Effect of Heat on Spores of Rough and Smooth Variants of *Bacillus stearothermophilus*[1]

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Received for publication 7 September 1962

Abstract

Fields, M. L. (University of Missouri, Columbia). Effect of heat on spores of rough and smooth variants of *Bacillus stearothermophilus*. Appl. Microbiol. **11**:100–104. 1963.— Spores of variants of *Bacillus stearothermophilus* were subjected to activating and lethal temperatures. Spore suspensions which were incubated longer contained a higher percentage of spores of the rough variant. The effect of sublethal heat on spore suspensions containing mixed variants (rough and smooth) was difficult to measure at sublethal temperatures (110 C), since the rough variant was not as heat-resistant. While the rough variant was activated in a shorter time, the smooth variant was not activated; when the smooth variant was activated, the rough was killed. A higher percentage of the smooth variant was forced into dormancy after being held at 50 C for 30 hr than the rough variant. When mixed populations were subjected to a lethal temperature (120 C), the curves only reflected the smooth variant. Since the curves which represented the smooth variant or mixtures containing the smooth variant were not linear, this was thought to be due to activation overbalancing the lethal effect. This research emphasized the importance of variants in explaining differences in spore resistance among spore suspensions of the same strain.

In a previous paper (Finley and Fields, 1962), heat-activation curves for two strains of *Bacillus stearothermophilus* were presented. In that study, National Canners Association (NCA) strain 1518 was activated less than strain M. Also, the degree of activation which varied between suspensions was attributed to heat-induced dormancy, although it was thought that other factors might be involved at the strain level. Variation within NCA 1518 was reported by Michener (1953) in his studies of the bactericidal action of subtilin on *B. stearothermophilus* spores. He reported that NCA 1518, which was obtained from Washington, D.C., contained two variants, a rough and a smooth.

In storage studies, which were performed in our laboratory, the data indicated that there appeared to be two types of spores in the population due to two peaks in the activation curve (Fields and Finley, 1962). For this reason, this study was instituted to determine whether or not rough and smooth variants were the cause of the peaks in the activation curves and what effect such variants would have on germination studies.

Materials and Methods

Study of stored spores for variants. Spores which had been used previously (Finley and Fields, 1962; Fields and Finley, 1962) were plated out without heat-shocking. The developing colonies were viewed at 45× to determine the colony types and to aid in isolation.

Separation of variants. Since the second suspension of NCA 1518, which was used previously (Finley and Fields, 1962; Fields and Finley, 1962), contained a higher percentage of rough colonies, it was used in plating out non-heated spores to isolate rough and smooth variants. When rough (surface) colonies on Dextrose Tryptone Agar (Difco) were noted, these were streaked on nutrient agar for further study to determine the purity of the isolate. Both the rough and smooth variants were isolated and streaked five separate times on nutrient agar to determine purity before they were used in spore production. Also, in streaking from the test tube (which originally came from NCA), which had dried out slightly, another variant, a white or opaque-rough, was isolated.

Spore production of variants. Distilled water was used to wash the cells of the variants from nutrient agar plates. These cells were used to inoculate petri plates containing nutrient agar. The plates were incubated at 50 C for 48 hr. A shorter incubation time was used to prevent as much heat-induced dormancy as possible (Finley and Fields, 1962). Since the rough variant did not produce spores within this incubation period on plain nutrient agar, the following medium was used in the spore production of the rough variant: nutrient agar enriched with 0.05% glucose and 30 ppm of $MnSO_4 \cdot H_2O$. This was the medium used previously (Finley and Fields, 1962) without the supplementary agar added. Plates of the rough variant were incubated for 48 hr.

Cleaning procedures. Both suspensions were set out at room temperature for 5 days for autolysis to occur. The vegetative cells of the rough variant underwent autolysis during this period but the vegetative cells of the smooth variant and the opaque-rough variant did not; therefore,

[1] Contribution from the Missouri Agricultural Experiment Station, Journal Series No. 2470, approved by the Director.

the lysozyme treatment previously described (Finley and Fields, 1962) was used. Both suspensions were washed in distilled water five times before they were stored at 5 C.

Heat-activation methods. The same procedure as described by Finley and Fields (1962) was used in this study. Dextrose Tryptone Agar was used as the plating medium. Incubation was at 50 C for 48 hr.

