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A Study of an Unusually Heat Resistant Variant of Bacilius Circulans

Roberta D. Demaret

DEMARET — HEAT RESISTANT VARIANT OF BACILLUS CIRCULANS

A STUDY OF AN UNUSUALLY HEAT RESISTANT VARIANT OF *BACILLUS CIRCULANS*

by

Roberta D. Demaret

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E. H. Castetter

DEAN

6/2/1954

DATE

Thesis committee

R. B. Johnson

CHAIRMAN

H. D. Tittmer

C. Clayton Hoff

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CHAPTER I. INTRODUCTION

The problem herein described was initiated while following the growth rate of a culture over a ten-hour period. A flask of polypeptone agar had been held in a 47° C. water bath to maintain it melted so that plates could be poured periodically. The plates poured during the last two to three hours were found to be heavily contaminated with a Gram-positive, aerobic, sporeforming bacillus. Aseptic technique had been used in pouring the plates; however, external contamination was possible over the long pouring interval, so the test was repeated incubating a flask of the sterilized medium in the water bath for seven to eight hours before pouring plates. Again growth was demonstrated, so the source of the microorganism could only have been one of the ingredients used to make the medium. The constituents of the medium were beef extract, polypeptone, and agar. A stained smear of the beef extract revealed many spores indicating this as a possible source of the microorganism. An unusual degree of heat resistance in these spores was evident because the medium had been autoclaved at 121° C. for 15 minutes.

The significance of a microorganism with spores resistant to ordinary sterilizing procedures is one of practical and theoretical interest. Standard methods are followed in preparation of media to insure sterility, and the existence of a microorganism capable of resisting those procedures would

indicate a change in procedure might be necessary with this microorganism. Theoretically, the degree of heat resistivity of the spores and the identification of the microorganism were important determinations as the possibility existed of finding here a new type of bacterium.

History.--Investigations of thermophilic bacteria have dealt with nearly every phase of their development, physiology, taxonomy, and practical importance. Only the most pertinent work will be considered here.

Sources of cultures.-- A thorough survey of the literature on thermophilic, aerobic sporeforming bacteria prior to 1920 was accomplished by Morrison and Tanner (1920, table pp. 346-353), who indicate that a large part of the literature deals with documentation of the occurrence of thermophils in various localities. The following paragraph is a summation of their report.

Miquel in 1879 was the first to isolate a bacterium of this type and to employ the term thermophil. Following this original isolation, a great deal of interest was aroused and numerous reports followed of similar bacteria isolated from varied sources. The bacteria were found in water, soil, milk, hot springs, masses of decaying plant materials, sewage, sea water, and environments not expected to support or contain thermophils. Of special interest was the report by Flugge who, as early as 1894, isolated a bacterium with spores which were resistant to hot water or steam for two hours. The

rapid accumulation of reports on thermophilic bacteria resulted in a degree of confusion existing between the terms thermophil and thermotolerant. In 1900, Sames suggested that a distinction between the two terms was necessary. He proposed restricting the term thermophil to microbes possessing a minimum growth temperature of 45°-50° C. and to designate those with a minimum growth temperature lower than 45° C. and a maximum growth temperature of about 50° C. as thermotolerant. About this time a theory as to the origin of these unusual life-forms was proposed. In 1903, Tsiklinsky stated that there was a probable relationship of thermophils to mesophils, the thermophils being merely variations of the common non-thermophilic organisms. In 1906, Blau reported that he obtained from soil four species which had extremely heat resistant spores. The thermal death time of the four species at 100° C. ranged from seven and one-half hours to 20 hours. This was the second report of unusually heat resistant spores.

Prickett and Breed (1928, p. 247) and Muller (as cited by Allen, 1953, p. 127) found thermophilic sporeforming bacilli in culture media, especially as a result of improper preparation of media during the first world war. The first two investigators treated their media in accordance with the procedure outlined in The Standard Methods of Water Analysis (1946, p. 185), incubating the plates at 56° C. and 65° C.

