Studies on Variants of *Bacillus stearothermophilus* Strain NCA 1518

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The heat resistance, fermentation reactions, nutritional requirements, and phage sensitivity of 18 selected morphological variants of *Bacillus stearothermophilus* NCA 1518 were studied. It was found that when smooth variants mutated to rough colonial morphology, there was no concurrent change in fermentation reactions, nutritional requirements, or heat resistance. The smooth variant, and the rough mutants derived directly from it, presented a uniform pattern of biochemical capabilities which differed from the pattern presented by the rough variants isolated from the same stock culture. This led to the conclusion that the smooth and rough types previously observed in stocks of *B. stearothermophilus* NCA 1518 either were carried in the stock since the original isolation or represent a very profound and uncommon mutation, or that one of the variants has been introduced into the stock culture from an extraneous source sometime in the past.

A number of investigations showed that the heat resistance of the spores of *Bacillus stearothermophilus* varies widely. Heat resistance of spores varied with the composition of the medium on which the spores were produced, the temperature at which they were produced, the medium in which they were heated, the particular strain involved, and the variant type within the strain. The National Canners Association (NCA) strain 1518 of *B. stearothermophilus* is widely used for determinations of heat resistance. A wide variety of thermal resistance values was reported in the literature (1, 5, 12). Some of these differences may be attributed to the existence of variant types within the stocks of *B. stearothermophilus* NCA 1518. Some of these variants, identified as rough (R) and smooth (S), according to their colonial morphology, were found by Fields (4) to differ in the thermal resistance of their spores. The differences in heat resistance and the composition and reaction of these variants to a number of different selective pressures were investigated by Fields and his co-workers.

Since there are spontaneous morphological mutations in microbial populations, and since there are observed differences in the heat resistance and biochemistry of the known morphological variants of *B. stearothermophilus* NCA 1518, investigation of the specific interrelationships involved with morphological mutations was desirable. The study helps in understanding the variations in heat resistance and also provides more insight into the extreme thermophilic and thermoduric nature of *B. stearothermophilus*.

The approach used in this investigation was to isolate a series of rough and smooth (morphological) mutants from the rough and smooth stocks of *B. stearothermophilus* NCA 1518. The mutant stocks were evaluated for heat resistance of the spores and biochemical capabilities of the vegetative cells as compared with the "standard" rough and smooth strains.

We show in this paper that, when a mutation from smooth to rough occurs in this strain, it is not necessarily accompanied by a change in heat resistance, biochemical capabilities, or nutritional requirements.

MATERIALS AND METHODS

Cultures. The source of *B. stearothermophilus* strain NCA 1518 used in these experiments was a spore suspension prepared by Finley and Fields (6). The original source of this culture was the Washington Laboratories of the National Canners Association. The identity of the rough (Rb) and smooth (S) stocks as *B. stearothermophilus* was confirmed by application of the standard taxonomic criteria of.

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Smith et al. (14) and by guanine plus cytosine (GC) ratios determined by M. Mandel.

Morphological variants were selected initially from the stock by optical scanning with a dissecting microscope with magnification from 7 to 30 ×. The substage mirror was adjusted to obtain oblique lighting.

Colonies of interest were purified by at least four serial single-colony picks on tryptic soy agar (TSA), then grown on nutrient agar, harvested in distilled water, coated onto 4-mm glass beads, and frozen at −18°C. The stocks were maintained in the frozen state throughout the balance of the experiments. Fresh cultures were used in all experiments. They were obtained by placing a bead from the frozen stock on a TSA plate, rolling it around, incubating the plate overnight, and picking a representative colony as the inoculum.

**Media.** TSA plates were used for scanning for morphological variants in most cases. A few experiments, however, were performed using dextrose tryptone agar (DT). Both media were obtained from Difco Laboratories.

**Fermentation reactions.** The fermentation reactions of the microorganisms were determined on a medium containing 1 g of (NH₄)₂HPO₄, 0.2 g of MgSO₄·7H₂O, 0.2 g of KCl, 0.2 g of proteose peptone, 15 g of agar (Difco), 15 ml of 0.04% brom cresol purple, and 1 liter of distilled water. The pH was adjusted to 7.0. The medium was autoclaved, 10% solutions of autoclaved carbohydrates were added aseptically to achieve a final concentration of 0.5%, and the medium was poured into petri plates. After the medium had solidified, the bacterial strains were streaked on the surface and the results were read after 48 hr of incubation at 55°C.

