Dipicolinic acid content and heat resistance of spores of Bacillus stearothermophilus and thermophilic bacteria from Yellowstone National Park
by James Alan Brierley

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in Bacteriology
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Abstract:
Thermophilic, aerobic sporeforming bacilli were isolated from hot springs and the Gardner River of Yellowstone National Park. These organisms were identified as Bacillus stearothermophilus, There have been conflicting reports of the correlation of spore dipicolinic acid content and heat resistance. Heat resistance of spores of Bacillus species has been reported to increase with an increase of incubation temperature. Spore crops of 3 selected strains, B. stearothermophilus ATCC 7954, and Yellowstone National Park isolates YNP 2-40 and YNP 3-6 were produced at two temperatures of incubation in order to determine if a correlation of these properties existed for the chosen strains. The spore dipicolinic acid content and heat resistance to 110 C were determined for each spore crop. B. stearothermophilus ATCC 7954 grown at 45 C and 60 C and YNP 3-6 grown at 45 C and 55 C responded to the increased sporulation temperature by producing spores with increased dipicolinic acid content, but there was no increase in heat resistance. YNP 2-40 was cultivated at 45 C and 55 C and produced spores of greater heat resistance at the higher temperature of sporulation, but there was no increase in spore dipicolinic acid content, These results do not indicate a direct relationship between spore dipicolinic acid content and heat resistance. Similar findings have been reported by other workers.

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ABSTRACT

Thermophilic, aerobic sporeforming bacilli were isolated from hot springs and the Gardner River of Yellowstone National Park. These organisms were identified as Bacillus stearothermophilus.

There have been conflicting reports of the correlation of spore dipicolinic acid content and heat resistance. Heat resistance of spores of Bacillus species has been reported to increase with an increase of incubation temperature. Spore crops of 3 selected strains, B. stearothermophilus ATCC 7954, and Yellowstone National Park isolates YNP 2-40 and YNP 3-6 were produced at two temperatures of incubation in order to determine if a correlation of these properties existed for the chosen strains. The spore dipicolinic acid content and heat resistance to 110 C were determined for each spore crop. B. stearothermophilus ATCC 7954 grown at 45 C and 60 C and YNP 3-6 grown at 45 C and 55 C responded to the increased sporulation temperature by producing spores with increased dipicolinic acid content, but there was no increase in heat resistance. YNP 2-40 was cultivated at 45 C and 55 C and produced spores of greater heat resistance at the higher temperature of sporulation, but there was no increase in spore dipicolinic acid content. These results do not indicate a direct relationship between spore dipicolinic acid content and heat resistance. Similar findings have been reported by other workers.

CHAPTER I

INTRODUCTION

The most resistant form of life known is the bacterial endospore. The nature of this resistance is unknown. Since the isolation of dipicolinic acid from the bacterial endospore by Powell (1953) many investigators have sought to determine if its presence is a factor in spore resistance.

Few reports concern the role of this material in thermophilic, aerobic sporeformers, and particularly strains from Yellowstone National Park. In this study recognized strains of Bacillus stearothermophilus were included in the taxonomic phases in order to classify the isolates. Subsequently the effect of sporulation temperature on spore dipicolinic acid content and heat resistance was determined.

CHAPTER II

REVIEW OF LITERATURE

Definition of Thermophilic

Gordon and Smith (1949) suggest that the term "thermophilic" be applied to denote organisms capable of growth at 55 C.

Two groups of thermophilic bacteria have been described by Imsenecki and Solnzeva (1945). One group, denoted as the stenothermal thermophiles, has an optimal growth range between 55 to 65 C, and does not show any growth after several days incubation at 28 to 30 C. The second group will also show optimum growth at 60 C, but growth may occur at lower temperatures such as 28 to 30 C. This group is denoted as eurithermal thermophiles.

Cameron and Esty (1926) separated organisms into the following groups. The mesophiles are those organisms which grow at 37 C but not at 55 C. The facultative thermophiles grow between 37 C and 55 C, and the obligate thermophiles will grow at 55 C but not at 37 C.