They found contaminants occurring in agar and beef extract; however, the spores were killed when the medium was autoclaved for 30 minutes (Prickett and Breed, 1928, p. 248).

In recent years the variety of sources of thermophilic bacteria has been extended even more. Egorova (1938, pp. 649-650) isolated them from soil at stations near the arctic Kara Sea and from soil of the islands in this sea. The bacteria were definitely classified as thermophils, their optimum development occurring at temperatures of 60° to 80° C., with the minimum growth temperatures of 36° to 40° C. All cultures fell into two groups, not named, containing only organisms bearing a terminal spore.

Definition of terms and theories of origin of these microorganisms.--As the literature on thermophilic bacteria accumulated, the definitions of the term thermophil varied. Bergey (1919, p. 301) began a study of the bacteria as a group. His definition of a true thermophil is a bacterium that will grow at temperatures above the maximum temperature pertaining to the great majority of bacteria, especially pathogenic forms. The minimum temperature of these bacteria is 40° to 45° C.; optimum, 60° to 70° C.; and maximum, 70° to 80° C. Facultative thermophils, on the other hand, develop at room temperature, display an optimum temperature of about 50° C., and a maximum temperature of about 60° C. The true thermophils of Bergey's study varied too much biologically

and morphologically for classification. The facultative thermophils were determined to be more closely related, possibly a single species.

The last two definitions to be proposed for thermophils are in close agreement. Cameron and Esty (as abstracted in Biol. Abs. 1926-1927, vol. 1, p. 326) designated two groups: the obligate thermophilic group growing at 55° C. but not at 37° C., and the facultative thermophilic group growing at both 55° C. and 37° C. The definition of Imšenecki and Solnzeva (1945, p. 540) is that stenothermal thermophils will not develop at the relatively low temperatures of 28° to 30° C. while eurithermal thermophils will grow at the latter temperatures.

The newer knowledge of bacteria which has developed in the past two decades would allow for the variability of bacterial species by using a species pattern for classification. Retaining the temperature range definitions, using either one of the last two terms described, a distinction can be made between microorganisms capable of growth at both high and low temperatures and at elevated temperatures only. The terminology and distinction used most commonly today is based on the work of Smith, Gordon, and Clark (1946, pp. 2-5) who accepted the definition of Cameron and Esty given above.

The majority of investigators dealing with thermophils have at least implied in their work the assumption

that the thermophilic organisms bear a more or less close relationship to those microbes which grow at ordinary temperatures. This view is expressed by Gaughran (1947, p. 218) and Gordon and Smith (1949, p. 338) as being generally accepted. Two other ideas have been proposed as to the origin of thermophilic microbes, but they have been considered only for general discussions. The proposition advanced by Weed and by Ambroz (as cited by Allen, 1953, p. 137) is that thermophilic microorganisms are survivors of a past geological age. Arrhenius (1927) argued for extra-terrestial origin of thermophilic microorganisms from the planet Venus with the sun's rays acting as a means of transportation.

Industrial and Public Health Aspects.--The majority of isolations of thermophilic bacteria since 1920 have come about as a result of their industrial importance as the causative agents of spoilage in canned foods or as a public health problem associated with the standard plate count for dairy products.

Donk (1920, p. 373) isolated a new species of thermophilic aerobic sporeforming bacteria from spoiled samples of canned corn and string beans. He proposed the name, Bacillus stereothermophilus. Later work by Smith, Gordon, and Clark (1952, p. 2) showed that a close relationship existed between this organism and Bacillus circulans. Cameron, Williams, and Thompson (1927, p. 64) determined that the sugar used in the canning process was the only

external source of contamination with this thermophilic microorganism. Their work was confirmed by Cameron and Williams (1928, p. 31) in a later investigation.