Heat-destruction studies. Thermal-destruction rates were performed by the classical method of Esty and Williams (1924) in sealed Pyrex glass tubes (internal diameter, 7 mm; outside diameter, 9 mm). Four tubes were run per heating time. The "come-up-times" were corrected by the method of Ball et al. (*unpublished data*). The number of survivors at zero time is the number of spores surviving this "come-up-time." The heated spores were plated out immediately after each run on Dextrose Tryptone Agar, and the plates were incubated at 50 C for 48 hr and counted. The number of spores (nonheated) was approximately 10^3 in each run.

Results and Discussion

Typical subsurface smooth colonies of NCA 1518, which appeared to be football-shaped, were obtained when the smooth variant was plated out on Dextrose Tryptone Agar. This shape was produced because of the position of the colony in relation to the surface of the agar. If the colony were tilted a little, it would appear to be more disc-shaped. The surface colony of the smooth variant was round with a central opaque spot. The vegetative cells were short (mean of 1.5 × 0.5 μ with maximum of 2.0 × 0.5 μ and minimum of 1.0 × 0.5 μ). This variant sporulated within 48 hr on nutrient agar.

Typical subsurface colonies of the rough variant of NCA 1518 (translucent) were observed. The edge of a surface colony of the rough (opaque variant) is shown in Fig. 1. The rough variants have colonies with an irregular margin (Fig. 1). The surface colonies of the rough variants tended to be larger than the smooth variant when grown on the same medium and under the same experimental conditions. The vegetative cells of the rough variant were considerably longer (mean of 3.6 × 0.5 μ with maximum length of 5.0 × 0.5 μ and minimum of 2.0 × 0.5 μ).

Figure 2 shows surface colonies of the rough-translucent and the rough-opaque variant of NCA 1518 after being streaked on nutrient agar for isolation. The main morphological difference in these two variants was in the appearance of the surface colonies.

These data indicated that the rough and smooth variants isolated in this study conformed to the criteria of Bisset (1955) and Braun (1953) for rough and smooth variants. Even though both strains had been compared previously