Isolation of Bacillus stearothermophilus

Donk (1920) found an organism in spoiled samples of canned corn and canned string beans which was a highly resistant sporeforming thermophile. He proposed the name Bacillus stearothermophilus, a name which has continued to the present.

Subsequently, this organism has been isolated from numerous sources. Marsh and Larsen (1953) isolated 24 cultures of aerobic, sporeforming bacteria capable of growing at 65 C from the hot springs of Yellowstone National Park. The characteristics of 21 of the strains were found to

conform quite closely to Bacillus stearothermophilus. They stated that the remaining strains appeared to represent a group intermediate between Bacillus stearothermophilus and Bacillus coagulans.

Walter and Northam (1952) isolated aerobic, sporeforming bacteria from 5 of 26 sites selected for sampling at Yellowstone National Park. Isolates were obtained only from springs where the water temperature was between 51 and 70 C and the pH between 6.5 and 7.8. They found 10 isolates from 3 springs which would grow at 55 C, thus designating them thermophilic. They tentatively identified the organisms as Bacillus stearothermophilus on the basis of limited studies.

Walter (1952) made 28 isolations of aerobic, sporeforming bacteria from hot springs near Bozeman, Montana and Yellowstone National Park. Six were identified as strains of Bacillus stearothermophilus. The others were strains of mesophiles.

Thermophilic bacteria are not limited to hot springs, spoiled canned food or an environment which would have a temperature that would be optimal for the organism. McBee and McBee (1956) found thermophilic, aerobic bacteria in 38 of 59 soil and water samples collected near Point Barrow, Alaska. In this region the soil is frozen for most of the year and seldom reaches a temperature of 10 C. McBee and Gaugler (1956) identified 15 of the aerobic, sporeforming bacilli which grew at 55 C isolated from Arctic soils and waters. Ten were identified as Bacillus stearothermophilus, which was the only species to grow at 65 C, 3 as Bacillus coagulans, and 2 as Bacillus licheniformis.

Dipicolinic Acid Content of Spores

Powell and Strange (1953) found a 30% decrease in spore dry weight during the germination process of spores of Bacillus megatherium (B. megaterium). The material lost during the germination process consisted mainly of amino acids, peptides, hexosamine attached to a nondialysable peptide, and a substance absorbing strongly in the ultraviolet, at wave lengths of 2650, 2700 and 2775 A. Powell (1953) isolated the ultraviolet absorbing compound in the form of its calcium salt and identified it as dipicolinic acid (pyridine-2,6-dicarboxylic acid). Dipicolinic acid had not previously been recognized as a constituent of living matter. It was found that calcium dipicolinate constitutes 15% of the spore dry weight of Bacillus megatherium (B. megaterium). The dipicolinic acid was not found in the vegetative cell, making it an exclusive property of the heat resistant spore.

Powell (1957) believed that the calcium dipicolinate is a constituent of resting spores and not a substance which is synthesized during germination. This conclusion was reached because the dipicolinic acid was not found only in the "germination exudate", but it was also found in extracts from disintegrated resting spores. The dipicolinic acid chelates with calcium. Thus it was suggested that the calcium dipicolinate is present in the spore in a chelated form. The dipicolinic acid may be incorporated throughout the spore protoplasm through further linkages of calcium with protein. This arrangement may contribute to the heat resistant properties of the spore.

Perry and Foster (1955) suggested a possible mechanism for the synthesis of dipicolinic acid. Vegetative cells and spores of species in the genus Bacillus contain α, ε,-diaminopimelic acid which, on removal of one amino group, cyclization and oxidation, would give rise to dipicolinic acid.

Curran, Brunstetter and Meyers (1943) found by spectrochemical analysis that bacterial spores were consistently high in calcium, and the vegetative cells relatively low in calcium. Thermophilic spores seemed to contain a higher proportion of calcium than the mesophilic spores. They also found that high concentrations of calcium were associated with enhanced heat tolerance and resistance. This information would tend to point toward the importance of the calcium dipicolinate chelate as a factor in heat resistance of the bacterial spore. Levinson, Hyatt and Moore (1961) compared certain species of Bacillus to demonstrate the role of the calcium: dipicolinate ratio to spore heat-resistance. The highest calcium:dipicolinate ratio was associated with the most resistant spore, and the lowest ratio was associated with the spores of lowest heat resistance.