Bacillus thermoacidurans is the name given by Berry (1933, p. 72) to an aerobic thermophilic sporeforming rod which he isolated from spoiled canned tomato juice. This microorganism was shown to be a facultative anaerobic thermophil which characteristically produced spores resistant to heat in the pasteurization process. Stern, Hegarty, and Williams (1942, p. 187) reported the first successful artificial cultivation of this microorganism. Attenuation or loss of ability to grow in tomato juice and to cause spoilage was noted as a characteristic of Bacillus thermoacidurans. Later Becker and Pederson (1950, p. 717) reported that Berry's sporeforming organism was not obligately thermophilic and that according to Smith, Gordon, and Clark (1952, p. 102) was identical with Bacillus dextrolacticus and Bacillus coagulans. Various workers agreed, as Berry reported (1933, p. 73) that Bacillus thermoacidurans originated in the soil and had no pathogenic potentialities.

The public health aspect of thermophilic microorganisms was evident in relation to milk counts as early as 1927 (Fay, p. 347). A disturbing factor in milk plate counts was the abundance of pin-point colonies. However, while thermophilic microorganisms were found in pasteurized milk, the

major cause of pin-point colonies was determined to be thermotolerant organisms (Fay, 1927, p. 353). Hansen (1932) fed guinea pigs nine species of thermophilic sporeformers isolated from pasteurized milk and was able to demonstrate no toxic effects. These microorganisms, while not pathogenic, were, however, definitely a nuisance.

The difficulty encountered by dairy workers with thermophilic microorganisms involved the coagulation of the milk in wooden holding tanks where the temperature of the milk was 71° to 77° C. Hussong and Hammer (1928, pp. 185-186) isolated from samples of coagulated milk an obligate thermophilic sporebearing rod which would produce the same type of change in litmus milk that true lactic acid microorganisms produce. This included coagulation due to the development of lactic acid and reduction of the litmus with comparatively little change in the protein. The name proposed for this organism was Bacillus calidolactis (Hussong and Hammer, 1928, p. 184).

Basis of study of microorganisms.--Prior to 1933, literature referring to the thermophilic bacteria as a group dealt primarily with four questions (Casman and Rettger, 1933, p. 78). These questions concerned: 1) classification and taxonomy; 2) demonstration of growth at a temperature higher than that for known organisms; 3) acclimatization of non-thermophils to higher growth temperatures, and vice

versa; and 4) environmental and other conditions which would favor resistance to heat. Casman and Rettger (1933, pp. 79-81) attempted acclimatization of Bacillus subtilis strains to high temperatures since previous reports indicated such a phenomenon was possible. They found, however, that the strains at their disposal had a fixed maximum temperature which could not be altered either by a gradual rise in temperature using serial transfers or by desiccation of both media and cells. They concluded (Casman and Rettger, 1933, p. 122) that the limitation of bacterial growth by temperatures higher than maximum was probably due to inhibition of activities of certain catalytic oxidation-reduction mechanisms involved in cellular respiration. Lamanna (1940, p. 596) determined that cultures having lower minimum and maximum growth temperatures were, as a rule, larger in cell size than those having higher minimum and maximum growth temperatures.

Gaughran (1947, p. 198) reported that the explanation of the ability of thermophilic microorganisms to grow at temperatures high enough to coagulate many proteins is a matter of speculation. Early theories regarded the protoplasm of the microorganisms as being of a peculiar type and later work indicated that dissolved gases and other constituents of the medium have an effect in promoting life at these high temperatures. The last proposition suggested that the microorganisms had a low grade of protoplasmic organization.

As indicated earlier in this paper, the relationship between mesophils and thermophils appears to be a very close one so that the two kinds of microorganisms actually fall into one genus. This has been adopted in classification systems used today.