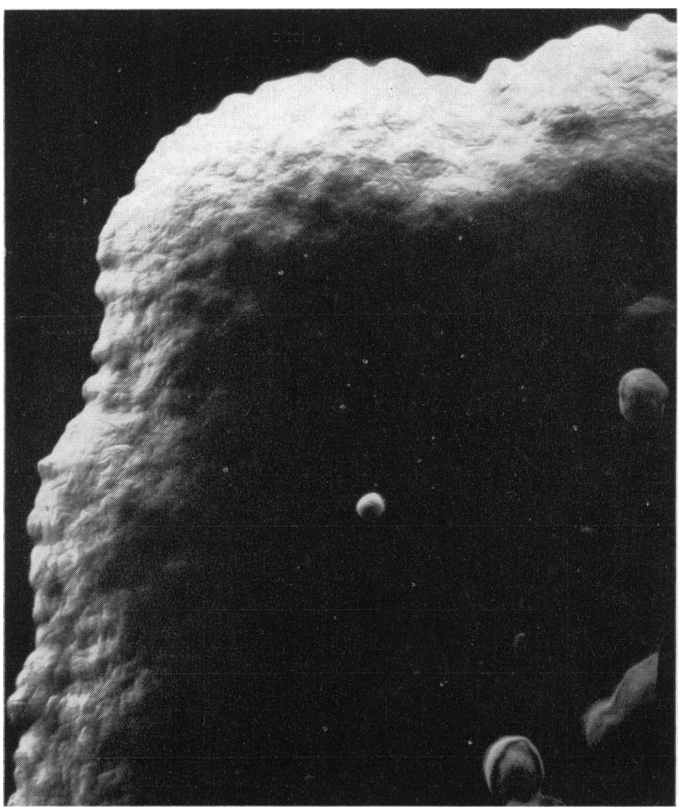

FIG. 1. *Edge of a surface, rough colony (opaque).* × *45.*

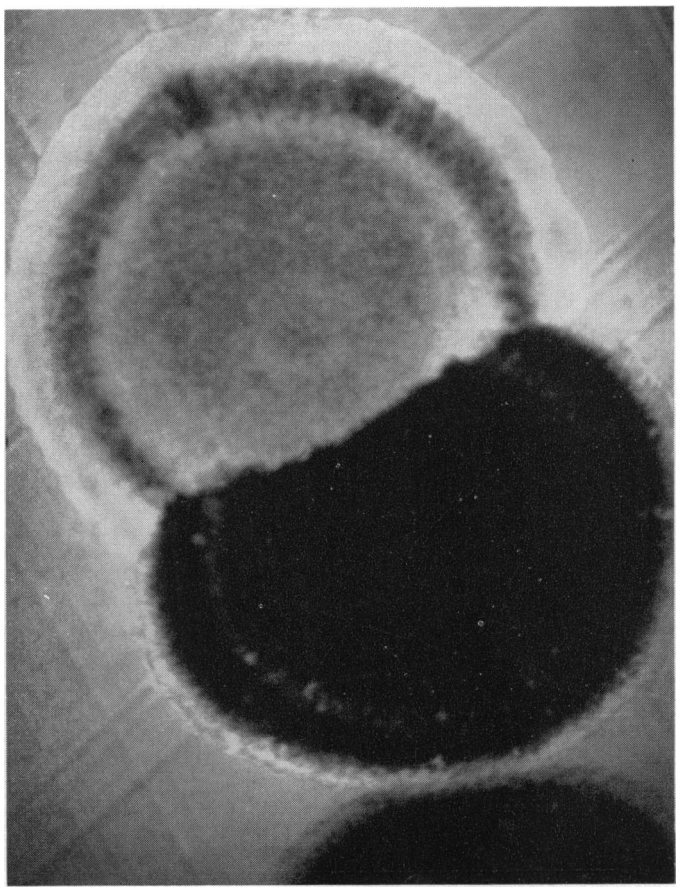

FIG. 2. *Surface colonies of the rough-translucent and rough-opaque variants of NCA 1518.* × *45.*

with all the characteristics of *B. stearothermophilus* as listed by Smith, Gordon, and Clark (1952), the rough and smooth variants were grown at 65 C through successive transfers to check on the possibility that the rough variants were contaminants. According to Smith et al. (1952), growth at 65 C is a stable characteristic of the species, and no other aerobic sporeforming bacillus can grow at this temperature. Both the smooth and the rough (translucent) and rough (opaque) grew well at 65 C, with the rough variants growing more rapidly than the smooth. After the validity of the variants was established, spores of each variant were produced as previously described. Activation of the rough variant (translucent) and smooth are shown in Fig. 3. The rough variant was activated less at 110 C than the smooth, and the highest activation achieved was at the "come-up-time" (166%). Thereafter, the per cent of germinating spores decreased with time until only 24% of the control (unheated spores) germinated at 16 min. The activation response of this variant was in sharp contrast to the smooth variant. A total of 11 min was required to achieve the same amount of activation response as the rough at "come-up-time." The highest degree of activation of the smooth variant, when heated by itself, occurred at 14 min at 110 C, a difference of 204% between the rough and smooth responses at this time or 244% of the control counts. When the smooth was heated with the rough, the highest activation also occurred at 14 min but the level of activation was 270% of the unheated control, or, in this case, there was a difference of 230% between the rough and smooth responses at 14 min. The previously mentioned 244% difference of the unheated control for the smooth variant was 59% greater than the activation achieved with NCA 1518 suspensions 1 and 2, reported earlier by Finley and Fields (1962). Even in the pure state of the smooth variant, NCA 1518 did not reach the degree of activation of strain M, which was previously reported (Finley and Fields, 1962), when the spores were heated at the same temperature and times. These data again illustrate the importance of strain difference in heat activation of bacterial spores.