**Nutritional requirements.** The nutritional requirements were determined by streaking the bacterial strains on agar media containing some or all of the ingredients. These media were modifications of the minimal medium of O'Brien and Campbell (11). The four media prepared by using the minimal medium were: (i) water, 100 ml; agar, 1.5 g; glucose, 0.1 g; sodium acetate, 50 mg; Na₂HPO₄, 25 mg; KH₂PO₄, 100 mg; NaCl, 100 mg; NH₄Cl, 100 mg; MgCl₂·6H₂O, 0.5 mg; CaCl₂·2H₂O, 0.5 mg; FeCl₃·6H₂O, 0.5 mg. (ii) The second medium contained the above ingredients plus biotin, 0.9 μg; riboflavin, 15 μg; nicotinic acid, 150 μg; pantothenic acid, 100 μg; pyridoxal, 7.5 μg; and folic acid, 6.0 μg. (iii) The third medium contained the above ingredients plus 19.5 mg of L-lysine. (iv) The fourth medium contained all of the above ingredients plus DL-methionine, 12 mg; arginine, 10.5 mg; histidine, 4.5 mg; isoleucine, 14.0 mg; valine, 14.4 mg; leucine, 19.5 mg; and glutamic acid, 10.0 mg. All glassware used to handle the media and ingredients were acid cleaned. Plastic petri plates (Fisher) were used.

**Oxygen requirements.** The oxygen requirements of the strains were determined in test tubes (20 by 100 mm) to which were added 20 ml of Difco tryptic soy broth (TSB) with 0.5% agar. The medium was sterilized, cooled, and allowed to solidify. The bacterial strains were inoculated by stabbing to the bottom of the tube.

**Bacteriophage sensitivity.** Bacteriophage sensitivity was determined by streaking the strains on a medium (RT agar) containing 10 g of tryptone, 8 g of NaCl, 1 g of yeast extract, 15 g of agar, 1 g of glucose, 0.22 g of CaCl₂, and 1 liter of distilled water. The sterilized glucose and CaCl₂ were added aseptically to the rest of the ingredients after they were sterilized and cooled.

The bacteriophages tested included TP-84 obtained from L. L. Campbell and three phages isolated in this laboratory, GH5, GH8, and GH9. The bacteriophage suspensions, at a titer of approximately 10⁹ plaque-forming units per ml, were spotted on heavy streaks of the test strains on RT agar. Plates were incubated for 24 hr at 55°C.

**Spore preparation.** Spores for heat resistance studies were produced by the following method. The strains were grown on TSA plates for 18 hr, harvested in 9 ml of TSB, and inoculated into Roux flasks, each containing 100 ml of nutrient agar fortified with MnSO₄·H₂O (30 μg/ml). After the surface of the agar was covered thoroughly, the excess broth was decanted. The flasks were stoppered loosely with cotton and incubated for 40 hr at 55°C. The spores in each flask were harvested in 100 ml of deionized, double-distilled (DDD) water. The spores were centrifuged at 2,000 × g for 5 min. The supernatant fluid was discarded. The pellet was resuspended in DDD water and centrifuged a second time at 2,000 × g. This was repeated a third time; then the pellet was suspended in 30 ml of DDD water and stored at 4°C in a sterile screw-top glass test tube containing a few sterile glass beads to facilitate mixing.

**Thermal destruction tests.** Thermal destruction tests were conducted according to the method of Eddy and Williams (3) by using DDD water as the heating menstruum. Concentrations of about 10⁴ to 10⁵ spores per milliliter were used. Two milliliters of each spore suspension was placed in Pyrex glass tubes (9-mm OD, 7-mm ID); the tubes were sealed by fusing the ends. The tubes were heated in an agitated oil bath at 120.5°C. Two tubes were used per heating time. Duplicate sets of determinations were made with each suspension. After an initial heating period of 3.25 min, the tubes were withdrawn from the oil bath at 1-min intervals and cooled immediately in ice water. The tubes were broken open, appropriate dilutions were plated in TSA, and the plates were incubated for 18 to 24 hr at 55°C. Statistical analyses were a regression analysis by the method of “least squares” and an analysis of variance (16).

