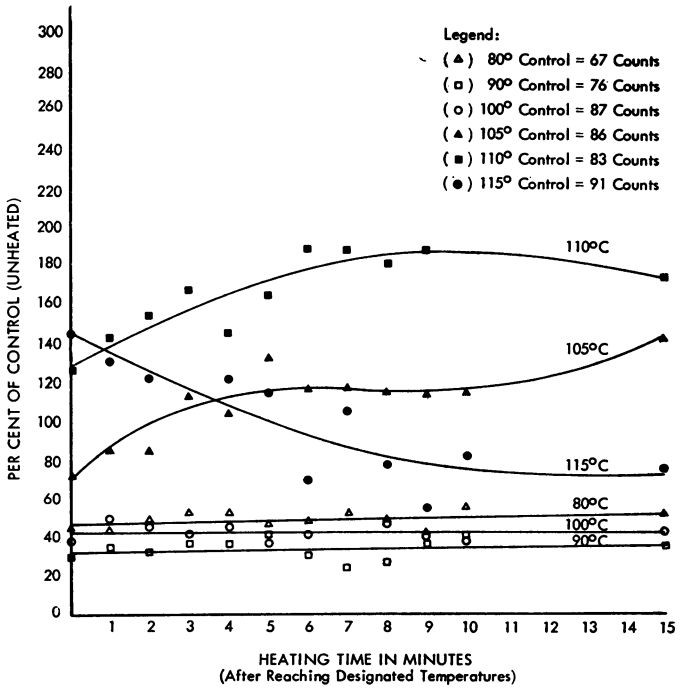

FIG. 1. *Heat activation of the first-suspension, strain 1518 spores in distilled water.*

This time-temperature relationship proved to be the maximal activation point for the suspension. Additional heating at 115 C only resulted in decreased counts.

When second-suspension spores of strain 1518 were subjected to temperatures of 105, 110, and 115 C, the results shown in Fig. 2 were obtained. The graphic presentations indicate that the two preparations of strain 1518 behaved differently. At 105 C, there was only a small amount of activation with the second preparation of spores. At 110 C, the peak activation was observed after heating for 8 to 10 min, but then only amounted to a 20% increase in viable counts over the unheated control. Maximal activation for the second suspension was obtained after heating the spores for 3 min at 115 C. This was a considerably higher heat requirement than was noted for maximal activation of the first-suspension spores. The increase in viable counts at the 3-min level was computed to be 60%.

The strain M spores responded somewhat more uniformly to heating. Figure 3 shows the heat activation responses of the first-suspension strain M spores. Results of heat treatment at 80, 90, and 100 C are similar to those previously reported for the first-suspension spores of strain 1518. However, the percentage of reduction in viable counts ranged only from 10 to 15%, and the 100 C treatment rather than the 90 C treatment was responsible for the maximal deviation. When the temperature was increased to 105 C, an activation increase of 150 to 160% was noted. This increase is considerably greater than any increase found for the strain 1518 spores, but still did not represent the maximal activation. Heating for 9 to 15 min at 110 C caused viable spore counts to increase about 260% over the unheated control. Approximately the same percentage activation was encountered initially with the 115 C heat treatment, but viable spore counts quickly dropped with increasing time. By the end of the 15-min observation period, the viable counts had decreased approximately 150%. Heating at 110 C for 9 to 15 min appeared to be the heat treatment inducing maximal spore activation for this suspension.

Presented in Fig. 4 are the activation responses of the second-suspension strain M spores. For the most part, the activation patterns are similar to those of the first-suspension spores, but there are noticeable differences in the magnitude of activation encountered. At 105 C, the maximal activation amounted to only a 60% increase in viable counts as compared to the 150% increase obtained for the first-suspension spores. The 110 C temperature, as it did with the first-suspension spores, induced maximal germination after heating 9 to 15 min. This increase in viable counts was computed to be approximately 230%, representing a 30% lower count than that computed for the first-suspension spores. The 115 C temperature resulted in a 220% decrease in viable counts over the 15-min observation period. Again, a difference in the magnitude of response was noted between the two suspensions; the decrease in viable spore counts for the second suspension was much greater (220% as compared to 150%) and at a much more rapid rate. In the case of both suspensions, however, maximal activation occurred after heating the spores at 110 C for approximately 9 min.