Not all the dipicolinic acid is chelated with calcium in the spore. Perry and Foster (1956) found in spore extracts of Bacillus cereus var. mycoides a relatively minor ultraviolet absorbing spot on certain chromatograms. It was identified as the monoethyl ester of dipicolinic acid, and this led the authors to believe that a portion of the dipicolinic acid in the spore exists in the esterified form. No significance was attached to this discovery concerning the heat resistance of the spore.

Church and Halvorson (1959) felt that biosynthesis of dipicolinic acid is usually considered to be indicative of the formation of heat resistant

spores. Spores of Bacillus cereus var. terminalis demonstrated a dependence of heat resistance on the dipicolinic acid content of the spores. The dipicolinic acid content was lowered by reducing the level of yeast extract in the sporulation medium. When the dipicolinic acid content of the spores was reduced by 60 to 70% the spores were found to be more heat sensitive. The dipicolinic acid content was increased by the addition of vitamins, suppressed by the addition of DL- or L-phenylalanine and relatively unaffected by the presence of various other amino acids.

In one instance the heat resistance was found to vary inversely with the dipicolinic acid content of the spore. Byrne, Burton and Koch (1960) noted that spores of Clostridium roseum produced in the presence of L-alanine had a lower content of dipicolinic acid. The spores were more resistant to heat than those with a higher level of dipicolinic acid, produced with no added L-alanine in the sporulation medium. The authors suggest that the heat resistance of spores of Clostridium roseum was not correlated with the concentration of dipicolinic acid.

Finley and Fields (1962) stated that the mineral-dipicolinic acid complexes in the spore are responsible either partially or totally for the thermal resistance of the spores of Bacillus stearothermophilus. They found that the presence of the phosphate ion in combination with heat apparently disrupts the mineral-dipicolinic acid complex, and lowers the heat resistance of the spores.

Heat Resistance in Relation to Temperature of Incubation

Williams (1929) made a comparison of the heat resistance of spores of

Bacillus subtilis. He found that the higher the incubation temperature used for sporulation the more heat resistant the spores became. Spores produced at room temperature (21 to 23 C) survived for 10 minutes at 100 C. When the sporulation temperature was increased to the optimum growth temperature of the organism (37 C) the spores survived 14 minutes at 100 C. The resistance continued to increase beyond the optimum growth temperature of the organism. Spores produced at 41 C survived for 16 minutes at 100 C.

Williams and Robertson (1954) noted a correlation between resistance of spores and the optimum temperatures for growth. Thus, the spore resistance of thermophilic organisms is greater than that of the mesophilic, which in turn exceeds that of the psychrophilic type. It was found that for each strain there is an increase in spore heat resistance with increasing incubation temperature. The data show that Bacillus stearothermophilus responds to increases in temperature of incubation up to near the maximum by producing spores of increased resistance to heat.

El-Bisi and Ordal (1956) determined the effect of the sporulation temperature on the thermal resistance of spores of Bacillus coagulans var. thermoacidurans. They noted that the thermal resistance of the spores produced at 45 C required heating for 18.8 minutes at 93 C for 90% destruction. Those produced at 30 C required only 6.06 minutes heating at 93 C for 90% destruction. These findings also suggest a direct increase in thermal resistance with the increase in growth temperature.

