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Heat resistance of rough and smooth variants of Bacillus stearothermophilus

James David Baldock
Iowa State University

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VARIANTS OF BACILLUS STEAROTHERMOPHILUS.

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HEAT RESISTANCE OF ROUGH AND SMOOTH
VARIANTS OF BACILLUS STEAROTHERMOPHILUS

by

James David Baldock

A Dissertation Submitted to the
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1969
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INTRODUCTION

The resistance of bacterial spores to heat is one of the most remarkable properties of all living forms. This property has offered a great challenge for experimentation and speculation and has been of prime concern to individuals concerned with sterilization procedures, particularly to those in the food industry in which exacting standards for thermal processing must be met to rid food of pathogenic and spoilage types of sporeformers.

Since the beginning of bacteriological science, academic interest in heat resistance has preoccupied many bacteriologists; work before 1920, however, is difficult to evaluate. Early investigators had not realized the true complexity of the problem of death by heating, nor had they taken into consideration the many variables involved in heating.

A search of the literature reveals considerable variation, inconsistencies, and contradictions in heat resistance data. A sizeable portion of this variation stems from inherent variability which is, to an extent, uncontrollable including such variations as that shown from species to species, variations within species and strain, and also variation within a given population. On the other hand, a large part of variability in heat resistance is attributed to controllable factors which operate outside the spore. Thus, heat resistance is not a fixed property, but is influenced by the environment of growth, the
subsequent handling of spores after production, the environment in which heat resistance is tested, and the subculture environment after testing.

Variation or variability is an accepted phenomenon occurring in all biological forms. Variation within a group or population can be represented by a normal frequency-distribution curve. According to general opinion, the heat resistance of bacterial spores is represented by such a curve, indicating that degrees of resistance within a spore population is normally distributed.

The investigation herein was divided into two parts; the first deals with factors which support the existence of variability in heat resistance of a given population. Attempts were made to select those individuals of extreme heat resistance and to produce from them populations possessing greater resistance than the average spores in the parent suspension. Another study, based on variability, was conducted which involved the fractionation of spores by centrifugation followed by heat resistance characterization. The influence of growth temperature on variability was studied in experiments where spores were produced at different temperatures and tested for thermal resistance.

The second part of this investigation dealt with another type of variability involving variation which normally breeds true, such as that exhibited by rough and smooth variants of certain microorganisms. Using *Bacillus stearothermophilus*
strain NCA 1518, studies were conducted to allow further characteriza-
tion of rough and smooth variants on a morphological, physiological, and immunological basis. These studies were carried out with the implication that the presence of variants in spore populations can be involved in certain variations and inconsistencies occurring in heat resistance data.
REVIEW OF LITERATURE

The Organism and Its Thermal Resistance

Since the time of the work Esty and Williams (1920), the study of heat resistance in bacterial spores has developed into a speciality area for the bacteriologist and, in particular, for the food microbiologist. Of particular concern in this area have been the spores of *Bacillus stearothermophilus* because these spores are the most heat resistant in the genus *Bacillus* and are responsible for "flat sour" spoilage in low acid foods (Fields and Finley, 1962).

Survival of *B. stearothermophilus* during heat treatment has been of interest since the organism was first described by Donk (1920); and because *B. stearothermophilus* is one of the most heat resistant organisms, the species is often used as a test organism in the evaluation of heat processes. Williams and coworkers (1937) found that this organism in its sporeform survived heat treatment at 120 C for 35 min in dilute phosphate buffer (pH 6.95). Reed *et al.* (1951) reported that a 99.99% decimal reduction of spores of NCA 1518 required from 19.3 to 29.6 min at 120 C in M/15 phosphate buffer solution (pH 7.0). Murrell and Scott (1966) observed that spores of NCA 1518 were about 50,000 times more heat resistant than were spores of *Clostridium botulinum* type E under very moist conditions;
however, this ratio fell to about 10 when $a_w$ values were less than about 0.5.

_B. stearothermophilus_ has been well characterized by Smith, Gordon, and Clark (1952) and by Wolf and Barker (1968). This thermophilic, aerobic sporeformer has the capacity to grow at 65 C which is considered a fairly reliable character of the species (Smith _et al._, 1952). Some strains are capable of growth at temperatures of 70 C or slightly higher (Finley and Fields, 1962). Smith _et al._ (1952) reported growth at 70 C in 45 of 87 cultures tested; at an incubation temperature of 65 C, they detected growth in all cultures.

**Variability in the strain**

Observations made before 1963 on the growth characteristics and heat resistance of _B. stearothermophilus_ did not take into consideration heterogeneity in the species. Smith _et al._ (1952), however, in their description of the colonies of this species listed rough and smooth morphology within the 98 cultures studied. Michener (1953) observed the occurrence of rough and smooth variants in NCA 1518. Fields (1963) reported differences in heat resistance and heat of activation obtained from the endospores of the variants. Spores of the smooth variant required 14 min of heating at 110 C for maximal activation while the rough type was fully activated during the period of "come-up-time" (Fields, 1963). D values (time required for 90% destruction at 250 F) of 2.32 and 1.42 min were
reported for the spores of smooth and rough variants respectively (Rotman and Fields, 1966).

The morphological information on the rough and smooth variants presented by Fields (1963) met with the criteria defined by Braun (1953) for rough and smooth variants. The rough variant produced both colonies and cells larger than the smooth variant when grown under the same environmental conditions (Fields, 1963).

Recent studies by Fields and his group on the growth characteristics of the variants have shown distinct physiological differences in oxygen demand, pH tolerance, and growth requirements (Fields, 1966; Hill and Fields, 1967a,b). The rough variant exhibited a lower nutritional requirement than the smooth variant (Hill and Fields, 1967b); however, the smooth grew faster than the rough type on nutritious media (Hill and Fields, 1967b). A low growth temperature (45°C) and an anaerobic environment favored the rough variant (Hill and Fields, 1967a). Initiation of growth at pH 6.0 and 8.0, instead of at pH 7.0, required only a few hours lag for the rough variant while the smooth variant needed more than 30 hr to readjust.

A study of glucose metabolism in rough and smooth variants revealed some differences. The smooth variant utilized glucose by way of the Embden-Meyerhof (EM) pathway and hexose monophosphate shunt (HMP), calculated at 95.8 and 4.2% participation, respectively. The EM pathway was found more active in the
smooth variant; however, the rough variant was shown to metab-
olize glucose via the EM pathway exclusively or in combination
with another pathway, other than the HMP pathway. Efficiency
levels of the tricarboxylic acid (TCA) cycle in the rough and
smooth variants were observed at 81 and 5%, respectively (Hill
et al., 1967).

Higher sporulation of the rough variant in pure and mixed
culture was reflected from studies of the two variants on pea
extract and pea agar (Fields, 1966).

Studies on amino acid content of spores from the variants
revealed two main differences. The smooth variant contained
higher levels of amino acids in all those examined except for
ornithine which was present in the rough form in twice the
amount found in the smooth type. In addition half cystine was
present in the smooth variant in amounts double that in the
spore of the rough variant (Fields and Rotman, 1968).

Much of the research on rough and smooth variants in a
population seems to emphasize variability as one approach to
giving a plausible explanation of inherent differences in spore
resistance among suspensions of the same strain. Williams and
Hennessee (1956) have shown that there is considerable difference
in the thermal death time of various strains of spores of B.
stearothermophilus.

Variability and Thermal Resistance Studies

Many of the investigations in bacteriology treat a pure
culture as if it were one ideal cell. The properties of this
statistical individual do not represent those individuals which differ from the majority. Variability of bacteria has become an accepted phenomenon in microbiology. Many workers who have undertaken studies with bacterial cells or spores have found cell variation and nonhomogeneity (Burke, 1923; Esty and Williams, 1924; Mitchell, 1951; Reed et al., 1951; Curran, 1952; Kaplan et al., 1953; Church et al., 1956; Vas and Proszt, 1957; Sherstoboev, 1961; Hansen and Riemann, 1963; Rotman and Fields, 1966).

One explanation for the existence of innate heterogeneity demonstrated in heat-survival curves (that show decreasing death rates) is offered by Alderton et al. (1964). They explain this type of variation in heat resistance as a non-hereditary phenomenon. Their data support the hypothesis that heating slowly evokes a protective response in the spore. The process of induction of increased heat resistance can be duplicated in acid treated spores which are stripped of their cations and heat treated in calcium buffer. On the other hand, by making conditions such that ion exchange can not occur between the spores and the external medium during moderate lethal heating, they are able to obtain straight log survivor curves. The explanation does not apply to extremely lethal heat treatments.

Evidence for variability or variation in a spore population is reported by some observers who cite inconsistencies or skips in thermal death curves which they attribute to inherent
variability resulting from the presence of individuals of varying heat resistance (Esty and Williams, 1924; Mitchell, 1951; Amaha and Ordal, 1957).

According to Braun (1947) bacterial variation can be classified into three groups, as follows: a) variation termed enzymatic adaptation, which may occur in the absence of propagation, b) variation arising from cell pleomorphisms which can be attributed purely to physiological modification, and c) rough and smooth manifestations, resulting from dissociation. Dissociation denotes a population phenomenon rather than a change of characteristics and applies to a spontaneous change in one or more members of a bacterial population and their subsequent establishment (Braun, 1947).

Heat resistance can vary within a given population independent of the luxuriance of growth and sporulation, according to Curran (1952). Some of the same environmental factors shown to affect or modify the degree to which a variant can establish itself within a population are factors which help determine resistance of a spore population. Two factors in common are changes in temperature of growth and differences in available nutrients (Curran, 1952; Braun, 1946). Thus, the distinction is made between variability due to heredity and variation attributed to growth conditions.
Selection and attempts to increase thermal resistance

The existence of a few extremely heat resistant individual spores is well documented in the literature (Esty, 1923; Esty and Williams, 1924; Vas and Proszt, 1957; Anand, 1961). Hinshelwood (1951) reasons that there is always the possibility and even probability of the occurrence of the occasional cell of abnormal resistance regardless of the inherent properties of a cell or cells in a population. He states that resistant survivors may arise from previous mutations and give rise to a specially endowed substrain in the total population. Thus, these cells may do more than just survive, they may even establish themselves, if the mutation is profound enough and environmental conditions are suitable for its continued existence.