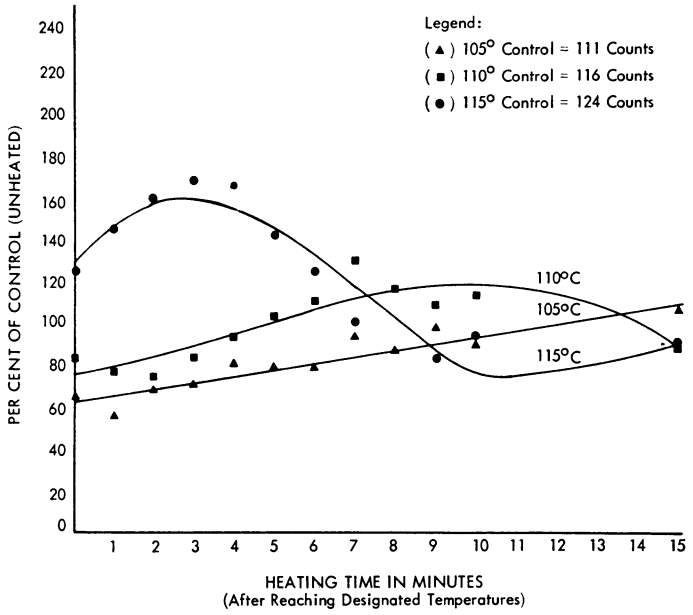

FIG. 2. *Heat activation of the second-suspension, strain 1518 spores in distilled water.*

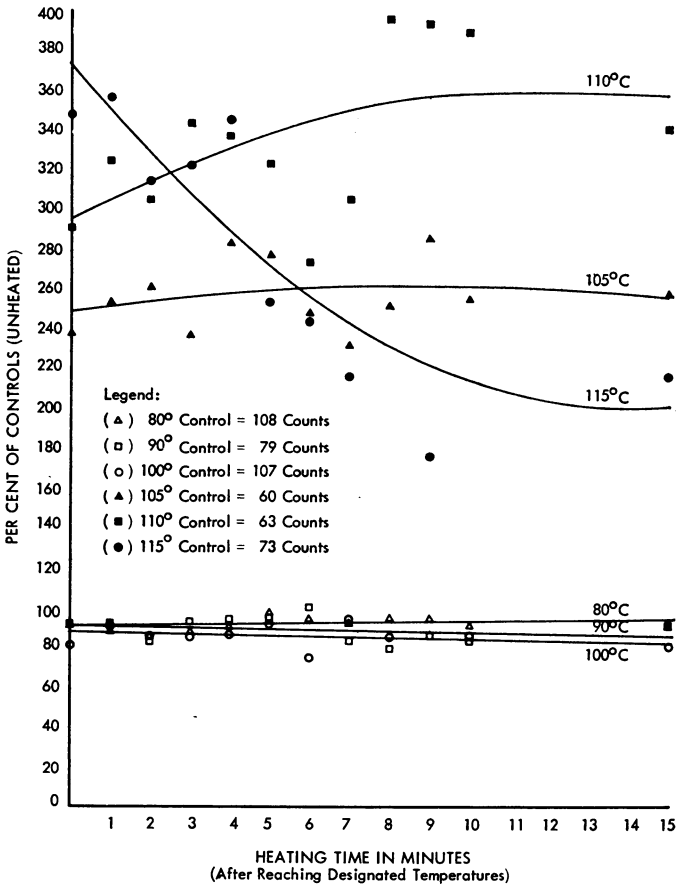

FIG. 3. *Heat activation of the first-suspension, strain M spores in distilled water.*

Effect of M/120 phosphate buffer at maximal and submaximal activation temperatures. Data presented in Table 1 show that the M/120 phosphate buffer had a highly significant influence on the heat activation responses of both the strain 1518 and M spores. This occurred regardless of the fact that second-suspension spores of strain 1518 were heated only at a submaximal activation temperature (110 C for 7 min). In all cases except one, plate counts derived from spores heated in phosphate were significantly lower than the corresponding distilled water controls. Even in the excepted case, the average phosphate plate count was lower, but the difference was too small to be considered statistically significant. The data also indicate that the presence of phosphate reduced the strain M plate counts much more on a percentage basis than it did the strain 1518 plate counts.