CHAPTER II. METHODS AND MATERIALS

Pure culture.--It was necessary to obtain a pure culture of the microorganism from the medium in order to determine its identity and properties. Because the spores of the bacillus are resistant to autoclaving at 121° C. for 15 minutes, it was postulated that a pure culture might be obtained merely by autoclaving the nutrient medium and subsequent plating. The medium consisted of the contaminated beef extract 0.3%, polypeptone 0.5%, and agar 2%. The pH of this and all other media employed in this study was adjusted to 7.5 using bromthymol blue as the indicator. The medium was subsequently autoclaved at 121° C. for 15 minutes. In order to promote germination of the spores, the flask of medium was held in a 47° C. water bath, rather than pouring plates immediately. Three plates of 20 ml. each were poured from the flask of medium at thirty-minute intervals for a period of two hours. After the medium in the plates was allowed to solidify at room temperature, the plates were incubated at a temperature of 53° C. To retard drying of the medium at such high incubation temperatures, the plates were placed in a large screw-cap jar along with a small beaker of cotton saturated with water. All media incubated at this temperature were so treated. Growth was slow, the first visible colonies appearing after four days, and so incubation was continued for seven days to allow for sufficient development of the

colonies. Gram stained smears were made of the colonies and transfers were made by streaking on polypeptone agar plates. After 48 hours of incubation, isolated colonies from these plates were transferred to polypeptone agar slants. Good growth occurred on the slants in 48 hours and these were stored in the refrigerator as stock cultures.

Preparation of spore suspensions and determination of spore count.--Spore suspensions were prepared from the stock culture by making subcultures on polypeptone agar slants containing 0.3% non-contaminated beef extract, 0.5% polypeptone, and 2% agar. Maximum sporulation was achieved by incubating these slants at 53° C. for seven days. The growth on the slant was aseptically washed into a tube containing five ml. of polypeptone broth composed of 0.5% polypeptone and 0.3% non-contaminated beef extract. The tube was shaken for two minutes to disperse clumps and to distribute the microorganisms uniformly throughout the fluid.

The number of spores in the suspension was determined by making a direct microscopical count using the Petroff-Hausser bacterial counting chamber. One ml. of the suspension was mixed with one ml. of 2% formaldehyde lightly colored with crystal violet, yielding a one to two dilution of the suspension. The number of spores contained in 20 squares of the counting chamber was enumerated under oil immersion and the average number of spores per square

determined. The volume of one square is 1/20 mm. X 1/20 mm. X 1/50 mm. deep = 1/20,000 cu. mm. This volume multiplied by 1/1000, converts the latter to cu. ml., and multiplied by the dilution, ½, yields 1/40,000,000, the volume of spore suspension in 1 square in cu. ml. The number of spores per ml. of suspension was thus determined by multiplying the average number of spores per square by 40,000,000.

CHAPTER III. EXPERIMENTAL RESULTS

Determination of heat resistivity of the spores.-- According to Bigelow and Esty (as cited by Williams, 1929, p. 441), determination of a thermal death time of thermophilic organisms is more accurate than a thermal death point. Accordingly, determination of the thermal death time was accomplished. The polypeptone medium was distributed in 500-ml. Erlenmeyer flasks in 100-ml. amounts, sterilized, cooled to 50-55° C. and inoculated respectively with 0.2 ml. of the spore suspension. The inoculated flasks were then autoclaved at 121° C. for varying lengths of time, immediately cooled to a temperature of 50-55° C., and incubated in the melted state at a temperature of 53° C. The accuracy of the thermometer on the autoclave was checked with Diack Control vials. These are so prepared that a color change to red occurs if the sterilizing temperature reaches 121° C. These vials confirmed the reading of the thermometer on the autoclave and precluded any possibility of error because of low autoclave temperature. When growth occurred, pronounced turbidity of the medium was noticeable usually within 48 to 72 hours incubation. Smears were made, Gram stained, and checked for the presence of the typical Gram-positive sporeforming rods. The flasks were incubated for a period of two weeks before being declared negative in those instances where no growth occurred. The results of this determination are outlined in Table I. The

Table I. Limit of Growth with Increasing Time Periods in Autoclaving

Number of spores inoculated	Time (min.) autoclaved	Result
100,800,000	20	growth
100,800,000	25	growth
100,800,000	30	growth
158,800,000	40	growth
158,800,000	50	growth
158,800,000	60	growth
98,800,000	75	no growth
158,800,000	90	no growth

thermal death time for 100,000,000-150,000,000 spores fell between 60 and 75 minutes at a temperature of 121° C.