Heat has been shown to be a mutagenic agent (Zamenhof and Greer, 1958; Zamenhof, 1960). The heat treatment of cells of Escherichia coli and spores of B. subtilis in a dry state under vacuum at 130 to 155 C for 16 min produced a large number of mutations and unstable genes resulting in phenotypic and genotypic change. The spores of mutants of B. subtilis were tested for heat resistance and found to possess equal resistance to spores of the wild type.

Lamanna and Malette (1965) contend that the heat resistance of spores is a result of a complex of characteristics both physiological and morphological. They support the idea that
heat resistance is subject to genetic law only in a general way and is not under the control of a simple genetic factor or gene per se. Thus, on these assumptions no single mutation can be expected to increase heat resistance or to readjust the overall endogenous nature of the spore to allow an increase. A mutation of a single gene could, on the other hand, lower heat resistance if this change causes an imbalance or weakening of the physiological or morphological systems. If heat resistance were lowered because of a single mutation, then a one step backward mutation could be expected to restore the original level of resistance (Lamanna and Malette, 1965).

According to Vas and Proszt (1957) individuals of extreme heat resistance are in low frequency in certain mesophiles. From a study of B. cereus, they concluded that a fairly constant fraction of spores (1 in $10^{-7}$ or $10^{-8}$) possesses extreme heat resistance. They suggested that the extremely heat resistant fraction forms part of the normal distribution of resistance and is not necessarily related to cells which arise by mutation.

Attempts have been made to demonstrate the existence of organisms of extreme resistance in a given population by selecting survivors of repeated heat treatments (Bigelow and Esty, 1920; Magoon, 1926; Williams, 1929; Sommer, 1930; Williams, 1936; Davis and Williams, 1948; Desrosier and Esselen, 1951; Vas and Proszt, 1957). The results have been variable. Bigelow and Esty (1920) were able to increase the heat
resistance of spores of an unidentified obligate thermophile by repeated culturing of survivors from sublethal heat treatments. Their results show an increase in survival time from 12 to 23 minutes at 120°C. Magoon (1926) reported an increase in thermal resistance of spores of *Bacillus mycoides* from 2 minutes to 44 minutes at 100°C, and Williams (1929) observed an increase in the resistance of spores of *Bacillus subtilis* from 38 to 54 minutes at 95°C, each after the selection of survivors of heated suspensions. Davis and Williams (1948) found that *B. globigii* exhibited a similar increase in resistance to heat after subculturing the survivors of heated suspensions. The conclusions of the experimenters is that a graded resistance to heat exists among spores of a population.

Other workers have been only partially successful and some have been entirely unsuccessful in selectively increasing bacterial resistance to heat. For example, Williams (1936), using cultures of *B. mycoides*, *B. subtilis*, and others, was able to increase the resistance of *B. mycoides* only; his results showed an increase from 10 to 25 minutes at 100°C after 10 selective transfers. He reasoned that the clumping tendency of this organism may have been a factor in the enhanced resistance. Sommer (1930) reported that *Clostridium botulinum* exhibited no tendency to increase in resistance to heat after careful selection. Desrosier and Esselen (1951), working with spores of P. A. 3579, were unsuccessful in increasing heat
resistance by selection. Vas and Proszt (1957) concluded from their study that the heat resistance of bacterial populations cannot be increased by subculturing the survivors of extreme heat treatments.

Rahn (1932, 1945) has criticized the evidence for an increase in thermal resistance by selection on the basis that the last survivors are no more resistant to heat than the average, having survived the heating periods by chance. Amaha and Sakaguchi (1951) concluded from their work that the main cause of increases in survival time can not be attributed to the selectivity of more resistant individuals, but instead to some fundamental causes affecting the physiochemical nature of the spore concentrations.

Fractionation of spores by centrifugation

Reynolds and Lichtenstein (1952) indicate that the order of death of P. A. 3679 spores when exposed to heat is not purely a matter of chance but is determined entirely or in part by a "gradation" resistance among individuals of the spore population in question. Confirmation of this hypothesis of a gradation within a spore population has been given by Yesair and Cameron (1936), who, by differential centrifugation of spore suspensions, correlated specific weight with heat resistance. They observed that the most heat resistant spores of a thermophilic anaerobe and a thermophilic flat sour organism were those which settled out first during centrifugation. This observation was
substantiated by the findings of Sugiyama (1951) who found sedimented spores contained fractions of different heat resistance, of which the first fraction to settle out without centrifugation was the most heat resistant. Anand (1961) found the spores of *B. stearothermophilus*, *B. brevis* (320), *B. subtilis*, *B. megaterium*, and *Cl. sporogenes* (3679) in the denser fractions to be more heat resistant than the spores of lighter fractions; however, he observed no marked difference between the spores of *B. cereus*, *Cl. sporogenes* (112), and *Cl. bifermentans* from denser or lighter fractions. Frank and Campbell (1957) found no differences in 3 fractions of spores of *B. coagulans* collected on the basis of density. Berlin et al. (1963) were not able to correlate density of different species of sporeformers with heat resistance. They were not comparing densities within a given population.

Along a similar line of study, Sherstoboev (1961) found that spores fractionated during centrifugation were highly nonhomogeneous on the basis of size and density. His study was based on sedimentation rates.

The techniques used in separating spores on a density basis has varied greatly since the relation of heat resistance to density was proposed by Shaw (1928). Much of the experimentation has been rather crude including: 1) simple centrifugation at various speeds to recover spores of progressively lower density (Yesair and Cameron, 1936), 2) simple
sedimentation without centrifugation (Sugiyama, 1951), 3) chemical density without centrifugation (Berlin et al., 1963).

In recent years density gradient centrifugation has been applied to spores with varying degrees of success. Its application to spores has been hindered to some degree because of the special requirements necessary in the supporting material. The supporting medium must possess the following qualities: 1) have high density up to 1.5, 2) be low in viscosity, 3) cause no aggregation of the spores, 4) be chemically and biologically inert so as to offer no toxic effects to spores or cells. Several density gradient compounds are available which can give high densities, but most have some undesirable characteristics which can cause aggregation or irreversible changes of spores (Lewis et al., 1965). Sucrose has been used by Church and Halvorson (1959) as a support material. Because of the high densities required for spore separation the material is not usually recommended. The high viscosity of heavy sucrose solutions offers another limitation. Lead chelates of ethylenediamine diacetic acid as a supporting material have been prepared and used successfully to separate distinct bands of spores by Lewis, Snell, and Alderton (1965). This material is not available commercially. A material called Urografin (an alternate name is Renografin) has been used by Tamir and Gilvarg (1966) for the separation and characterization of vegetative cells and spores. They demonstrated that this
substance is a suitable supporting medium for isopycnic density separation of spores.

**Growth temperature and its relation to heat resistance**

In reviewing the literature on the effect of growth temperature on thermal resistance, two main divisions of thought became evident. A large portion of the workers have indicated that a direct increase in thermal resistance of spores results from an increase in growth temperature (Esty and Williams, 1920; Eckford, 1927; Williams, 1929; Curran, 1934; ZoBell and Conn, 1940; Lamanna, 1942; Williams and Robertson, 1954; El-Bisi and Ordal, 1956; Lechowich and Ordal, 1962). A direct relation between the temperature at which spores of *B. subtilis* and *B. stearothermophilus* were produced and their heat resistance was noted by Williams (1929) and Williams and Robertson (1954). With *C. botulinum* spores Sugiyama (1951) observed a similar effect except that the resistance reached a maximum when incubated at 37 C and fell when cultures were kept at 41 C. Theophilus and Hammer (1938) observed that growth temperatures above or below the optimum yielded spores of reduced heat resistance.

Lechowich and Ordal (1962) after obtaining a direct correlation between growth temperature and heat resistance examined the chemical changes associated with temperature-induced heat resistance in *B. coagulans* and *B. subtilis*. The
DPA and calcium contents increased correspondingly with increase in heat resistance in spores of B. subtilis, whereas in spores of B. coagulans there was a decrease in total DPA, but the ratio of cation concentration to DPA concentration increased as their resistance to thermal destruction increased. They concluded from their studies that the cation concentration played a dominant role in heat resistance.
MATERIALS AND METHODS

Bacteriological Procedures

Organism

Rough and smooth variants of B. stearothermophilus NCA 1518 (ATCC 7953) were obtained from M. L. Fields, University of Missouri, Columbia, Mo. The letters "R" and "S" will be used to denote rough and smooth in future reference to variant forms.

Media

Several media were used in various phases of this study. The media and their uses are listed below:

1. Nutrient agar (NA) (Difco) fortified with 0.05% dextrose and 30 ppm Mn++ (Fields, 1963) used for sporulation and recovery.
2. Nutrient broth (NB) (Difco) fortified with 0.05% dextrose and 30 ppm Mn++ used for growth study.
3. Dextrose tryptone agar (DTA) (Difco) used for colonial study and purity checks of R and S variants.
4. Trypticase soy agar minus the agar (TSA-A) (BBL) with added dextrose (0.25%) used for pH study.
5. Trypticase soy broth (TSB) (BBL) used for production of inocula, antigen cultures, and growth study.
6. Trypticase soy agar (TSA) (BBL) used for sporulation and size studies.

Spore production

Spores were produced on the surface of fortified NA. Large prescription bottles (32 oz.) containing 125-150 ml of medium
placed on their sides served as sporulation flasks. Bottles were equipped with special rubber stoppers designed to permit ventilation. Each stopper was drilled and a 2 inch by 12 mm glass tube plugged with cotton was inserted in the opening.

These flasks were inoculated with 0.5 ml of heavy cell suspension taken directly from 18-20 hr cultures grown in TSB at 55 C. Flasks were incubated at 55 C for 48 hr at which time maximal sporulation was obtained.

**Procedures for cleaning spores**

Sporulation of 10% of the population was usually considered high enough to warrant harvesting and cleaning. The growth was removed from the agar surface by washing with sterile H₂O. Addition of 5 mm sterile glass beads on the agar surface aided in dislodging the growth. The spores and cells were centrifuged and resuspended in sterile 0.85% saline adjusted to pH 7.0. To remove vegetative cells from the spores, the suspensions were exposed to lysozyme (0.5 mg per ml suspension) at 50 C for 2 hr at pH 7 with gentle shaking according to the procedure of Finley and Fields (1962).