Variability within the data is apparent and at first glance there is a tendency to associate it with increasing age of the spores. However, no definite patterns or trends were established concerning age in these experiments.

Discussion

Decreased plate counts or deactivation occurred when spores were subjected to sublethal temperatures of 100 C or less. This phenomenon, which Brachfeld (1955) called heat-induced dormancy, was transient, however, since subjecting the spores to a more drastic heat treatment (105 to 115 C) resulted in true activation. Thus, there is evidence that sublethal heat is capable of inducing two totally different, but closely related, responses in *B. stearothermophilus* spores. Greater heat-induced dormancy was noted in the first-suspension spores of strain 1518 than in the first-suspension spores of strain M, indicating that the degree with which heat-induced dormancy is produced is strain dependent. Second suspension spores were not subjected to the lower, sublethal temperatures; consequently, within-strain variation with regard to this phenomenon was not directly assessable. However, the fact that second-suspension spores of both strains were activated less than the first-suspension spores does indicate a deeper dormancy existed from the outset in the second suspensions. The only apparent explanation for this indicated variance between suspensions is that the prolonged incubation involved in producing the second spore crops established conditions conducive to inducing dormancy for a longer period of time. Brachfeld has shown heat-induced

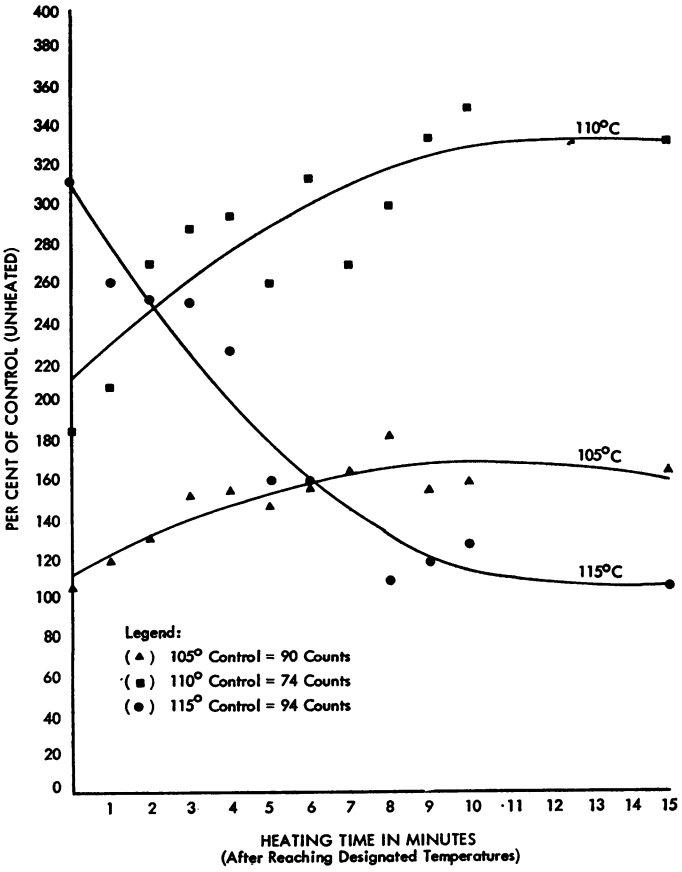

FIG. 4. *Heat activation of the second-suspension, strain M spores in distilled water.*

TABLE 1. *Effect of M/120 phosphate on heat activation of Bacillus stearothermophilus spores*