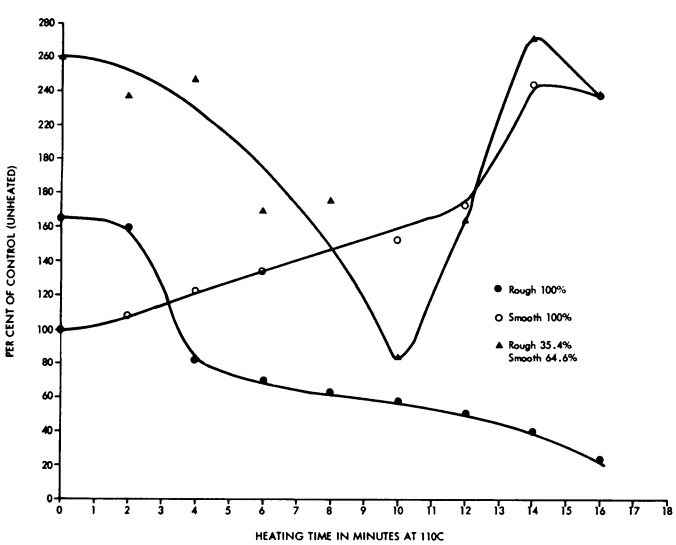

FIG. 3. *Effect of heat on newly harvested spores of the rough and smooth variants of strain NCA 1518. Time after reaching 110 C.*

When the spores from rough and smooth variants were mixed, a curve which contained features of the rough and smooth was obtained. These data indicate the importance of knowing the nature of the spore population in determining not only the time and temperature which will yield the maximal response, but also how to interpret such a curve. In a mixed population, part of the spore population was being activated, while the other part was being inactivated.

To determine whether a greater activation could be achieved at a lower temperature, spores of the rough variant (translucent) were heated at 102 C for various times. At this temperature, 90% of the control spore counts occurred at 14 min, and the remaining counts for the other times were less than 80%. As shown in Fig. 4, a difference of 8 C produced a difference of 76% in the maximal amount of spores germinating at these two temperatures. Heat-induced dormancy was produced in agreement with previous work (Finley and Fields, 1962).

Heat-induced dormancy was also produced in both variants when the rough (translucent) and smooth were held at 50 C for 30 hr (Table 1). Greater heat-induced

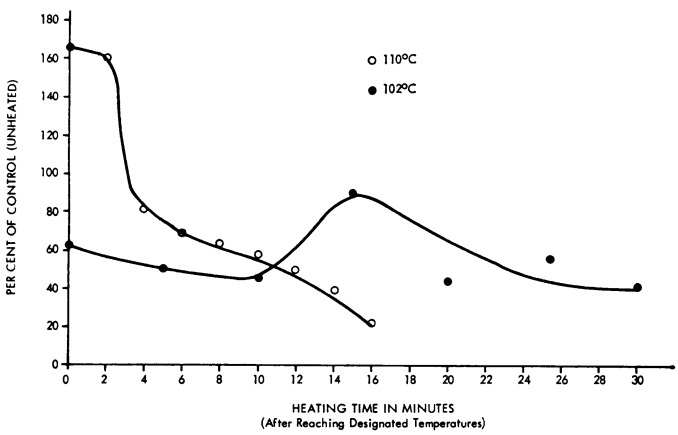

FIG. 4. *Effect of temperature on rough (translucent) variant of NCA 1518.*

TABLE 1. *Heat-induced dormancy in smooth and rough variants of Bacillus stearothermophilus spores*

Variant	Control (no heat-shock and not held at 50 C for 30 hr)		Held at 50 C for 30 hr (no heat-shock)		Held at 50 C for 30 hr (heat-shocked)*	
	Plate count†	Per cent of control	Plate count†	Per cent of control	Plate count†	Per cent of control
Smooth....	124	100	13	10.5	262	211.4
Rough.....	75	100	15	20.0	71	94.7

* Smooth spores were heat-shocked at 100 C for 14 min and rough spores at "come-up-time" (9 min), a time to reach 110 C.

† Three tubes were run per variant; duplicate plates were poured per tube.

dormancy was noted in the smooth variant (89.5%), while only 80% of the rough variant spores were forced into dormancy by the heat.

The response of the white or opaque-rough variant at 110 C is shown in Fig. 5. The maximal percentages achieved during the same heating time for the rough-translucent variant and rough-opaque variant were 166% and 43%, respectively. In addition to the difference in response to heat, the rough-opaque variant did not grow as well as the rough-translucent variant on nutrient agar.

To determine the effect of variant distribution in the spore population when the spores were submitted to lethal heat, thermal-destruction studies were performed. As indicated in Fig. 6, the smooth variant was resistant and the rough variant was susceptible to heat at 120 C.