Further removal of vegetative debris was accomplished by differential centrifugation on an International Refrigerated centrifuge (Model PR-2) equipped with a swing-out rotor. A speed of 2000 rpm for 20 min was sufficient to pellet the spores. The debris, mostly in the supernatant, was removed by careful pipetting with a propipette. This step was repeated
several times. Prior to each differential run the suspension was dispersed by shaking on a Vortex test tube mixer for 10-15 min. Addition of a few sterile glass beads aided the dispersion.

As a final part of cleaning spores, density gradient separation was used according to the method of Tamir and Gilvarg (1966). Gradients were prepared from Renografin (3,5 diacetamino - 2,4,6 triiodobenzoic acid) which is an X-ray contrasting agent sold by Squibb and Co. The compound was obtained commercially as a 60 or 76% solution. Spores to be cleaned were sonicated for 0.5-1.0 min and suspended in 10 ml of distilled water at pH 10 in order to disperse and prevent any aggregation of materials in the suspension. After the gradients were cooled to 4 C the spore suspensions were layered carefully on top to avoid disturbing the gradient.

Because of density differences between R and S spores, different gradient ranges and centrifugation procedures were used. R spores were separated from vegetative debris on a gradient ranging from 54 to 63% Renografin which was made with the aid of a Beckman density gradient former. Tubes containing R spores were spun at 2800 rpm (approximately 1800 x g) for 3 to 4 hr at 8 C on an International refrigerated centrifuge (Model PR-2) equipped with a swinging bucket rotor.

An alternate procedure for cleaning R spores was also used. R spores were placed on a Renografin 60 solution as obtained directly and centrifuged at 15,000 rpm for 30 min at
14 C on a Beckman ultracentrifuge (Model L-2) equipped with a swinging bucket rotor (SW-25.2). The non-gradient centrifugation procedure was used successfully, because spores were pelleted and the vegetative debris remained on top. Either procedure yielded clean R spores.

For cleaning S spores, a gradient was prepared ranging from 57% Renografin at the bottom of the tube to 45% Renografin at the top. The gradient was made on a Beckman gradient former and cooled to 4 C. S spores, after being sonically dispersed, were layered on top of the gradient and spun at 15,000 rpm for 30 min at 5 C.

After centrifugation the various interphases and layers were examined under phase contrast microscopy (970×) for segregation of spores and debris. Spore layers were centrifuged and washed several times in sterile distilled water and stored at 4 C.

Determination of Thermal Resistance

The thermal death time tube technique described by Bigelow and Esty (1920) and later revised by Esty and Williams (1924) was used in thermal resistance studies (National Canners Association Research Laboratories, 1968).

Cleaned spores were prepared for heating by first exposing them to sonic vibration for several seconds to aid in the elimination of clumps. Suspensions were standardized
to predetermined concentrations with a Bausch and Lomb 340 spectrophotometer. A concentration of approximately $10^4$ spores per ml, was obtained when a suspension with an absorbancy ($A_s$) reading of 0.4 at 435 nm was diluted 1:400 in M/15 phosphate buffer solution (pH 7). Suspensions containing $1.5 - 2.5 \times 10^7$ spores per ml were obtained by suspending spores in the heating menstruum and adjusting to 0.8 $A_s$ at 435 nm.

Two ml aliquots of a spore suspension were placed in thermal death time (TDT) tubes. Tubes were sealed with an oxygen flame. If sealed tubes were not used immediately the tubes were shaken on a platform shaker, followed by individual shaking on a Vortex test tube mixer just before use to resuspend spores and disperse clumps.

Sealed TDT tubes, in small wire holders, were immersed completely in a thermostatically controlled oil bath (American Instrument Co., Silver Spring, Maryland) and heated at 121.1 ± .05 C (Figure 1). Tubes were removed in pairs at 1 min intervals and immediately chilled in an ice bath. One minute was allowed for come-up time before removal of the zero tube.

Contents of the TDT tubes were subcultured on fortified NA after appropriate dilutions. For ease in counting colonies, heated spores were placed on a layer of pre-poured fortified NA; then a few ml of melted NA were added and swirled, mixing the spores. After allowing the agar to solidify, another layer of agar was poured, thereby trapping most survivors
Figure 1. Oil bath (American Instrument Co.) used in thermal experiments showing wire holders containing thermal death time tubes (A)
below the surface (Ballock et al., 1968). Plate counts from duplicate plates were taken after 48 hr of incubation at 55 C.

**Increasing Thermal Resistance**

Attempts were made to increase heat resistance of the R variant of *B. stearothermophilus* NCA 1518 by subculturing surviving spores withstandng extended periods of heating. Spore suspensions containing $1.5 - 2.5 \times 10^7$ spores per ml were prepared and heated according to the procedure as stated previously.

Four successive heat treatments followed by subsequent selection and subculturing of survivors were completed. In each thermal death time run the majority of survivors was selected and placed on TSB at 55 C for 48 hr. Purity of subcultures was determined by streaking on DTA plates. Growth at 70 C was the final confirmation of purity.

For production of spores from the pooled survivor-subcultures, fresh cells from 18 hr TSB cultures were added to several sporulation bottles containing 125-150 ml of fortified NA. After 48 hr of incubation, spores were harvested, cleaned, and prepared for heating as stated previously.

**Sporulation Temperature and Thermal Resistance**

Triplicate spore crops of R spores were produced at three different incubation temperatures (55, 60, 65 C) on fortified
NA for the evaluation and comparison of their heat resistance. Spores were produced, harvested, cleaned, and heated as stated in previous sections.

Density Gradient Separation and Thermal Resistance

S spores were separated on a discontinuous gradient made from Renografin. Three solutions were prepared from Renografin 60 at the following concentrations: 45, 50, 55 percent. Ten ml portions were carefully layered at room temperature. Gradients were cooled to 4 C. Freshly sonicated S spores were placed on top of the gradients. Tubes containing the gradient were centrifuged at 15,000 rpm for 30 min at 14 C on an ultracentrifuge (Beckman Model L-2) equipped with an SW-25.2 swinging bucket rotor. The separation procedure was patterned closely after the work of Tamir and Gilvarg (1966).

The bands were removed and checked by phase contrast microscopy. Separate spore bands were centrifuged and washed several times in sterile distilled water. Suspensions were placed at 4 C and later heated as given in a previous procedure.

Characterization of Rough and Smooth Variants

Growth studies

Temperatures ranging from 40 to 70 C were selected for establishing growth rates and temperature optima. Two media were employed: TSB, a highly nutritious medium, and fortified NB, a moderately nutritious medium.
Quantitation of growth was based on readings in absorbancy or optical density (log Io/I). Monod (1949) stated that optical density remains proportional to bacterial density throughout the positive phase of growth of the culture when cultures are well dispersed. Growth was measured in Pyrex culture tubes (200 x 25 mm) which had been previously standardized on a Bausch and Lomb Spectronic 20 at 560 nm with a solution of CoSO$_4$·7H$_2$O according to a procedure of the National Bureau of Standards (1953).

Tubes of broth were inoculated with one drop of an 18 hr culture grown in TSB or with spores produced on fortified NA incubated at 55 C for 48 hr. Heat activation of the spores was necessary for maximum germination (Fields, 1963). Spores in sealed conventional TDT tubes were placed in a 121 C oil bath for 2 min, heating them from room temperature to a final temperature of 121 C in the 2 min period as determined with a thermocouple. This period of heating was sufficient to heat shock the R and S spores without causing any significant lethality.

Duplicate tubes were inoculated with a predetermined number of vegetative cells or spores. One uninoculated tube was incubated at each temperature for use as a control and blank for absorbancy reading at 530 nm. Absorbancy readings were taken at intervals of 3 to 4 hr. Prior to each reading tubes were shaken on a Vortex test tube mixer.
pH study

Trypticase soy agar (BBL) minus the agar (TSA-A) was prepared as Hill and Fields (1967a) described, except that 0.25% dextrose was added. The unheated, hydrated medium was centrifuged to remove the agar. Commercial trypticase soy broth was not used because of the buffering effect of the dipotassium phosphate present in the broth (TSB) but absent in the agar (TSA). This buffering ingredient was considered undesirable in the study of pH changes.

Cultures of variants growing in TSA-A broth at 55 C were monitored continuously with a Beckman Expandomatic pH meter equipped with a Bausch and Lomb recorder. Samples were checked for purity periodically on DTA plates. Variants were used in both pure and mixed cultures.

Size and sporulation study

For the determination of cell and spore size and percentage sporulation, 150 ml or 250 ml square media bottles were filled with 40 ml of fortified NA or TSA and placed on their sides. Each bottle was ventilated with a cotton plugged glass tube, inserted in a rubber stopper.

Triplicate bottles were inoculated with young cells (18-20 hr) incubated at temperatures ranging from 37 to 65 C. Three replicates were performed at all temperatures. After 48 hr of incubation cells and spores were examined microscopically. Purity checks on DTA plates were made along with pH
determinations. Photomicrographs were taken of unstained wet smears of cells and spores using a phase microscope. From the photomicrographs, measurements of the cells and spores were made with a Vernier caliper, whereby dimensions were converted to actual sizes. For each mean size twenty to thirty measurements of lengths and widths were made at the various temperatures. Other data were collected including: relative sporulation levels, effect of growth medium on size, and degree of degeneration.

Antigenic study

Antigen preparation Typical R and S colonies were selected and checked for species and variant purity. After establishing homogeneity, subcultures were incubated at 55°C in TSA shake flasks for 9 hr at which time cells were washed in 0.85% saline three or four times after subsequent centrifugation and stored in normal saline at 4°C.

R and S spores used as antigens were produced and cleaned as described previously. To insure homogeneity of spore antigens special attention was given to density gradient separation as a means of removing vegetative debris from the spores after lysozyme treatment. After cleaning and washing several times spores were stored in normal saline at 4°C.