**RESULTS**

**Morphological variants.** Three types of colonies arose when the stock spore suspension (6) of B. stearothermophilus NCA 1518 was inoculated onto DT agar. One type of colony
was smooth (designated S) as described by Fields (5). A rough type giving an acid reaction on DT agar was designated Ra. Another rough type produced an alkaline reaction on DT agar as described by Fields (5) and was designated Rb. After purification by single-colony picks, these strains were used as the “standard” stocks.

It was noted that the growth of the microorganism was more luxuriant, and the differences between rough and smooth types were more readily apparent on TSA than on DT agar. Therefore, TSA was used in subsequent experiments. It also was noted that TSA colonies of the rough variants looked smooth when they were closely crowded together; hence, only well isolated colonies were considered in screening. In screening to isolate rough mutants from the S stock, it was found that rough sectors occurred in approximately 1 out of every 450 colonies. Rough sectors of smooth colonies propagated as stable, rough variant types when transferred. Ten of these were isolated and designated S1 through S10. Approximately 50,000 colonies of Ra and Rb were scanned without finding any smooth variants. Of these 50,000, about 29,000 were treated with nitrosoguanidine (100 µg/ml, 30 min) prior to plating in an attempt to induce mutation from rough to smooth, but none was observed.

Some variations in the colonial types of Ra and Rb were noted and isolated. These included Rbi, a variant of Rb which was somewhat less rough in colonial morphology; Rbw, a variant of Rb which lacked the yellow pigmentation typical of Ra and Rb; Ro, an opaque type; Rax, an extra-rough variant of Ra; and Rai, a less rough variant of Ra.

The Ra and Rb strains had a strong tendency to autolyze after they had reached their maximum cell concentration or were held at room temperature, whereas the S strain cells were quite stable.

Fermentation reactions. The fermentation reactions were positive for all strains with sucrose, fructose, glucose, and maltose, although rates of fermentation varied in some cases.

The fermentation reactions of all strains were negative for L-arabinose, D-arabinose, adonitol, dulcitol, galactose, inulin, myo-inositol, lactose, rhamnose, and sorbitol. Reactions varied with cellobiose, raffinose, xylose, and mannitol. All S strains (S, S1 to S10) were negative for the fermentation of cellobiose, rhamnose, and xylose but were all positive for raffinose. The Rax strain did not ferment either cellobiose or mannitol. All other R strains fermented these sugars. All R strains fermented xylose but not raffinose. Fermentation reactions were initially checked in a broth medium. The Ra and Rb strains and their derivative strains, however, were so highly aerobiotic that they gave false negatives; therefore, an agar medium was used. Growth in stabbed tubes of TSB containing 0.5% agar was positive at the surface only for all R strains and positive to the bottom of the tubes for all strains derived from S.

Nutritional requirements. The nutritional requirements of all R strains were very minimal, whereas all S strains were more demanding. All of the amino acids in the modified O’Brien and Campbell (11) medium, however, appeared to be inhibitory to both groups of variants. Variant Ra2, with its biotin requirement, was the only one of the R series to require anything more than the mineral salts and acetate in the medium.

Bacteriophage sensitivity. All strains were sensitive (in varying degrees) to the four phages tested except that strains Ra, Rai, Rax, Rb, Rbi, and Rbw were not sensitive to GH8 and Rbw was not sensitive to TP-84. The phage sensitivity patterns provide added evidence that the variants were bonafide descendants of the parent strains, and therefore all strains were valid B. stearothermophilus.

Heat resistance. Two sets of thermal destruction rate experiments were run. The first experiment utilized one spore suspension of each variant except S (for which two suspensions were prepared and tested). Two determinations were made with each spore suspension. When the log₁₀ of the survivors was plotted against time for these determinations, the results were found to be parallel for the duplicate determinations. Fits to linearity (r²) ranged from 0.9048 to 0.9966. Slopes of the regression equations (b) and D values (number of minutes for a 90% reduction in survivors), which are reciprocals of the b values, are presented in Table 1. Analyses of variance for the data indicated a least significant difference (LSD) of 0.0471 for the b values.

The results are arranged in sequence of decreasing heat resistance in Table 1. The only strain with a b value significantly different from all other strains was Rai. However, the b values for the two replicate suspensions of S varied significantly. Because of this, a second set of thermal destruction rate determinations was made using two replicate spore suspensions of each strain tested.

The replicate spore suspensions in the
second experiment were consistent for four out of six pairs. Two pairs of suspensions showed significant differences between replicates according to the F test. The LSD for the b values in this experiment was 0.0192. The results of this experiment are shown in Table 2.