Identification of the Microorganism.--The following gives the characteristics of taxonomic importance used in determining the species' identification:

Vegetative rods: 0.4 μ to 0.7 μ by 2.4 μ to 6.4 μ, averaging 0.5 μ by 4.2 μ, based on measurement of 60 cells; some slightly bent; ends rounded or pointed; usually not in chains but a few filaments found; actively motile; Gram variable.

Spores: 0.8 μ to 1.6 μ by 1.4 μ to 2.4 μ, averaging 1.2 μ by 1.7 μ, based on measurement of 60 spores; oval; subterminal to terminal; spore wall thick and stainable.

Sporangium: swollen; clavate.

Colonies: flat; thin; transparent; undulate; tendency to spread.

Polypeptone slants: Growth spreading and indistinct.

Acetylmethylcarbinol production: negative.

Indole production: negative.

Hydrolysis of starch: positive; wide zone of hydrolysis.

Nitrites from nitrates: positive.

Utilization of citrates: negative.

Reduction of 0.1% methylene blue: no growth. (Usually positive, but this variation found by Smith, Gordon,

and Clark [1952, p. 83].)

Casein hydrolysis: negative.

Polypeptone broth: very poor growth, faint turbidity with some flocculent sediment.

Gelatin hydrolysis: positive.

Urease: negative.

Fermentation reactions: acid without gas in 48 hours from sorbitol, inulin, salicin, glycerol, maltose, mannitol, arabinose, sucrose, fructose, galactose; acid without gas in one week from dextrin, xylose, raffinose; acid without gas in three weeks from rhamnose and starch; no action on lactose or dulcitol.

Temperature range of growth: scant at 20° C. to 28° C.; good at 37° C.; optimum 47°-55° C.; scant at 57° C.

Oxygen requirement: aerobic to facultative.

Morphology was determined from Gram-stained preparations prepared from slants incubated for 24 hours and for seven days. Measurement of 60 cells and 60 spores was accomplished by use of a calibrated ocular micrometer. Motility was determined by observing the organisms under high dry power in a hanging drop preparation of a broth culture incubated for four hours at 53° C. Biochemical activity was determined utilizing media prepared in accordance with the Manual of Methods for Pure Culture Study of Bacteria (1953, Leaflet II, pp. 2-24). According to Smith, Gordon, and Clark (1946

p. 42), a superior base medium for carbohydrate fermentation tests was a modification by Ayers, Rupp, and Johnson of an ammoniacal nitrogen medium consisting of:

carbohydrate	5.0 gm.
$(NH_4)_2HPO_4$	1.0 gm.
KCl	0.2 gm.
$MgSO_4$	0.2 gm.
Agar	15.0 gm.
Distilled water	1000 ml.

The pH was adjusted to 7.0 and 20 ml. of a 0.4% solution of bromcresol purple added as indicator. The results of fermentation tests recorded above were based on the use of this medium, incubated at 53° C. for a total period of three weeks. The temperature range of growth was determined by making subcultures from the stock cultures to polypeptone agar slants and incubating at 20° C., 28° C., 35° C., 37° C., 53° C., and 57° C. for seven days. Oxygen requirement was determined by pouring deep tubes of a polypeptone agar medium containing the contaminated beef extract and observing colony formation at different levels in the agar.

Studies on the number of surviving spores.-- It was reported by several investigators (Magoon, 1926, p. 255; Morrison and Rettger, 1930, p. 338; Williams, 1936, p. 596; Davis and Williams, 1948, p. 558) that individual spores of a particular

species exhibit considerable variation in heat resistance. Consequently, an attempt was made to determine the actual number of spores of this microorganism which survived autoclaving. The same procedure as for the determination of the thermal death time was employed using variations in medium constituents, in the time of pouring plates and in incubation temperature and conditions.