In order to expose internal cellular and spore antigens, brief periods of sonic vibration were used to disrupt a portion of the population as judged from periodic examination with phase contrast microscopy.
Antisera production

Antigens were injected into the marginal ear veins of eight young rabbits separated into pairs. Each pair received either a cell or a spore antigen of the R or S variant type at intervals of 3 to 4 days for one month. Each rabbit received 7 to 8 ml of antigen during the injection period. From the anesthetized rabbits about 30 ml of blood was drawn via a cardiac puncture. Serum was prepared according to the procedure given by Campbell et al. (1963).

Titers and agglutination responses were determined by using the Microtiter System (Sever, 1962). Microtiter plates were covered with a glass plate and incubated at 37 C for 3 hr and read after which time they were left overnight at 4 C and examined again. Readings were made in the Microtiter wells with a dissecting microscope (18×).
RESULTS AND DISCUSSION

Increasing Thermal Resistance by Selection

After four successive heat treatments, each followed by subsequent selection and subculturing, heat resistance of *B. stearothermophilus* was increased (Figure 2, Table 1).

Table 1. Comparison of heat resistance of R spores subcultured from heat resistant survivors*

<table>
<thead>
<tr>
<th>Trial</th>
<th>99.999% Decimal Reduction time (min)</th>
<th>F value&lt;sup&gt;a&lt;/sup&gt;</th>
<th>k value&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>7</td>
<td>1.12</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>11</td>
<td>0.95</td>
</tr>
<tr>
<td>3</td>
<td>9.25</td>
<td>13</td>
<td>0.55</td>
</tr>
<tr>
<td>4</td>
<td>11.25</td>
<td>16</td>
<td>0.46</td>
</tr>
</tbody>
</table>

*Average of duplicate runs.

<sup>a</sup>F value = time required to destroy the population at 121.1°C in M/15 neutral PO₄<sub>4</sub> buffer.

<sup>b</sup>k value = slope calculated from: \((\log N_1 - \log N_2) / t_2 - t_1\) when \(N_1\) is the number of spores at time \(t_1\) and \(N_2\) is the number remaining at time \(t_2\).

Table 1 lists the decimal reduction time (99.999%), the \(F_{250}^{250}\) values, and \(k\) values for the series of treatments. The \(k\) value, which is the slope or death rate constant, was calculated for each treatment on the straightest part of the curve. Because of nonlinearity of the curves, \(D\) values were not used.
Figure 2. Survival of R spores of *B. stearothermophilus* NCA 1518 produced from survivors of sequential heat treatments (I - IV) at 121.1°C.
The decimal reduction times obtained for the four treatments were subjected to an analysis of variance (Snedecor, 1956). The differences among the four heating treatments were highly significant when analyzed by analysis of variance (Table 2).

Table 2. Decimal reduction time* (99.999%) for R spores of B. stearothermophilus produced from survivors of sequential heat treatments**

<table>
<thead>
<tr>
<th>Series</th>
<th>Runs 1</th>
<th>Runs 2</th>
<th>Runs 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.25</td>
<td>5.00</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>8.50</td>
<td>9.00</td>
<td>9.25</td>
</tr>
<tr>
<td>3</td>
<td>8.75</td>
<td>9.25</td>
<td>10.00</td>
</tr>
<tr>
<td>4</td>
<td>11.25</td>
<td>11.75</td>
<td></td>
</tr>
</tbody>
</table>

*Data expressed in minutes.

**Heated in M/15 neutral PO₄ buffer at 121.1 C.

Several investigators have shown increased resistance in at least one species of sporeforming bacteria (Bigelow and Esty, 1920; Magoon, 1926; Williams, 1929; Davis and Williams, 1948). Other workers who have found no tendency for enhanced heat resistance include Sommer (1930), Desrosier and Esselen (1951), and Vas and Proszt (1957). Williams (1936), working with several different species of sporeformers, found an increase in heat resistance in only one. He was skeptical of the increase and attributed it to the clumping tendency of the spores in the
suspension. Rahn (1945) theorized that heat resistance was not increased by selecting the last survivors, contending that survivors were no more resistant than the average because of the logarithmic order of death. A similar stand was made by Amaha and Sakaguchi (1951) from their work on heat resistance.

The theory proposed by Rahn (1945) assumed uniform resistance in the cells or spores and that death occurred as a result of a single event resulting in an exponential decline in viable cells. Jordan et al. (1947), on the other hand, assumed non-uniformity of resistance and a non-logarithmic order of death. The second theory is compatible with heat resistance data frequently reported as non-logarithmic since it takes into consideration the normal or graded distribution of heat resistance in a population. Licciardello and Nickerson (1963) state that the existence of nonlinear survival curves requires more recognition and should be studied and related on a mathematical basis to thermal process calculations.

Vas and Proszt (1957) concluded that a certain frequency of extremely heat resistant individuals exists in a spore population. They stated that the heat resistant fraction in a population of B. cereus did not arise from mutation, but represented a part of the natural distribution of resistance. These workers were unable to show increased resistance in survivors of heat treatments. According to them, resistant cells were detected only at levels of 1 in $10^7$ to $10^8$ spores.
Use of the R variant, which sporulated more readily than the S, proved satisfactory for production of the numbers of spores required for preparing such concentrated suspensions.

A continual surveillance of the R survivors from heat treatments revealed only cells of the R type. Such surveillance was an absolute requirement since an apparent increase in heat resistance would have been observed if S spores were present in the suspension, because of the greater heat resistance attributed to S spores (Fields and Finley, 1962; Rotman and Fields, 1966).

An increase in heat resistance of an extreme thermophile is of practical concern to industry or anyone concerned with sterilization processes, since the possibility of developing enhanced resistance always exists. This study has shown that heat resistance can be increased in the R variant of B. stearothermophilus; in fact, in the fourth heat treatment, the resistance had reached that of the S variant which is usually considered more resistant than the R form. If the heat resistance of the S form were increased a population of extremely heat resistant spores would exist, many able to survive the usual sterilizing procedures. Such selection has not been observed in nature; but, in a food plant, conditions for selection are theoretically possible.

Effect of Sporulation Temperature on Thermal Resistance

Sporulation temperature markedly affected heat resistance of rough spores (Table 3, Figure 3). With S spores no attempts
were made to study the effect of growth temperature since sporulation levels were low at temperatures above 55 C.

Table 3. Comparison of heat resistances of R spores produced at three different temperatures*

<table>
<thead>
<tr>
<th>Sporulation temperature</th>
<th>99.9% Decimal Reduction Time</th>
<th>F value</th>
<th>k value</th>
</tr>
</thead>
<tbody>
<tr>
<td>55</td>
<td>4.25</td>
<td>7</td>
<td>0.60</td>
</tr>
<tr>
<td>60</td>
<td>7.00</td>
<td>9</td>
<td>0.48</td>
</tr>
<tr>
<td>65</td>
<td>8.50</td>
<td>12</td>
<td>0.30</td>
</tr>
</tbody>
</table>

*Average of 3 replicates.

aF value = time required to destroy the population at 121.2 C in a M/15 PO₄ neutral buffer.

bK value = slope, calculated at 1 to 7 min from:
\[(\log N_1 - \log N_2)/t_2 - t_1\] when N₁ is the number of spores at time t₁ and N₂ is the number remaining at time t₂.

Differences in heat resistance were encountered between triplicate harvests at the same temperature; but the trend was always the same as shown in Table 4.

For the most part increased sporulation temperature has been correlated with increased heat resistance (Esty and Williams, 1920; Eckford, 1927; Williams, 1929; Curran, 1934; ZoBell and Conn, 1940; Lamanna, 1942; Williams and Robertson, 1954; El-Bisi and Ordal, 1956; Lechowich and Ordal, 1962). In a few instances resistance was reported to increase only until
Figure 3. Survival curves of R spores of B. stearothermophilus NCA 1518 produced at sporulation temperatures of 55°C, 60°C and 65°C. Heat treatment was at 121.1°C.
the optimum growth temperature was reached; beyond that point, heat resistance was lowered (Theophilus and Hammer, 1938; Sugiyama, 1951).

Table 4. Comparative thermal resistance of crops of R spores grown at three different temperatures*

<table>
<thead>
<tr>
<th>Sporulation temperature</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>55</td>
<td>5.75</td>
<td>4.0</td>
<td>5.00</td>
</tr>
<tr>
<td>60</td>
<td>8.50</td>
<td>6.5</td>
<td>6.75</td>
</tr>
<tr>
<td>65</td>
<td>12.00</td>
<td>8.0</td>
<td>8.50</td>
</tr>
</tbody>
</table>

*Values expressed as 99.9% decimal reduction.

Lechowich and Ordal (1962) found increases in the ratio of cations, especially calcium, to DPA as heat resistance was increased. As a result of increased incubation temperatures they suggested that enhanced heat resistance from increased growth temperature can be correlated with the ability of the cations, especially calcium, to enter into intricately folded combinations of cations, DPA and proteins. They hypothesized that calcium might enter into combination with DPA and proteins or solely with protein, thereby stabilizing the structural integrity of the spore proteins. The exact relationship of calcium and other cations to heat resistance in bacterial spores has yet to be established.
Density Separation

Effect of density on thermal resistance

Density separation of S spores on a discontinuous gradient was accomplished, yielding spores with different levels of resistance to heat as depicted in Figure 4. The spores that pelleted had a survival time 2 min longer than the lighter band. Comparison of the heat resistances of these spores is made in Table 5.

Table 5. Comparative heat resistance of "light" and "heavy" S spores of B. stearothermophilus separated on a density gradient

<table>
<thead>
<tr>
<th>Fraction</th>
<th>99% Decimal Reduction</th>
<th>F value&lt;sup&gt;a&lt;/sup&gt;</th>
<th>k value&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heavy</td>
<td>6.25</td>
<td>11</td>
<td>0.53</td>
</tr>
<tr>
<td>Light</td>
<td>4.50</td>
<td>9</td>
<td>0.58</td>
</tr>
</tbody>
</table>

<sup>a</sup>F value = number of minutes required to kill the spore at 121.1 C in a M/15 neutral PO<sub>4</sub> buffer.