### Table 1. Regression values and results of LSD test for thermal destruction experiment

<table>
<thead>
<tr>
<th>Strain</th>
<th>b</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rai</td>
<td>-0.4588</td>
<td>2.18 (a)</td>
</tr>
<tr>
<td>Rbi</td>
<td>-0.3629</td>
<td>2.76 (b)</td>
</tr>
<tr>
<td>Sr10</td>
<td>-0.3221</td>
<td>3.10 (b) (c)</td>
</tr>
<tr>
<td>Rax</td>
<td>-0.2895</td>
<td>3.45 (c) (d)</td>
</tr>
<tr>
<td>Rbw</td>
<td>-0.2707</td>
<td>3.69 (d) (e)</td>
</tr>
<tr>
<td>Rb</td>
<td>-0.2707</td>
<td>3.69 (d) (e)</td>
</tr>
<tr>
<td>Sr4</td>
<td>-0.2590</td>
<td>3.86 (d) (e) (f)</td>
</tr>
<tr>
<td>Sr5</td>
<td>-0.2393</td>
<td>4.18 (e) (f) (g)</td>
</tr>
<tr>
<td>Sr7</td>
<td>-0.2312</td>
<td>4.32 (e) (f) (g)</td>
</tr>
<tr>
<td>S(1)</td>
<td>-0.2228</td>
<td>4.49 (f) (g)</td>
</tr>
<tr>
<td>Ra</td>
<td>-0.2145</td>
<td>4.66 (f) (g) (h)</td>
</tr>
<tr>
<td>Sr8</td>
<td>-0.2110</td>
<td>4.74 (g) (h)</td>
</tr>
<tr>
<td>Sr9</td>
<td>-0.2038</td>
<td>4.91 (g) (h)</td>
</tr>
<tr>
<td>Sr6</td>
<td>-0.2026</td>
<td>4.94 (g) (h)</td>
</tr>
<tr>
<td>Sr1</td>
<td>-0.1979</td>
<td>5.05 (g) (h)</td>
</tr>
<tr>
<td>Ro</td>
<td>-0.1964</td>
<td>5.09 (g) (h) (i)</td>
</tr>
<tr>
<td>Sr2</td>
<td>-0.1728</td>
<td>5.79 (b) (i)</td>
</tr>
<tr>
<td>Sr3</td>
<td>-0.1725</td>
<td>5.80 (b) (i)</td>
</tr>
<tr>
<td>S(2)</td>
<td>-0.1505</td>
<td>6.64 (i)</td>
</tr>
</tbody>
</table>

* LSD = least significant difference; b (column 2 heading) = regression values; D = number of minutes for a 90% reduction in survivors. Values followed by the same letter (in parentheses) were not significantly different according to the LSD test. The LSD for the b values was computed to be 0.0471.

### Table 2. Regression values and statistical analysis for thermal destruction experiment 2

<table>
<thead>
<tr>
<th>Strain</th>
<th>Between</th>
<th>b Avg</th>
<th>D rep 1</th>
<th>D rep 2</th>
<th>D avg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sr3</td>
<td>7.75</td>
<td>-0.0864</td>
<td>9.53</td>
<td>13.61</td>
<td>11.57</td>
</tr>
<tr>
<td>S</td>
<td>1.10 ns</td>
<td>-0.1538</td>
<td>6.16</td>
<td>6.85</td>
<td>6.50 (a)</td>
</tr>
<tr>
<td>Sr10</td>
<td>0.64 ns</td>
<td>-0.1628</td>
<td>5.69</td>
<td>6.34</td>
<td>6.14 (a)</td>
</tr>
<tr>
<td>Ra</td>
<td>0.28 ns</td>
<td>-0.2233</td>
<td>4.63</td>
<td>4.32</td>
<td>4.48 (b)</td>
</tr>
<tr>
<td>Rb</td>
<td>2.72 ns</td>
<td>-0.2349</td>
<td>3.87</td>
<td>4.52</td>
<td>4.26 (b)</td>
</tr>
<tr>
<td>Rai</td>
<td>6.21</td>
<td>-0.2595</td>
<td>2.89</td>
<td>4.09</td>
<td>3.85 (c)</td>
</tr>
</tbody>
</table>

* Rep = replicate; LSD = least significant difference; ns = not significant.
* The data from Sr3 were excluded from the computation of the LSD because of the marked variation between replicates.
* Significant at 5% level.
* Values followed by different letters (in parentheses) were significantly different by the LSD test. The LSD for the b averages was 0.0192.