The first variation in procedure involved inoculation of 80 million to 396 million spores into 100 ml. of media of the following composition: 1) polypeptone agar, 2) polypeptone agar plus 1% starch, 3) polypeptone agar plus 0.5% sterile glucose solution added aseptically after the final autoclaving, 4) polypeptone agar plus 1% starch and 0.5% glucose, 5) ammoniacal nitrogen base medium with 0.5% glucose using glass distilled water as solvent, and 6) ammoniacal nitrogen base medium with 1% starch and 0.5% glucose using glass distilled water as solvent. After the final autoclaving and cooling of the flasks, plates were poured immediately retaining a small volume of melted agar in the flasks for incubation with the plates at 53° C. One set of polypeptone agar plates was incubated in the presence of 10% carbon dioxide. This atmosphere was produced by adding 10 ml. of 20% sulfuric acid to 2 g. of sodium carbonate contained in a small beaker set in a large screw-top jar with the plates.

The second variation of proceedure involved inoculation of 99 million to 532 million spores into 250 ml. of media of the following composition: 1) polypeptone agar; 2) polypeptone agar, using in place of the beef extract a beef infusion prepared by placing 500 g. of lean ground beef in one l. of distilled water, extracting in the refrigerator overnight, filtering through cheesecloth, boiling for ten minutes, cooling, and filtering through filter paper; 3) polypeptone agar plus 0.1% yeast extract; 4) tryptone glucose beef extract agar plus 0.1% yeast extract; and 5) polypeptone agar using the contaminated beef extract. After the final autoclaving and cooling of the flasks, two plates of 10 ml. each were aseptically pipetted from each flask, and the flasks were held in a 47° C. water bath to maintain the melted state. At intervals of 30, 60, and 90 minutes, and two, three, four, five, six, seven, and eight hours, two plates of 10 ml. each were aseptically pipetted from each flask. One set of plates from each flask was incubated at 37° C. and the other set of plates was incubated at 53° C., along with the small volume of fluid remaining in each flask.

At the end of 72 hours incubation, all trials except the last three resulted in only microscopic colony formation in the plates, observable by using a binocular dissecting microscope, and pronounced turbidity developed in the melted medium. Gram stained smears of the agar in

the plates and of the fluid medium revealed typical Gram-positive sporeforming rods. At the end of six weeks incubation of some of the plates, they were steamed in the autoclave only long enough to melt the agar, and the fluid medium was poured, as aseptically as possible into a sterile flask for incubation in this melted state at 53° C. In 72 hours, pronounced turbidity of this remelted medium resulted and smears revealed typical Gram-positive sporeforming rods.

In the last three trials, plate counts were made. The amount of suspension added to each flask inoculated contained 159 million spores. The first plates poured from the flasks immediately after the final autoclaving and cooling had the following counts: the polypeptone medium containing the contaminated beef extract had ten colonies on each plate; the two inoculated media containing 0.1% yeast extract had counts of 42 and 39 colonies per plate incubated at 53° C. Generally, the results of the plate counts showed more colonies developed in plates incubated at 37° C. than in plates incubated at 53° C. The number of colonies in the plates made from the polypeptone medium containing the contaminated beef extract was exceeded three or four times by the number of colonies in the plates made from the two inoculated media.

An attempt was made to determine the dilution of spores which would not contain any of the unusually heat

resistant members. A 1:10,000 dilution of a suspension containing 267 million spores per ml. was made by pipetting one ml. of the suspension into 99 ml. of sterile distilled water, shaking the dilution bottle, and mixing one ml. of this 1:100 dilution with another 99 ml. of sterile distilled water. After shaking the second dilution bottle containing the 1:10,000 dilution of the suspension, one ml. was inoculated into each of two flasks containing 50 ml. each of sterile polypeptone agar and the same proceedure as for the thermal death time determination was followed. Both flasks showed pronounced turbidity within seven days and smears demonstrated the typical Gram-positive sporeforming organism.