Lechowich and Ordal (1962) studied the relationship of the cation and dipicolinic acid content of bacterial spores to their resistance to thermal destruction. The heat resistance of the spore crops of Bacillus

subtilis and Bacillus coagulans was varied by different incubation temperatures. The survivor curve of the Bacillus subtilis 45 C spore suspension was characterized by a slow rate of destruction during the first 45 minutes of heating, after which the rate of death increased. In contrast, the thermal destruction rate of the 30 C Bacillus subtilis spore suspension was essentially logarithmic. The spores produced at 45 C had both a higher dipicolinic acid and cation content than those produced at 30 C. These data suggest a relationship between an increased thermal resistance and the amount of dipicolinic acid and cations within the spore. The survivor curves obtained for the Bacillus coagulans spores also indicated that as the sporulation temperature increased, the thermal destruction rate decreased. A difference in the dipicolinic acid content of the spores of this organism compared with the heat resistance was noted. In the case of Bacillus coagulans there did not appear to be a direct relationship between an increase in the dipicolinic acid and cation content and the temperature of spore production. Although there appeared to be a slight increase in the calcium and magnesium content for the more resistant spores, the dipicolinic acid content was found to decrease with an increased heat resistance of these spore suspensions.

CHAPTER III

MATERIALS AND METHODS

Isolation of Thermophilic Bacteria

Various techniques have been used for the isolation of thermophilic aerobic sporeforming bacteria from hot springs (Marsh and Larsen, 1953, Walter and Northam, 1952), and from soil and water samples (McBee and McBee, 1956). The following technique was used in the present study for collecting water samples from thermal and sub-thermal sites at Yellowstone National Park.

Sterile tin cans held in a universal extension clamp were dipped into the water and scraped across the bottom surface to include a small quantity of sediment. This material was then transferred to sterile thermos bottles or plastic 16 ounce bottles (Nalgene). A similar technique was used with sterile 100 ml water blank dilution bottles. The top 2/3 of the bottles was covered with aluminum foil to insure sterility. The entire bottle was dipped into the test area and the sample was transported to the laboratory in this container.

At the time and site of sample collection the temperature and approximate pH were recorded. The latter was determined by the use of pH paper strips (Oxyphen Products Co., Forest Hills 75, New York).

The samples were returned to the laboratory facilities at Montana State College, Bozeman, on the same day that they were collected. The pH of each sample was again determined using the pH paper strips and comparing the results with the readings from a Beckman pH meter, Model G.

The following technique was used to isolate thermophilic aerobic

bacteria. One ml of each sample was plated (pour plate technique) on nutrient agar and nutrient agar plus 0.5% glucose. A 2.5% agar concentration was used for the isolation media. Porcelain petri dish tops were employed in an attempt to decrease accumulation of surface moisture and to prevent spreading of colonies during the incubation period. The plates were inverted and placed in two pound coffee cans with a damp paper towel in the bottom of each can. Incubation was at 55 C in a hot air incubator for 12 to 15 hours.

Representative isolated colonies were picked and streaked onto nutrient agar plates and again incubated at 55 C for 12 to 14 hours. One isolated colony was picked from each plate and streaked on nutrient agar slants which were incubated at 55 C. The slants served as stock cultures. Transfers were made from each stock slant to another nutrient agar slant, which was incubated at 65 C in a water bath. Those cultures which grew at 65 C were held for further tests.

Spore Production

In order to obtain large crops of spores for dipicolinic acid analysis and heat resistance studies, a technique similar to the one used by Perry and Foster (1956) was employed for spore production by the selected organisms. The sporulation medium of Lee and Ordal (1963) was modified and used. The medium consisted of nutrient broth, 2.5% agar, 0.05% $MnSO_4$ and 0.1% $CaCl_2$ made up with distilled water. One hundred twenty five ml of medium were poured into sterile rectangular Pyrex glass pans (10" x 6" x 1 3/4") covered with aluminum foil. Each pan with medium was incubated inverted

for at least 12 hours at respective sporulation temperatures prior to inoculation. This was done for the purpose of determining if contamination were present.

The inoculum of isolated strains from Yellowstone National Park and the American Type Culture Collection was incubated in screw cap tubes (15 x 125 mm) containing 5 ml of nutrient broth, for 20 to 24 hours at 50 C. Each plate was inoculated by spreading 1 tube of inoculum over the surface of the medium with a sterile glass rake. The plates were then incubated inverted for 48 hours at respective sporulation temperatures of 45, 55 or 60 C. Hot air ovens were employed for incubation.

Following the period of incubation the growth was examined for spores. A smear was made from each plate and stained by the Bartholomew and Mittwer "cold" method (Society of American Bacteriologists, 1957) and examined microscopically.