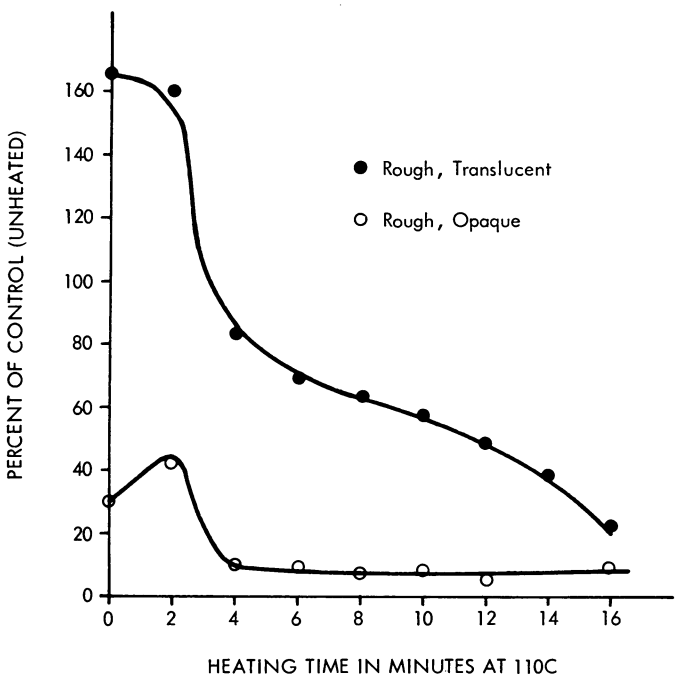

FIG. 5. *Effect of heat on the rough (translucent) and rough (opaque) variants of strain NCA 1518. Time after reaching 110 C.*

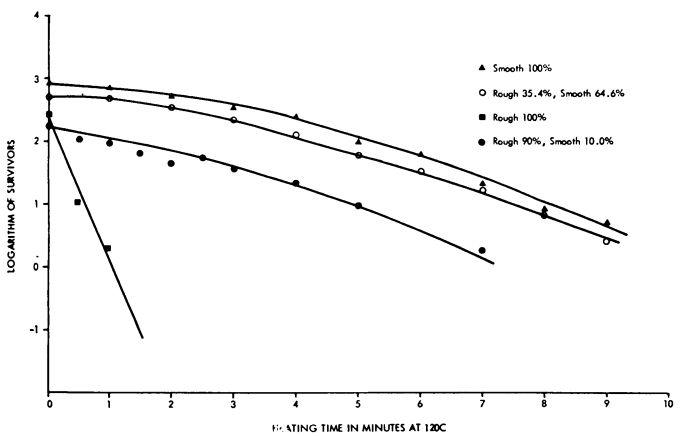

FIG. 6. *Effect of lethal heat on the rough (translucent) and smooth variants of NCA 1518. Time after reaching 120 C.*

The sensitivity of the rough variant is in agreement with the findings of Michener (1953). Since the rough variant was so sensitive to lethal heat, most of this part of a mixed population was destroyed during the "come-up-time," and, essentially, the two curves which were mixtures only reflected the smooth portion or the resistant part of the spore populations. Colonies on the plates of the run which contained 90% rough and 10% smooth (percentages determined on unheated spores) were inspected at 0, 0.5, 1, and 1.5 min of heating time at 120 C and found to contain the following percentages of rough colonies, respectively: 29.7, 16.0, 9.4, and 2.1.

The three curves, which represent the smooth or mixtures of smooth and rough, are similar in slope but differ only in concentration. Since the spore load was approximately 10^8 for all runs (based on unheated counts), the main role of the rough-translucent variant was as a diluent of the smooth or resistant spores. All of the curves (except the 100% rough) showed an initial slow rate of kill at the beginning of the curve. The results presented here and previously (Fields and Finley, 1962) indicated that the slope of the curve is due to activation overbalancing the destruction of the less resistant spores.