CHAPTER IV. DISCUSSION

Experimental evidence was reported by Laubach, Rice, and Ford (1916, p. 519) as well as by Smith, Gordon, and Clark (1946, pp. 2-5; 1952, pp. 2 and 106) that dissociants were easily obtained in the cultures of Bacillus circulans tested. Such a variety of reactions were demonstrated that the members of the species were considered a complex composed of aggregates of cultures that resembled in some respects certain other closely related species, particularly Bacillus macerans on one hand and Bacillus stereothermophilus on the other hand. Among the thermophils examined by Gordon and Smith (1949, p. 338), two distinct groups were determined resembling Bacillus stereothermophilus and Bacillus coagulans. Relating the experimental results to these reports, and basing this decision on the use of a species pattern to determine identification, this culture is a facultative thermophilic variant of Bacillus circulans with unusually heat resistant spores.

It was apparent from the thermal death time determination that some spores of this culture of microorganisms had an unusually high degree of heat resistance. From experimental results of the plate counts, the survival number was determined to be a small fraction of the inoculum. However, the ability of even a few selective spores of this microorganism to survive 60 minutes autoclaving at a

temperature of 121° C. is remarkable. Williams and Zimmerman (1951, p. 65) showed that the heat resistance of vegatative cells was neither reflected nor related in the resistance of the spores. Referring to this culture, agreement with this statement lies in the extreme heat resistance of a small number of these spores as compared to the complete destruction of vegatative cells by ordinary sterilizing proceedure. Considering the fact that the inoculum used in the determination was large, this may have increased the length of time necessary to effect complete sterility. According to Williams (1929, p. 440) the greater the number of cells per unit volume exposed to heat, the longer will be the period of time required for sterilization. The heat resistance of these spores, however, is probably a characteristic of this strain of microorganism, as a previous attempt to increase heat resistance of spores to this degree had met with little success (Williams, 1936, p. 597). More work using variations in spore inoculum numbers would increase the accuracy of the results.

The attempts to determine the survival number of spores were hampered by factors preventing germination of the spores in a solidified agar medium. Because growth always occurred when the same inoculated medium was left in the fluid state, or when the solidified agar was remelted, attempts were made to determine and eliminate these factors.

Morrison and Rettger (1930, p. 332) determined that germinating spores are vitally active bodies having metabolic requirements that are the same as or more exacting and specific than those of vegetative cells. To determine the requirements for germination in a solidified agar medium, several different factors were tested. The availability of nitrogen in the medium was assured in using the ammoniacal nitrogen base medium for one test, but this was not effective. The presence of carbohydrate and starch to enhance spore metabolism and inactivate possible toxic lipids in the medium did not meet the requirements. The effect of incubation in an atmosphere of 10% carbon dioxide to stimulate multiplication by increased carbon dioxide tension was tested without result. Holding the fluid agar at 47° C. in a water bath to decrease the lag period in germination of the spores was not in itself effective.

There seem to be two possible explanations of this phenomenon. One explanation is that the conditions in the beef extract containing the organism cannot be duplicated because of the many metabolic products of putrefaction which accumulate with such a large number of organisms present. Preparation of a fresh beef infusion medium does not meet these requirements. The other explanation is that the number of spores inoculated into different media is so large that colonies are unable to develop beyond the size of

microcolonies because of crowding and competition for nutrients. Incorporating 0.1% yeast extract in the polypeptone medium provides the best nutrient medium among those tested for germination of the spores. Using a much smaller inoculum in this medium would probably give a more accurate determination of the survival number of spores.

CHAPTER V. SUMMARY

An organism isolated from a jar of beef extract was determined to have spores possessing unusual heat resistant properties. A study of the microorganism yielded the following results:

1. The identification of the microorganism was determined to be a facultative thermophilic variant of Bacillus circulans.

2. The thermal death time for 100 million to 150 million spores per 100 ml. of medium at 121° C. was determined to fall between 60 and 75 minutes.

3. The survival number of spores was determined to be a small fraction of the inoculum, but these spores had an unusually high degree of resistance to heat.

4. Inability to duplicate nutritional factors in the beef extract combined with an excessive number of spores in the inoculum appear to be the reasons for lack of germination of the spores in inoculated, solidified agar plates.

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