**DISCUSSION**

The rough and smooth colonial types sometimes found in NCA strain 1518 of *B. stearothermophilus* have been known for some time (9) and were studied in this laboratory and other laboratories for a period of years (4, 5, 7, 8, 13, 14). On the basis of these studies, there was reason to believe that mutations in colonial morphology occurred in the population. There was also some basis for suspecting that when the mutation from smooth to rough colonial type occurred there was a concurrent drop in heat resistance and a change in a number of nutritional and biochemical characteristics. The results of the study reported herein showed that this was not necessarily true.

The fermentation reactions, oxygen requirements, and nutritional requirements of the strains tested in these experiments demonstrated two distinct patterns. In one group were strain S from the stock culture and all of the Sr strains isolated directly from strain S. In the other group were the R varieties isolated from the stock culture and their variants. Members of each group were all highly consistent in their fermentation patterns and oxygen requirements. Nutritional experiments produced a somewhat less clear-cut but reasonably consistent picture. There were a number of variables in the nutritional experiments, such as inoculum size, criterion for occurrence of growth, and oxygen requirements which made the results difficult to read and interpret and limited their significance. It is apparent, however, that under the conditions of these experiments the R strains were much less demanding in their nutritional requirements than the S strains.

The phage sensitivity data likewise fell into two groups, with the S strains more susceptible than the R strains to the phages used. However, strain S was used for the propagation of the phages.

The heat resistance data obtained by Fields (4) and Rotman and Fields (13) indicated that the spores of the smooth variety of *B. stearothermophilus* 1518 were more heat-resistant than the spores of the rough variety. This was confirmed for the strains obtained directly from the original spore suspension. However, some of the rough strains derived directly from strain S were just as heat-resistant as their smooth parent strain, whereas other rough strains derived from strain S showed significantly lower heat resistance than their parent strain. Some difficulties were encountered in consistently obtaining duplicate spore suspen-
sions having the same heat resistance from the same strains, although the experimental conditions gave readily repeatable results for any one spore suspension. The results indicated that, although the heat resistance of the S strains was higher on the average than the R strains, there were more unrelated variables which greatly reduced the predictability of the relative heat resistance between given spore suspensions of strains from the two sources. The variation in heat resistance between some spore suspensions of the same strain, apparently prepared in exactly the same manner, was noted as a frequent occurrence by the National Canners Association (10).

From these results, it can be concluded that when there is a mutation in this strain from smooth to rough, the nutritional and biochemical capabilities of the mutant strain usually remain essentially unchanged from those of its parent. The heat resistance of the mutant, likewise, does not appear to be directly affected by the morphological mutation. The difficulty of isolating smooth mutants from the rough stocks precluded any evaluation of whether this change could possibly accompany an increase in heat resistance.

Although both the smooth and the rough strains obtained from the stock spore suspension conform to the classical taxonomic criteria for *B. stearothermophilus* and have a GC base ratio appropriate to that species (52.0%), their characteristics were sufficiently different that they appeared to be distinct strains. This was borne out by the fact that of the 10 rough variants isolated from the smooth strain, none matched the rough stock strains in biochemical capabilities or phage sensitivity, whereas all matched their parent strain. It was not possible to determine whether the two variants were carried in the stock cultures since the original isolation of the strain, were due to a mutation since then, or were introduced from some extraneous source. This does, however, accentuate a point made by Braun (2), that it is desirable to have a homogeneous population in stock cultures and that this is not attainable when the stock cultures are continually passed by arbitrary mass transfer. The proper maintenance of stock cultures should involve periodic restreaking of the cultures for purity and retesting to see that the purified cultures continue to have essentially the original characteristics. This could become a laborious and cumbersome procedure for a culture collection of any size maintained in a vegetative state. With modern methods of lyophilization or freezing, however, the labor involved is markedly reduced, because one set of reliable cultures can be prepared this way for use over a period of years. The technique of freezing spores on glass beads, as was done in these experiments, proved to be a convenient method of maintaining a stock in such a way that genetic stability was assured for the duration of the study.

The variability in the heat resistance of duplicate spore suspensions of the same strain, as observed in this experiment and as encountered by other workers (10), tends to indicate that this type of variation might be one major reason for the variety of thermal resistance values published in the literature for *B. stearothermophilus* strain NCA 1518.

ACKNOWLEDGMENTS

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