<sup>b</sup>k value = slope = (log N<sub>2</sub> - log N<sub>1</sub>)<sub>t<sub>2</sub> - t<sub>1</sub></sub> when N<sub>2</sub> is the number of spores at time t<sub>1</sub> and N<sub>1</sub> is the number remaining at t<sub>2</sub>.

These results agree with the findings of several workers (Yesair and Cameron, 1936; Sugiyama, 1951; Anand, 1961). In a similar study, Anand (1961) correlated heat resistance and specific gravity of individuals in populations of five different
Figure 4. Survival curves of two fractions of S spores of B. stearothermophilus NCA 1518 designated as "light" (L) and "heavy" (H). Heat treatment was at 121.1°C.
species including *B. stearothermophilus*; he was not able to obtain the same relation in populations of three other species. Frank and Campbell (1957) found no differences in heat resistance of three fractions of *B. coagulans*.

Findings of Tamir and Gilvarg (1966) from isopycnic gradient centrifugation of spores provided some insight into the nature of the heterogeneity of spores. Besides showing a wide spectrum of densities in a population of *B. megaterium*, they observed that lighter spores required little or no heat activation for germination, whereas heavier spores germinated slowly unless heat activated. Heat of activation was related directly to heat resistance in studies on *B. stearothermophilus* NCA 1518 by Fields and Finley (1962) and Fields (1963). They found that spore suspensions requiring higher temperatures for activation were more heat resistant.

Spores grown in the absence of calcium are lighter than spores grown in the presence of calcium according to Tamir and Gilvarg (1966). Calcium forms a complex or salt with dipicolinic acid which has been shown to affect heat resistance (Church and Halvorson, 1959; Lechowich and Ordal, 1962). In view of the high density of calcium dipicolinate (1.71 g per cm³) Tamir and Gilvarg (1966) reasoned that spores produced in the absence of Ca++ will be less dense than those produced on regular medium. The reasons for the existence of light spores in a population are theoretical. Three suggestions have been
made to account for the development of spores of low density: release of spores from parent cells before maturation, induced germination in some spores in a spore population and/or a certain deficiency in a medium (Tamir and Gilvarg, 1966).

Apparently calcium dipicolinate is the main constituent influencing spore density. A complex of dipicolinic acid, calcium, and amino acids may stabilize the proteins of spores and contribute to their heat resistance as suggested from studies by Powell and Hunter (1956) and Young (1959).

**Density gradient separation as a cleaning technique**

A suitable gradient for separating vegetative debris (or cells) from spores has been used which allowed large scale purification of S spores needed for heat treatments and preparations of antigens. The separation of R and S spores was accomplished in one step because of density differences. S spores were found lighter than R spores. With gradient cleaning low yielding sporulating cultures were salvaged which normally would have been discarded.

The gradient material, Renografin, presented no noticeable toxic effects or changes in the heat resistance. Tamir and Gilvarg (1966) reported that spores and cells of *B. megaterium* were unaffected by contact with Renografin for periods up to 24 hr. They found no toxic effect or germination as judged from heat destruction information.
Characterization of Rough and Smooth Variants

**Cultural characteristics**

The cultural characteristics of *B. stearothermophilus* were studied extensively by Smith *et al.* (1952) and by Wolf and Barker (1968). The morphological and physiological characteristics obtained in this study are presented in Table 6. The R and S variants exhibited quite different characteristics; however, they did fall within the broad physiological patterns described by the above workers. The one exception to their description was the starch reaction for which the R culture was negative. From a study of 230 cultures, Wolf and Barker (1968) described the starch reaction in *B. stearothermophilus* cultures as strongly positive.

The temperature of incubation for the biochemical tests was 55°C. The negative results encountered with the R variant could have been "false negative" readings since growth temperature can inhibit certain biochemical reactions (Alford, 1960). Alford suggested that "false negative" reactions can be eliminated by making the tests at temperatures lower than the temperature required for most rapid growth. Our results show that the R variant grows fastest from 60 to 65°C; thus, 55°C would be a suitable temperature for determining biochemical characteristics if Alford's reasoning is correct.

The S type of colony appeared round, convex with a smooth texture, while the R colony was typically rough and somewhat
Table 6. Some morphological and physiological characteristics of rough and smooth variants of *B. stearothermophilus* NCA 1518

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Rough</th>
<th>Smooth</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell morphology*</td>
<td>single to filaments</td>
<td>single cells</td>
<td>NA (fortified)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>48 hr at 55 C</td>
</tr>
<tr>
<td>Cell size*</td>
<td>4.6 × 0.6µ</td>
<td>3.1 × 0.8µ</td>
<td>NA (fortified)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>48 hr at 55 C</td>
</tr>
<tr>
<td>Spore size*</td>
<td>1.8 × 1.1µ</td>
<td>1.6 × 1.0µ</td>
<td>NA, 24 hr uncrowded</td>
</tr>
<tr>
<td>Colonies on DTA</td>
<td>irregular and rough</td>
<td>pinpoint, round, and smooth</td>
<td></td>
</tr>
<tr>
<td>Subsurface colonies</td>
<td>compact, rosette or disc-shaped</td>
<td>loose knit, cottony</td>
<td>NA, 24 hr uncrowded</td>
</tr>
<tr>
<td>Colony size</td>
<td>2.0 - 2.5 mm</td>
<td>1.0 - 1.7 mm</td>
<td>DTA, 55 C uncrowded</td>
</tr>
<tr>
<td>Nutrient agar growth</td>
<td>good to excellent</td>
<td>fair to good</td>
<td></td>
</tr>
<tr>
<td>Nutrient broth</td>
<td>&quot;ropy&quot;, viscous</td>
<td>uniform turbidity</td>
<td></td>
</tr>
<tr>
<td>NaCl broth (3%)</td>
<td>negative</td>
<td>negative</td>
<td></td>
</tr>
<tr>
<td>Hydrolysis of starch*</td>
<td>negative</td>
<td>positive</td>
<td>Smith, Gordon &amp; Clark, 1952</td>
</tr>
<tr>
<td>Hydrolysis of casein*</td>
<td>negative</td>
<td>positive</td>
<td>Smith, Gordon &amp; Clark, 1952</td>
</tr>
</tbody>
</table>

*Considered by Smith et al. (1952) to be key characteristics.*
<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Rough</th>
<th>Smooth</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrolysis of gelatin*</td>
<td>negative</td>
<td>positive</td>
<td>Smith, Gordon &amp; Clark, 1952</td>
</tr>
<tr>
<td>Anaerobic growth</td>
<td>limited growth</td>
<td>positive</td>
<td>DTA agar stabs 48 hr, steamed &amp; covered with paraffin</td>
</tr>
<tr>
<td>Growth at 70 C* on NB</td>
<td>good</td>
<td>little, if any</td>
<td>48 hr</td>
</tr>
<tr>
<td>Growth at 70 C on TSB</td>
<td>good</td>
<td>good</td>
<td>48 hr</td>
</tr>
<tr>
<td>pH on TSA-A</td>
<td>7.2 - 7.5</td>
<td>4.9</td>
<td>48 hr</td>
</tr>
</tbody>
</table>
flattened with irregular edges (Figures 5, 6, and 7). These observations agreed with those of Michener (1953) who reported finding R and S variants in *B. stearothermophilus* NCA 1518 and with those of Fields (1963) who has further studied and described these variants in detail.

Several factors affected the colonial form including growth medium, pH, and age. On nutrient agar, the S colony appeared typical while the R grew in a smooth-like manner, but still more flattened, and darker than the S. The R colony as it appeared on both TSA and on DTA was considered typical.

The pH of the agar medium influenced morphology of the R colony more than that of the S type. At a pH above 7.5 the R colony grew in a more flattened manner with a serrated margin. On DTA plates R colonies induced a color change in the medium indicative of early acid production followed by another color change after 24 to 48 hr attributable to the release of basic metabolites (Hill and Fields, 1967b). In purple zones where the pH was above 7.0 the R colonies seemed to lose their typical appearance, gaining instead, a smoother appearance over the surface and periphery; those colonies growing at a pH below 7.0 remained typical even after 24 to 48 hr.

Another factor affecting R and S manifestations on DTA was age. Colonies 12 to 24 hr of age were usually typical; however, upon continued growth for another 24 to 48 hr the colonies frequently lost their distinctive R and S characters.
Figure 5. Typical surface colonies of the smooth variant of *B. stearothermophilus* NCA 1518 (90×)
Figure 6. Typical surface colonies of the rough variant of B. *stearothermophilus* NCA 1518 (90×)
Figure 7. A rough and a smooth variant colony of *B. stearothermophilus* NCA 1518 growing together (90x)
With age, S colonies began to look irregular and attain a ground glass appearance when viewed under a dissecting microscope; while the old R colony became flattened and sometimes acquired a smooth appearance. The morphological changes probably were attributable to age, build up of metabolic wastes, and other changes connected with cultures in latter stages of growth.

Subsurface colonies of the R and S variants displayed a morphology distinctly different from that of surface colonies. At 48 hr of age the R type grew as a loosely knit unit appearing "cottony" over the surface (Figure 8). In contrast, the S variant grew as a dark compact colony demonstrating a rosette or a disc or saucer-like appearance (Figure 9). Fields (1963) had previously described the disc-shaped colony. The R type was approximately twice the size of the S colony after 48 hr of incubation.

The growth of R and S variants in broth cultures revealed another striking difference (Figure 10). The ropy, viscous nature of the R variant was attributed to slime or capsule production complemented by the filamentous nature of the organism. In contrast, the S form produced even turbidity in broth. The S variant apparently produced little or no capsular material. This material has been shown to be the site of specific antigens (Knaysi, 1951). Antigenic differences were observed between the two types. In the genus Bacillus, Hanby and Rydon (1946) suggested that the capsule (slime) is made of
Figure 8. Rough colonies of *B. stearothermophilus* NCA 1518
Top - A surface and a subsurface colony at 17 hr of age (90×)
Bottom - A subsurface colony at 36 hr of age (90×)
Figure 9. Subsurface smooth colonies of *B. stearothermophilus* NCA 1518
Top - 36 hr of age, rosette type (90×)
Bottom - 36 hr of age, saucer type (90×)
Figure 10. Broth tubes showing ropy growth in rough variant (A) and uniform growth in smooth variant (B) of *B. stearothermophilus* NCA 1518
a polypeptide of D-glutamic acid. The fact that the R form did not produce mucoid colonies on the surface of agar indicated that slime production depended on environment. In broth the ropy, viscous growth suggested that a higher level of capsular material resulted from environmental influence. Perhaps oxygen tension was the main factor involved, since the production of slime in *B. anthracis* was shown to be favored by increasing CO₂ pressure (Knaysi, 1951); however, any number of environmental factors may influence slime production (Knaysi, 1951).