Age of suspension in days	Strain; suspension*	Mean colony count		Mean difference‡	Per cent difference
		Distilled water	M/20 120 phosphate†		
37	1518; I	147.2	115.2	32.0***	21.7
41	1518; I	55.2	48.7	6.5	11.8
43	1518; I	88.8	66.7	22.1***	24.9
48	1518; I	59.2	43.2	16.0***	27.0
51	1518; I	50.7	32.2	18.5**	36.5
57	1518; I	70.5	50.2	20.3***	28.8
31	1518; II	120.3	73.3	47.0***	39.1
34	1518; II	152.8	76.2	76.6***	50.1
35	1518; II	147.0	88.2	58.8***	40.0
36	1518; II	125.2	70.8	54.4***	43.5
37	1518; II	124.7	69.8	54.9***	44.0
38	1518; II	112.5	70.0	42.5***	37.8
37	M; I	215.3	122.2	93.1***	43.2
41	M; I	199.8	61.3	138.5***	69.3
43	M; I	186.3	86.3	100.0***	53.7
48	M; I	186.0	89.8	96.2***	51.7
51	M; I	176.7	80.0	96.7***	54.7
57	M; I	188.8	89.5	99.3***	52.6
31	M; II	170.8	106.5	64.3***	37.6
34	M; II	248.8	76.8	172.0***	69.1
35	M; II	248.7	101.0	147.7***	59.4
36	M; II	240.0	103.7	136.3***	56.8
37	M; II	234.0	80.3	153.7***	65.7
38	M; II	233.0	94.5	138.5***	59.4

* Strain 1518 spores were heated at 110 C for 7 min; strain M spores were heated at 110 C for 9 min.

† Mean of six replicates.

‡ (**) = Significant at 1.0% level as determined by the "t" test; (***) = significant at 0.1% level as determined by the "t" test.

dormancy to be operative in the incubation temperature zone of this thermophile. This, plus the fact that strain 1518 spores are more susceptable to induced dormancy, provides an explanation why the second-suspension spores of this strain required a more drastic heat treatment (115 C for 3 min) for maximal activation than did the less dormant spores of the other strain 1518 suspension (110 C for 7 to 10 min) and the two strain M suspensions (110 C for 9 to 15 min). Except in the case of this one suspension, the heat activation requirements reported herein closely parallel those reported by Titus (1957) for a companion strain of *B. stearothermophilus*, but are at variance with the heat activation requirements determined by Curran and Evans (1945) for strain 1518. Curran and Evans were able to achieve activation in their strain 1518 spores after heating for 10 min at 95 C. At no time was activation encountered in the present study until the temperature of the heating menstruum exceeded 100 C.

As indicated above, the degree of activation was quite variable between the two strains and, to a lesser extent, between suspensions of the same strain. Differences in the degree of heat-induced dormancy can probably account for most of the suspension variation, however, other factors may be involved at the strain level. During the taxonomic studies of both strains, it was noted that strain 1518 consistently grew at 70 C, whereas strain M grew at 65 C, but not at 70 C. This inability of strain M to grow at 70 C may indicate that there is a comparable intrinsic difference in the heat activability of the spores of these two strains. Williams and Hennessee (1956) have shown that there is considerable difference in the thermal death times of various strains of *B. stearothermophilus* spores. Thus, if one accepts the hypothesis of Desrosier and Heiligman (1956) that the factors which affect heat activation are related to the factors which are associated with the progress of thermal death, one would expect different strains of this organism to vary in their degree of activation in relation to their F values.

Comparative plate count data obtained from spores suspended and heated in distilled water and in M/120 phosphate buffer at both maximal and submaximal activation temperatures showed that the phosphate menstruum had a definite, inhibitory effect on germination and outgrowth. Consequently, the findings of our study are not in agreement with the findings of Williams and Hennessee (1956). The comparative data also indicated that there is a direct relationship between the magnitude of heat activation and the magnitude of phosphate inhibition. This was illustrated by the fact that the more pronounced inhibition occurred in the strain M spores which were heat-activated to a much higher degree than were the strain 1518 spores.

The inhibitory role of phosphate in this study may be closely akin to the phosphate effects noted by Ordal and Lechowich (1958) in their studies on the thermal destruction of spores of *Bacillus coagulans* var. *thermacidurans*. These workers observed that, whenever the phosphate concentration of the heating menstruum was increased or decreased beyond M/40, the death rate of the spores was accelerated. Their tentative hypothesis for this twofold effect was that the higher concentrations of phosphate increased the death rate because the phosphate anion accelerated the release of thermostabilizing cations (calcium and manganese) from the spore, whereas the lower concentrations of phosphate increased the death rate because of osmotic and ion concentration differences between the spores and the heating menstruum. In either case, the presence of the phosphate ion in combination with heat apparently disrupts the mineral-dipicolinic acid complexes in the spore which are responsible either partially or totally for the thermal resistance of the spore. Under such circumstances, the 110 C heat treatments used in this study could have had a lethal effect on a portion of the spore populations.

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