After these studies, the spore suspensions previously used (Finley and Fields, 1962) were evaluated in terms of actual percentages of rough and smooth variants in these spore populations (Fig. 7 and 8). Figure 7 shows two types of activation curves. The curve for first-suspension spores

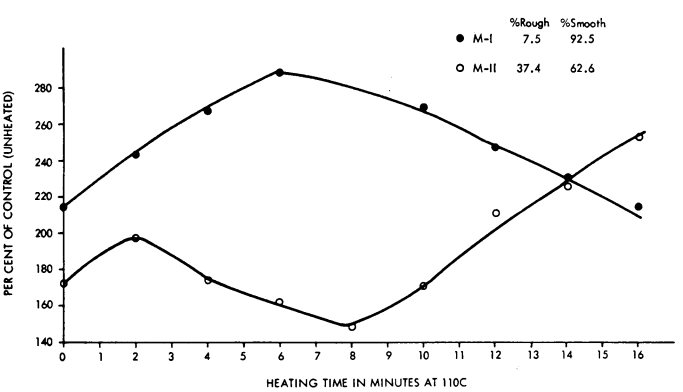

FIG. 7. *Effect of heat on rough and smooth variants of strain M (after spores were stored). Time after reaching 110 C.*

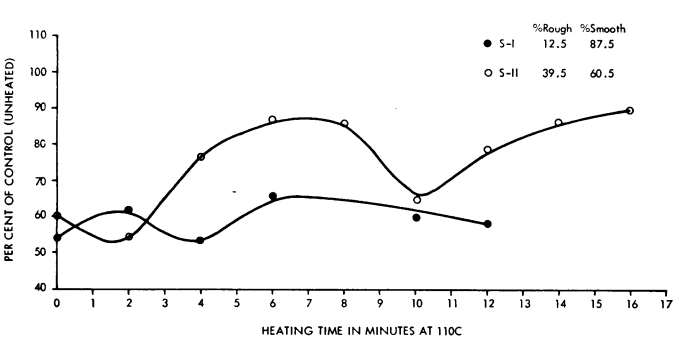

FIG. 8. *Effect of heat on rough and smooth variants of NCA 1518 (after spores were stored). Time after reaching 110 C.*

of strain M reflects the purity of the suspension. Apparently, the 7.5% rough is not enough to produce an effect which can be noted. With the second-suspension spores of strain M, the 37.4% rough component was reflected by the peak which occurred at 2 min, and the percentages of the smooth component of the spore population increased from 8 to 16 min. The data also indicate (as previously reported for these suspensions, Finley and Fields, 1962) that the smooth portion of the population in the second suspension was in a deeper dormancy (Fig. 7). This is illustrated by the fact that the greatest activation for the first suspension occurred at 6 min but at 16 min for the spores of the second suspension.

The activation curves for the first and second suspensions of NCA 1518 also indicate that the population was composed of two fractions (Fig. 8). The colonies which developed from unheated spores of both strain 1518 and M were observed at 45 ×. Both rough and smooth colonial types were found. The peaks in the curve for the first-suspension spores of NCA 1518 occurred at about 2 and 6.5 min, and the maximal per cent of spores germinating in the second suspension occurred at about 7 and 16 min, again reflecting the higher percentage of rough in the second suspension. The data in Fig. 7 and 8 also indicate that, after storage, the response with NCA 1518 was less than the unheated control, although strain M, regardless of suspension, still could be activated.

The occurrence of rough variants in spore populations is probably linked with population pressure. According to Braun (1953), population pressure can act as a selection pressure, permitting the establishment of any variant that can grow and remain under the environmental conditions that exist. Both the M strain and NCA 1518, which were produced and used in a previous study (Finley and Fields, 1962), were incubated longer than the second suspensions, and both contained a higher percentage of the rough variant. One of the problems encountered with this thermophile was the lack of spore production; therefore, increased incubation time was used to improve spore production. When the spores which were used in the previous study were produced (Finley and Fields, 1962), manganese ions were added to the sporulation media to enhance sporulation (Ordal, 1957); manganese ions may have had an influence upon the metabolite accumulation, which brought about population changes. For example, with *Brucella*, it has been shown that Mn^{++} affects the metabolism of the smooth variant and allows alanine to accumulate and favor the establishment of the rough variant in initially smooth cultures (Braun, 1953).

Certainly these factors, as well as prolonged incubation, need further research. This study emphasizes the importance of variants in explaining differences in spore resistance among spore suspensions of the same strain of bacteria. This fact has not been stressed or mentioned by food microbiologists before, and it should have more consideration.

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