The growth was washed off the plates with sterile distilled water. Sterile wooden tongue depressors held with surgical forceps were used to scrape the agar surface. A total of 250 ml of water was used to wash the growth off each set of 4 plates. Eight plates were used at each temperature in order to obtain adequate quantities of spores. Each suspension was then filtered. A glass column (2 x 1/2 in) with glass wool firmly packed to a 2 inch height was used to remove pieces of medium scraped off with the growth.

A method similar to the one employed by Long and Williams (1958) was used to obtain spores free of vegetative cells and other debris. Each 250 ml suspension was centrifuged, using a Servall centrifuge (Ivan Sorvall,

Inc., Norwalk, Conn.) at 4,080 g for 30 minutes. The sediment was resuspended with 10 ml of sterile distilled water. This suspension was then sedimented at 12,000 g for 20 minutes. The spores were washed by slurrying 5 ml quantities of sterile distilled water several times over the surface of the sediment to remove vegetative debris. The sediment was then resuspended in 30 ml of sterile distilled water by shaking with a Burton Clinical Shaker (Burton Manufacturing Co., Los Angeles, California) until a homogeneous suspension was obtained. Each spore suspension was washed at least 6 times, or until microscopic examination revealed spores free of most vegetative material. The "clean" spore suspensions were stored in 1 ml of distilled water at -18 C.

Dipicolinic Acid (DPA) Determination

The method of Janssen, Lund and Anderson (1958) was used to determine the quantity of dipicolinic acid in the spore crops. The harvested spore crops were sedimented in screw cap tubes (15 x 125 mm) and the supernatant water poured off. A vacuum of 25 inches of mercury was established by a water aspirator for each tube. The vacuum was held until the spores became dry and flaked from the sides of the tube. Each spore crop was placed at 55 C for 1 hour prior to analysis.

Duplicate samples of dried spores from each crop containing between 4 to 20 mg were placed in screw cap tubes (15 x 125 mm), suspended in 5 ml of sterile distilled water and autoclaved at 18 lb/in^2 for 15 minutes. Each suspension was cooled, acidified with 0.1 ml of 1.0 N acetic acid, transferred to respective centrifuge tubes and left at room temperature for 1

hour. After the period of settling the suspension was centrifuged at 1500 g for 10 minutes. Four ml of supernatant were pipetted into a clean 15 x 125 mm test tube. A pipette with a pipette controller was used to remove the extract without disturbing the sediment. One ml of freshly prepared reagent consisting of 1% $Fe(NH_4)_2(SO_4)_2.6H_2O$ and 1% ascorbic acid in 0.5 M acetate buffer at pH 5.5 was added to the 4 ml of supernatant.

The color density was measured within 2 hours with a Klett-Summerson colorimeter (Klett Mfg. Co., New York) with a number 42 blue filter, and compared with a series of dilutions of a prepared DPA standard.

Heat Resistance Determination

The heat resistance of spores produced by certain selected organisms was determined. A "double pan" hot bath was used consisting of 2 stainless steel pans, the outer of 20 inch diameter and 6 inch depth, and the inner of 11 inch diameter and 7 inch depth. Aluminum foil was used to cover the area between the pans. Veterinary mineral oil was placed in each pan to a level of 3 1/2 inches. The oil in the inner pan was circulated by a Sargent Cone Drive stirring motor (E. H. Sargent and Co., Chicago) with a tygon propeller. The bath was heated with a Chromalox Heavy-duty electric table range (Wards National Science, Rochester, New York). The bath was heated to 110 C (Fields, 1963) and manually controlled. The temperature varied within a range of ± 1 C.

Spores were suspended in M/120 phosphate buffer pH 7 (Williams and Robertson, 1954) at a concentration of approximately 10^2 to 10^4 per ml. A series of sterile Pyrex screw cap tubes (15 x 125 mm) containing 5 ml of

the spore suspension was placed in a test tube rack in the hot oil bath. One tube for each organism tested was removed every 5 minutes over a period of 25 minutes and immediately placed in an ice bath. Serial dilutions were made of each suspension and plates poured. Plate count agar (Difco) was used and incubation was at 55 C for 24 hours. Counts were made at the end of this period.