Response to oxygen levels was noted in broth cultures of R and S variants; the R variant grew near the top in the broth usually adhering to the growth vessel forming a rim at the top of the broth, whereas the S form grew throughout the medium. This observation, in addition to another characteristic, from cultures grown anaerobically, suggested higher oxygen requirements for the R variant than for the S variant. The actual growth of the R variant under anaerobic conditions was only slight in 48 hr while under the same conditions the S form produced good growth. The fact that the S variant was found tolerant of anaerobic conditions is supported by observations of Hill et al. (1967) who reported that the S variant has an active Embden-Meyerhof (EM) pathway and relatively weak tricarboxylic acid (TCA) pathway. On the other hand, the latter workers have shown that the R variant has a less active EM pathway and a very active TCA pathway. Under aerobic conditions
the EM pathway usually operates in conjunction with the TCA cycle. The EM pathway can operate separately under anaerobic conditions (Dawes and Large, 1968).

**Growth studies**

Growth curves for R and S variants at temperatures ranging from 40 to 70 C on a nutritionally "rich" and relatively "poor" medium are presented in Figures 11 and 12. TSB represented the rich medium; whereas fortified NB was representative of the poor medium. With low levels of inocula growth at 45 C was limited. With higher levels of inocula the lag at 45 C was shortened, but still the lag usually persisted longer than 48 hr indicating a relationship between temperature and the duration of the lag phase. The growth temperature-quantity distribution curves in Figure 13 show the effect of temperature upon growth levels. Standard curves have been established for certain thermophiles showing that turbidimetric measurements were proportional to numbers of cells per ml (Allen, 1953; Fields, 1966). Allen (1953) observed that turbidity and cell count measurements of growth coincided during the logarithmic phase of growth in *B. circulans*; but, they deviated when growth became slower. Fields (1966) showed a similar correlation with growing cultures of R and S variants of *B. stearothermophilus NCA 1518*.

Figures 11 and 12 represent concentrations of bacterial cells in the growth medium. These results were basically
Figure 11. The growth of rough and smooth variants of *B. stearothermophilus* NCA 1518 in trypticase soy broth at various temperatures
Figure 12. The growth of rough and smooth variants of *B. stearothermophilus* NCA 1518 in nutrient broth at various temperatures.
ABSORBANCY AT 530 m\(\mu\)
Figure 13. The effect of growth temperature and medium upon growth levels of rough and smooth variants of *B. stearothermophilus* NCA 1518 at the end of 24 and 48 hr growth periods
similar to those described by Neilson et al. (1959) in which log of cell count was plotted against time. According to Monod (1942) these yields become less as the temperature increases. (Monod, 1942). Growth rates are considerably lower at the lower growth temperatures ca. 45-50 C; however, a lower growth temperature can increase the total cell count if sufficient time is permitted. These results represent actual cell counts only when cultures were measured in the logarithmic phase.

Certain other experimental limitations exist in using turbidity as a measure of quantitative growth. Cell lysis which occurs at the end of growth, can decrease the turbidity. Microscopic examinations showed that some increase in turbidity resulted from cellular debris. The degree of lysis in the rough variant of B. stearothermophilus is particularly dependent on oxygen level which will be discussed subsequently. In a 48 hr growth period lysis occurred in all cultures grown at temperatures above 50 C. In a study on the growth of B. circulans at 60 C, Allen (1953) reported some lysis, but at levels much less than that found in B. subtilis.

Other experimental problems in turbidimetric measurements which were considered included: changes in light scattering per cell, the presence of spores in the cell population, and changes in shape and size. Microscopic observation of older cells revealed intracellular granules which were more
refractile than the protoplasm which contained them. Henrici (1928) has observed that these granules in B. megaterium disappear during active growth and reappear when growth decelerates. Few, if any, spores were produced in R or S cultures on TSB. Changes in shape and size did occur during growth of B. stearothermophilus, particularly in the R variant.

Wilson (1922) stated that the viable growth does not always coincide with that of total growth. No attempt has been made to relate viable cell count (plate counts) to total count (microscopic count). Allen (1953) observed that high mortality is characteristic of thermophilic bacteria growing at elevated temperatures. The viable count in B. circulans was shown to parallel the total count, being slightly less until the end of the logarithmic phase at which time the viable count started to fall in a logarithmic manner (Allen, 1953).

One of the reasons for a drop in cell crop as growth temperature was raised has been attributed to a reduction of efficiency of the synthetic activities responsible for the net rate of cell formation (Mitchell, 1951). At higher temperatures the processes involved in growth tend to speed up just as the rate of a given chemical reaction is increased when temperature is raised. At higher temperatures, growth rates are not increased proportionally to the increase in temperatures because of the extra energy the organism must expend to overcome the destructive effects of heat. Since the
disorganizing or denaturing effects outweigh the increases in rate processes in synthesis affecting the growth systems, higher levels of nutrition are necessary to maintain a culture with the capacity to produce cells both viable and nonviable (Mitchell, 1951; Allen, 1953).

By using a rich, buffered medium (TSB) greater cell densities were obtained. NB does not offer certain nutrients necessary for growth at 70 C. In the comparisons of DTA and NB the deficiency in NB was evident in two ways: 1) reduction of growth level of the S variant and 2) lowering of the optimal growth temperature about 5 C below the optimum obtained with TSB (Table 13).

Between the R and S variants, the main differences observed were: 1) the R variant grew better than the S variant on a medium containing less available nutrient; nevertheless, the S form outgrew the R form on a rich medium in the first 24 hr period. In a 48 hr period the difference was reduced as the R continued to gain; 2) the S variant on either type of medium had a lower optimal temperature of growth than did the R variant.

Concentrations of R and S cells at a particular absorbancy reading are not necessarily equal as pointed out by Fields (1966). Differences in cell size and length do exist between the variants; however, relative comparisons of cell concentration during the growth study can be made from turbidimetric measurements.
In most of the growth curves for R and S strains a "wavy" or multiple log phase effect was detected. This phenomenon was more pronounced in the R cultures than in the S cultures and appeared as double or triple growth cycles. In the S culture the effect was minor occurring usually after high cell concentrations were reached. A similar variation in growth curves was reported by Monod (1942) which he called the "diauxie effect". This effect was observed in cultures of Escherichia coli grown on a synthetic medium containing glucose and sorbitol as a carbon source. As the glucose was utilized the organism went into a lag phase awaiting the appearance of an inducible enzyme for the utilization of sorbitol. The evidence indicated that each cycle corresponded to the exclusive utilization of one of the constituents of the mixture, resulting from an inhibitory effect of one of the compounds on the formation of the enzyme attacking the other (Monod, 1942).

Any one of several factors may be responsible for the diauxie effect in the growth curve of the R variant. The most obvious explanation is the utilization of the carbohydrate and subsequent enzymatic adaptation to allow a utilization of peptones present in the medium. The R variant is a weak hydrolyzer of proteins since neither gelatin or casein were attacked (Table 6); this does not mean that the R variant could not adapt and utilize peptones. The R variant apparently
can metabolize certain protein constituents as evidenced by accumulation of basic substances in the growth medium (Hill et al., 1967). Hill and Fields (1967b) have also observed the diauxie effect in the growth of the R variant in minimal broth and TSA-A, but not in NB. Hill and Fields (1967b) suggest that the diauxie effect might be due to two different "rough variants" instead of a response to two separate carbohydrates. No data have been presented thus far to support the existence of two populations or types of R variants.

**pH study**

pH readings plotted continuously against time are depicted in Figure 14 for R and S variants in pure and mixed cultures grown at 55°C. The pH of the control (uninoculated flasks containing TSA-A) underwent only minor changes. The S variant produced conditions for a rapid pH change while the R showed only slight acidity in the early stages of growth followed by a continued increase in pH throughout the incubation period. These findings are similar to those reported by Hill and Fields (1967b).

The mixed culture exhibited a pH pattern similar to the S variant. The ratio of S to R which was 3:2 remained fairly constant indicating no inhibition by either variant. Fields (1966) reported that the S variant dominated the culture in a mixed population in pea extract.
Figure 14. pH changes in rough and smooth variants in pure and mixed populations of *B. stearothermophilus* NCA 1518 on TSA-A
R - rough. S - smooth. M - mixed. C - control
The high pH developed by the R variant has been attributed to the production of basic substances and a very active tricarboxylic acid (TCA) cycle (Hill et al., 1967; Hill and Fields, 1967b). The same workers have shown that the S variant does not produce significant amounts of basic substances and it has a relatively inactive TCA cycle.

Effect of temperature and medium on size and sporulation

Size of cells and spores The mean length, width, and volume obtained from over 1500 measurements taken from photographs of cells and spores are plotted against growth temperature in Figures 15 and 16. Spore size of both variant types increased with an increase in growth temperatures (Figure 16). R vegetative cells, on the other hand, increased in length while S vegetative cells were influenced very little (Figure 15).

Comparisons of the sizes of R and S variant cells and spores, produced under the same environmental conditions, revealed inherent differences in size (Figures 17, 19, 20). Spores of the R variant were larger than S spores (Figures 16 & 20). R cells were longer than S cells; however, length differences alone did not always reflect volume differences. At 55°C and below the volume of the S cells was greater than the R cells; conversely, at higher temperatures the R cell volume exceeded the S cell volume. The S cells were slightly wider than the R cells at all growth temperatures (Figure 15).
Figure 15. Effect of growth temperature on length, width, and volume of vegetative cells of the rough and smooth variants of Bacillus stearothermophilus NCA 1518 grown on nutrient agar fortified with 0.05% dextrose and 30 ppm Mn++. Cell volume calculated from:

\[ V = \pi \left( \frac{w}{2} \right)^2 (l-w) + \frac{4}{3} \pi \left( \frac{w}{2} \right)^3 \]

where \( w \) = width of cell and \( l \) = length of cell. The formula is for the volume of a cylinder with a hemisphere on each end (Huntington and Winslow, 1937)
Figure 16. Effect of growth temperature on length and width of spores of the rough and smooth variants of Bacillus stearothermophilus NCA 1518 grown on nutrient agar fortified with 0.005% dextrose and 30 ppm Mn++
Figure 17. Phase-contrast photomicrographs of young unstained vegetative cells of *Bacillus stearothermophilus* NCA 1518 from the surface of dextrose tryptone agar produced at 55°C (1,300×)

a. Cells of the smooth variant

b. Cells of the rough variant
Thus, the differences in cell size between the variants and within the variants were influenced directly by temperature of growth. Filamentous cells, which were observed to a limited degree above 50 °C, were not included in these measurements.

Increase in size of vegetative cells of the R variant occurring with elevated growth temperature as shown in Figure 15 might be attributed to the accumulation of slime or capsular material; Duguid (1948) reported that capsular material could continue to accumulate while protoplasmic growth was inhibited.

Several workers have reported changes in cell size within a given population during the various phases of growth (Huntington and Winslow, 1937; Knaysi, 1940; Burrows, 1963). They report, in general, that cells increase in size during the lag period of growth reaching a maximum size in physiological youth, followed by a decrease during the logarithmic phase. By the time the stationary phase is reached, the cells are uniformly smaller. In this study, the changes in size, which were noted, were not related to changes associated with the various phases of growth. Measurements were taken at the end of 48 hr; this amount of time was considered sufficient for all cultures to reach stable morphological cell types. Even at a growth temperature as low as 45 °C, cells were most likely into or beyond the stationary phase after 48 hr of incubation. In fact, Neilson et al. (1959) after incubating B. stearothermophilus
at 45 C found the stationary phase occurred in about 12 hr when starting with an initial cell concentration of $10^6$ per ml. In our work heavy inocula, containing between $10^6$ and $10^7$ cells per ml, was used to insure a short lag phase and a reasonably early stationary phase.

**Filament formation** When R and S cultures were grown on TSA different responses were observed. The R variant produced cells of a highly filamentous nature (Figure 18b). When cultures of the S variant were grown on TSA, a small portion of the constituent cells was observed as chains made up of two or three cells (Figure 18a). Unlike the filamentous forms of the R variants the S cells were septate. The only time septate filaments were observed in the R cultures was in young cultures grown on DTA (Figure 17). Normally the S variant was composed of single cells which had separated completely after cell division.

Morphological variants which are filamentous because of the continuance of axial growth without corresponding cell division are known as "long forms" (Duguid and Wilkinson, 1961). This morphological variant occurs in growing or ageing culture because of certain environmental conditions which influence cell metabolism. Morphological variation can be induced by inorganic salts, acidity and alkalinity, amino acids in excess, antibacterial agents and starvation from lack of growth factors (Duguid and Wilkinson, 1961).
Figure 18. Phase-contrast photomicrographs of unstained vegetative cells from 48 hr cultures of Bacillus stearothermophilus NCA 1518 produced on trypticase soy agar at 50 C (2,200x)

a. Cells of the smooth variant showing septate filaments

b. Cells of the rough variant showing filamentous morphology
Figure 19. Phase-contrast photomicrographs of unstained vegetative cells and spores from 48 hr cultures of *Bacillus stearothermophilus* NCA 1518 produced at 55°C on nutrient agar fortified with 0.05% dextrose and 30 ppm Mn++ (2,000×)

a. Cells and spores of the smooth variant

b. Cells of the rough variant
Figure 20. Phase-contrast photomicrographs of unstained vegetative cells and spores from 48 hr cultures of *Bacillus stearothermophilus* NCA 1518 produced at 65 °C on nutrient agar fortified with 0.05% dextrose and 30 ppm Mn++ (2,100×)

a. Cells and spores of the smooth variant

b. Cells and spores of the rough variant
The physical nature of the medium on which the R variant was grown was an important factor affecting the type of filament produced. Filamentous growth occurred to a much greater extent on TSA than on NA. According to Bisset (1955) a medium which offers less resistance on the surface and has a low surface tension and high concentration of electrolytes can enhance the growth of long filaments. The filamentous nature of the R variant on TSA offers some growth advantage over the S variant. On the surface of agar the R colony forms a more efficient colony because of a greater proportion of cell contact with the substrate (Bisset, 1955); conversely, this efficiency is lost in an S colony because of complete cell division which eliminates filament production and close contact with the growth medium.

The reason(s) for filament formation relates to certain conditions of growth which interfere with cell division. As pointed out previously certain physical conditions can affect the degree or type of filamentation. The formation of nonseptate filaments are associated with more severe conditions than that of septate production (Duguid and Wilkinson, 1961). Manganese deficiency has been related to the production of nonseptate filaments (Duguid, 1948). The presence of manganese might be the variable encountered in this experiment since filamentous growth occurred to a greater extent on TSA than on NA fortified with 30 ppm Mn++. The degree of filament formation in fortified NA was greater as growth temperature was increased, indicating
perhaps a change in cell metabolism at elevated temperatures affecting cell division. In this study another possible reason for filament formation in cell growth on TSA may be associated with an excess of certain amino acids. Starvation or lack of growth factors in TSA is considered unlikely.

Sporulation On fortified NA after 48 hr of incubation the R variant produced spores at all growth temperatures from 45 to 65 C and, in every case, in greater numbers than in the S variant. At 55 C the R variant reached an estimated sporulation level of 75 to 80% of the population which was the highest percentage attained; at the same growth temperature the S variant sporulated in the 25 to 60% range. Few spores were observed in the S variant at temperatures below or above 55 C on fortified NA. In mixed R and S cultures R spores predominated, almost completely, the spore population. These findings were similar to those given by Fields (1966) in which he reported greater sporulation levels in the R than in the S variant cultures when grown on pea extract or pea agar. Our findings from studies on mixed cultures also supported his work.

The production of spores depends on a number of environmental factors including; temperature, pH, oxygen tension, carbon sources, nitrogen source, and growth factors (Ordal, 1957). The effect of growth temperature on sporulation was studied by Blau in 1906 (Mitchell, 1951). In a study of 13 members of the genus Bacillus he found that the formation of
spores and spore germination were sensitive to temperature in the same way that vegetative cells are. The maximum temperature for vegetative growth, spore formation, and germination was shown to be not more than 3°C apart for any of the cultures studied. Holzmuller in 1909 (Knaysi, 1948) concluded that for any given strain, spore formation has the same optimum as that for growth and germination. The latter worker reported that germination takes place within a narrower interval than growth, and spore formation in a narrower interval than germination.

Of interest in this study was pH of the variant cultures at the end of sporulation. The average pH reached after 48 hr of incubation at 55°C on fortified NA was 6.0–6.2 for the S variant and 7.2 for the R variant. Knaysi (1948) concluded that the optimal pH for spore formation lies between 6.9 and 7.4 for many sporeformers; however, Fabian and Bryan (1933) found that sporulation in four species of Bacillus was not materially affected within a favorable growing range of pH 5.0 to 7.5. The range of optimal pH for sporulation is the same as that for growth and for germination of the spores (Knaysi, 1948).

The effect of aeration or molecular oxygen on sporulation was determined. The S variant did not respond to aeration; instead, aeration decreased spore formation by one-half the level produced in closed flasks. On the other hand, the R variant sporulated better when aeration was provided via
ventilated plugs in sporulation flasks. Fields (1966) attributed increased sporulation in the R variant of NCA 1518 to higher oxygen levels when sporulation on pea agar was compared with sporulation in pea extract (broth). He observed that 1000 times more spores were produced on the agar than in the broth. Knaysi (1945) stated that the absolute necessity of molecular oxygen for sporulation of Bacillus species has not yet been conclusively proved, although its necessity for the quick and efficient formation of large numbers of spores has been well established in aerobic species.

Growth media played a significant role in the formation of spores of B. stearothermophilus. Virtually no spores were formed on TSA; however, TSA supported excellent vegetative cell growth. Fortified NA served as an excellent sporulation medium, but exhibited lower cell densities than TSA. Spore formation has been shown to take place sooner in a medium of lower nutrient value than a richer medium (Henrici, 1928; Knaysi, 1948). Knaysi (1945) concluded that the formation of spores occurs in cultures of healthy cells facing starvation. Holzmuller (Knaysi, 1948) has contended that depletion of nutrients induces spore formation; however, sufficient experimental evidence to support this has not yet been presented. Knaysi (1945) emphasized that the formation of spores cannot be related to one independent variable, because the factors are so interrelated that proper evaluation of any one factor is not possible unless
its relation to other factors is explained. According to Knaysi (1948) the following factors are all interrelated; temperature, the concentration of oxygen, the state and density of the population, the utilization of nutrients, the accumulation of by-products of metabolism, and the simultaneously developed pH.

**Autolysis** The effect of aeration on vegetative growth was significant in R cultures. R cultures grown in tightly closed sporulation bottles showed a large degree of autolysis at the end of 48 hr of incubation. In cultures where aeration was provided via cotton-plugged stoppers the degree of lysis was greatly reduced; however, the amount of autolysis was proportional to the temperature of growth. Both variant types showed higher levels of autolysis at 65 C than at lower temperatures. Degree of lysis after 48 hr of incubation was independent of the level of nutrition in the growth medium as judged from growth on fortified NA and TSA.

**Antigenic characteristics**

Four antisera from cells and spores of both R and S variants were obtained with titers ranging from 10 to 20. Low titers proved beneficial to the experimental approach since the Microtiter system only worked with a serum of a low titer or with a diluted serum. Since titers were low, adsorption was eliminated.
The agglutination reactions are shown in Table 7.