CHAPTER IV

EXPERIMENTS AND RESULTS

Description of Thermophilic Organisms

Three trips were made to Yellowstone National Park to obtain water samples which might contain organisms capable of growth at 65 C. Forty-three different samples were collected from 16 locations. (Table I).

TABLE I

Number of samples collected from various sources in Yellowstone National Park and selected isolates demonstrating growth at 55 C and 65 C

Date Collected	Source	Number of Samples	Isolates grown at 55 C	Isolates grown at 65 C
10-26-62	Opal Spring	1	0	0
	Jupiter Spring	1	2	0
	Cavern Spring	1	1	0
	Middle Terrace	1	1	0
	Upper Terrace	1	2	0
	Cupid Spring	1	0	0
	Beaver Lake	1	0	0
	Beryl Spring	4	5	0
	Gardner River	1	3	0
	Terrace Spring	3	13	0
11-2-62	Cavern Spring	4	5	1
	Upper Terrace	2	0	0
	Terrace Spring	13	27	4
11-16-62	Cavern Spring	4	6	3
	Gardner River	3	3	3
	Hot River	2	5	3
	Totals	43	73	14

Seventy three representative colonies were selected which grew at 55 C and of these, 14 grew at 65 C.

The thermophilic bacteria, which grew at 65 C, were isolated from areas with a pH varying between 6.7 and 7.7, and with temperatures between 51 C and 65 C (Table II). Three of the thermophilic isolates were obtained from samples from the Gardner River, which had a temperature of about 2 C. However, 2 of these isolates were lost after transfer to nutrient agar slants.

TABLE II

Temperature and pH comparison of sources of thermophilic isolates from Yellowstone National Park

Date samples collected	Culture No.	Source	Temp.	pH determination		
				paper-lab	paper-lab	meter-lab
11-2-62	YNP 2-1	Terrace Spring	54	6.6	7.0	7.5
	YNP 2-3	Cavern Spring	55	6.8	6.8	6.8
	YNP 2-19	Terrace Spring	51	7.2	7.4	7.7
	YNP 2-20	Terrace Spring	54	6.8	7.0	7.5
	YNP 2-40	Terrace Spring	54	6.8	7.0	7.5
11-16-62	YNP 3-2	Hot River	52	6.6	6.6	6.7
	YNP 3-3	Hot River	53	6.6	6.8	6.8
	YNP 3-6	Gardner River	2	6.0	6.4	7.3
	YNP 3-9	Cavern Spring	63	6.6	6.8	7.3
	YNP 3-10	Cavern Spring	63	6.6	6.8	7.3
	YNP 3-13	Hot River	53	6.6	6.8	6.8
	YNP 3-14	Cavern Spring	51	7.0	7.2	7.8

The 12 isolates are aerobic, sporeforming bacilli that are capable of growing at 65 C. These strains were compared with 4 strains of Bacillus stearothermophilus, 7953, 7954, 8005, and 10149, obtained from the American Type Culture Collection. The methods recommended by Smith, Gordon and Clark (1952) were used in determining morphological, cultural and physiological characteristics. The results are shown in Table III.

The Yellowstone National Park isolates YNP 2-1, YNP 2-3, YNP 2-19, YNP 2-40, YNP 3-2, YNP 3-13, and YNP 3-14 key directly to Bacillus stearothermophilus when comparing the determined characteristics with those given in Bergey's Manual of Determinative Bacteriology (Breed, Murray and Smith, 1957). The remaining strains YNP 2-20, YNP 3-3, YNP 3-6, YNP 3-9, and YNP 3-10 lack the essential key characteristic of the ability to hydrolyze starch. However, these strains do grow at 65 C and Smith, Gordon and Clark (1952) state that B. stearothermophilus is the only aerobic sporeformer capable of growth at 65 C. Thus, it is believed that these strains are probably B. stearothermophilus that lack the ability to hydrolyze starch.