Table 7. Agglutination reaction between R and S variant cell and spore antisera and the antigens of both intact and disrupted cells and spores of *B. stearothermophilus*

<table>
<thead>
<tr>
<th>Antiserum prepared against</th>
<th>Antigen</th>
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<tbody>
<tr>
<td></td>
<td>R cell</td>
<td>R spore</td>
<td>S cell</td>
<td>S spore</td>
</tr>
<tr>
<td>R cell</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>R spore</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>S cell</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>S spore</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*+ or - indicates positive or negative reaction.*

Cross agglutination reactions occurred from which the following conclusions were made: 1) R and S cells are antigenically different; 2) R and S spores share common antigens with their parent vegetative cells; 3) R cells and S cells possess antigens in common with the opposite spore type; 4) R and S spores are antigenically similar.

R and S cells of other species are often characterized by having different antigenic constituents (Knaysi, 1951; 1956). The antigenic differences between R and S types are associated with the slime or capsular material (Burrows, 1963). The layer
is usually associated with the smooth form, but in our study the material was believed to be a distinguishing characteristic of the R variant especially when grown in broth. The S variant possibly produced the substance and excreted it into the medium. If this were the case, capsular material would have been lost in the preparation of the antigen.

The R cell antiserum reacted with the R spore antigen; however, this did not occur with the S variant. The negative agglutination response was attributed to the low titer in the antiserum against the S cell. The S cell antiserum did, nevertheless, give the expected positive agglutination response with the S cells. The reaction of S cell antiserum and R cell antiserum with spores of the opposite type suggested that the vegetative antigens indigenous to spore preparations were in common with both variant cells. R and S cells apparently share certain somatic antigens.

Cross agglutination reactions between R and S spores were explained on the basis of strain similarity. From 39 strains of *B. polymyxa*, Davies (1951) found spore antigens common to all strains. Doak and Lamanna (1948) reported similar spore antigencity between closely related organisms of different species.

The location of the spore antigens held in common with vegetative cells is inside the spore (Doak and Lamanna, 1948; Walker, et al., 1967). These internal spore antigens are
revealed if prolonged immunization is carried out or if specially treated spores are used as the antigen (Doak and Lamanna, 1948). Alkali treatment of spores is shown to expose the parent vegetative cell antigen (Doak and Lamanna, 1948). In this study sonic vibration was effective in disrupting spores for the purpose stated above.

**Proposal for strain and variant designation**

The literature before 1963 on *B. stearothermophilus* NCA 1518 does not take into consideration the presence of R and S variants in the strain. These variants differ in heat resistance, environmental and nutritional requirements for growth, sporulation requirements, morphology, and in their biochemical nature (Michener, 1953; Fields, 1963; Fields, 1966; Rotman and Fields, 1966; Hill and Fields, 1967a,b; Hill et al., 1967). Our study provides information supporting much of the work by Fields and his group, and we have obtained additional information helping to further characterize the variants in terms of their growth and cultural characteristics, morphologies and antigenic characteristics. Since these differences between R and S variants are considerable, the recommendation is made that strain designations be used to include the type of variant (e.g. 1518S or 1518R). This same recommendation applies to studies where other strains of bacilli containing stable variants are used. With complete strain and variant designations more accurate comparisons can be made on results from
studies involving heat resistance, growth and sporulation. This practice would help to prevent, to a degree, discrepancies which are so common in the literature, particularly in heat resistance studies.
SUMMARY AND CONCLUSIONS

Increasing Thermal Resistance by Selection

Spore suspensions of the R variant of *B. stearothermophilus* NCA 1518 were heat treated in such a manner to permit the selection of the most resistance spores. Survivors were allowed to produce the next generation. Increased heat resistance was achieved through four series of treatments as evidenced by thermal death time determinations.

Effect of Sporulation Temperature on Thermal Resistance

In a study of the influence of sporulation temperature on heat resistance of R spores, a direct correlation was established. Heat resistance was increased in the R variant as sporulation temperature was raised from 55 to 65 C.

Density Separation and Thermal Resistance

Spores of the S variant of *B. stearothermophilus* NCA 1518 were separated on a gradient and tested for heat resistance. Those spores which were pelleted during centrifugation showed greater heat resistance than those spores left suspended in the gradient.

Density gradient separation in a suitable suspending material proved to be a valuable cleaning technique in the
preparation of spores by allowing separation of a cellular debris from spores in the suspension.

Characterization of Rough and Smooth Variants

Several morphological and physiological characteristics of the R and S variants of B. stea rothermophilus were studied. Numerous differences were observed between the variants. Most of the differences were within the species description pattern of B. stea rothermophilus; however, the R variant gave a negative reaction on starch which did not fit the descriptions given by Smith et al. (1952) or Wolf and Barker (1968). Morphological and growth characteristics of the R and S were strikingly different. Differences exist in the following areas: cell and colonial morphology, growth in broth, response to oxygen level, and capsular production.

Growth Studies

Growth studies on the R and S variants on NB and TSB provided information on the requirements and responses to various temperatures. The R variant exhibited a tendency to outgrow the S variant on a less nutritious medium (NB); however, on TSB, a rather rich medium, the S variant grew at a faster rate than the R variant. Growth on NB gave a 5°C lower optimal growth temperature than that obtained on TSB. On both media, the S variant had a lower optimal temperature for growth than
the R variant. In the S variant growth at 70 C was dependent on the high nutrition offered by TSB, while the R variant was not as demanding in growth requirements as the S form. Growth of the R variant on TSB presented a "diauxie effect". The phenomenon was only shown by the R variant on TSB. The effect in the S variant was insignificant.

A general conclusion is presented from the results of growth on two media and their relationship to growth temperatures. Growth conditions play a major role in the establishment of one variant over the other. When growth conditions are extremely favorable the R variant offers little competition to the S form, but when the growth medium is near minimal and when the temperature is above 55-60 C the R variant can easily establish itself over the S variant.

pH Study

The pH change during the growth of R and S cultures at 55 C on TSA-A broth was monitored continuously for 120 hr or longer. Different patterns for the variants were obtained. The R variant showed only slight acidity in the early stages of growth followed by a gradual increase throughout the remainder of the growth period. The S variant produced conditions for rapid pH change, decreasing to a pH reading of 4.9 and remaining stationary throughout the incubation period.
Mixed cultures in a 3:2 ratio of $S$ to $R$ in TSA-A broth produced a pH pattern similar to that of the $S$ variant.

**Effect of Temperature and Medium on Size and Sporulation**

Photomicrographic measurements on cells and spores produced at various growth temperatures were made. At least 20 to 30 measurements were taken for each mean; the overall mean was the average of several such means. Graphic plots of the means, along with computed volumes, against temperature revealed the following relationships: 1) Spore size of $R$ and $S$ variants was related directly to growth temperature; increases in growth temperature resulted in larger spores; 2) $R$ cells increased in length and width (volume) as growth temperature was raised; 3) $S$ cell length was not influenced while width was decreased slightly as temperature of growth was increased.

A comparison of relative size discloses a number of relationships. Firstly, spores of the $R$ variant are larger than those of the $S$ variant. Secondly, $R$ cells are longer than $S$ cells, while the $S$ cells are slightly wider at all growth temperatures. The volume of the $S$ cells compared to that of the $R$ cells is greater at 55°C and below, while it is less than that of the $R$ cell at temperatures above 55. Temperature of growth greatly influences the size of variant cells and determines which variant will have the larger cell volume.

Nonseptate filament formation was characteristic of $R$ growth on TSA. The $S$ variant seldom produced chains or
filaments; however, growth on TSA exhibited slight septate filamentation of 2 or 3 cells. Filament production was minimal on fortified NA, and was dependent upon elevated growth temperatures.

Sporulation was influenced by temperature, especially with the S variant where good yields were observed only at 55 C. On the other hand, the R variant formed spores at all temperatures from 45 to 65 C; however, the best yields were obtained at 55 C.

pH of the variant cultures was determined after growth for 48 hrs at 55 C. Cultures which had sporulated at normal levels (50-70% of the population) gave pH readings consistent with the type of variant present. The characteristic pH was 7.2 for the R variant and 6.0-6.2 for the S variant.

The effect of aeration on sporulation was studied with the aid of phase-contrast microscopy. The S variant did not respond to aeration, in fact, this variant formed more spores in closed containers than in aerated containers.

Two media, NA fortified with 30 ppm Mn++ and TSA, were compared as sporulation media. Fortified NA supported good sporulation from moderate vegetative cell growth. TSA supported luxuriant vegetative cell growth without spore formulation.

The degree of autolysis was influenced by the level of aeration in the R culture. Low levels of aeration enhanced
the autolytic cycle. Higher temperatures of growth also enhanced the lysis. The level of nutrition did not affect the degree of autolysis.

Antigenic Characteristics

The R and S cells of NCA 1518 did not show cross-agglutination reactions with heterologous antiserum which was in keeping with the accepted notion that R and S are different antigenically. The R and S spores cross reacted as well with heterologous antiserum as with homologous antiserum. Anti-R and anti-S spore sera reacted with their parent cell antigens. Cross reaction occurred between anti-R and -S cell types with the heterologous spore types. From these results the following conclusions are made: a) R and S cells are antigenically different; b) R and S spores share common antigens with their parent cells; c) R and S spores are antigenically similar; d) R and S cells possess antigens in common with the opposite spore type.

Variant Type in Nomenclature

A proposal is presented which recommends that bacterial strain designations include variant types. This proposal is of utmost importance to research in bacteriology where discrepancies and inconsistencies can develop from improper or insufficient bacterial nomenclature. Studies with the R and
S variants of strain NCA 1518 have shown wide morphological and physiological differences which offer strong support to the establishment of a combined strain and variant designation.

From the investigations on variants of *B. stearothermophilus* NCA 1518 the following conclusions are presented:

1. Heat resistance of R variant can be increased by selection of survivors from heat treatments.
2. Heat resistance of the R variant can be enhanced by raising the sporulation temperature.
3. Heavier spores of the S variant separated on a density gradient are more resistant than the lighter spores.
4. When growth conditions are favorable the S variant out-grows the R variant, but when the growth medium is minimal and when the temperature is above 55 C the R variant surpasses the S variant in growth.
5. The vegetative cells and spores of the R variant are larger than those of the S variant.
6. The R variant produces spores at temperatures from 45 to 65 C, optimum is at 55 C.
7. The S variant sporulates well only at 55 C.
8. The R and S spores share common antigens with their parent cells.
9. The R and S cells are antigenically different.
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