CHARACTERIZATION OF SOME THERMOPHILIC BACTERIA FROM THE HOT SPRINGS OF YELLOWSTONE NATIONAL PARK[1]

CONNELL L. MARSH AND DON H. LARSEN[2]

Departments of Chemistry and Bacteriology, University of Nebraska, Lincoln, Nebraska

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Extensive enzymatic studies have been conducted on cell-free fractions from a thermophilic bacterium designated as National Canners Association strain 2184 and tentatively identified as *Bacillus stearothermophilus* Donk (1920), (Militzer *et al.*, 1949, 1950, 1951, 1952; Georgi *et al.*, 1951; Marsh and Militzer, 1952). Preparatory to extending these investigations to other thermophilic bacteria, 24 strains of aerobic, sporeforming bacteria, which grew at 65 C or higher, were isolated from the thermal areas of Yellowstone National Park.

Gordon and Smith (1949) in their taxonomic study of thermophilic bacteria reported that strains of *B. stearothermophilus* were the only organisms in their collection capable of growth at 65 C. A preliminary attempt to characterize the Yellowstone strains, along with 6 National Canners Association strains, showed that a number of them differed significantly from the characters ascribed to *B. stearothermophilus*.

The objectives of this paper are to report on the characterization of the Yellowstone isolates and to call attention to the possibility that sufficient differences may exist among strains of thermophilic bacteria capable of growth at 65 C to warrant definite strain, if not species, designation.

MEDIA AND METHODS

The media and methods employed were essentially those described by Gordon and Smith (1949) except for a few modifications and additions which are described below.

Lysozyme sensitivity. The observations on lysozyme action were included because Militzer and co-workers (1949) employed this method of obtaining the cell granule preparations used in their enzymatic studies. National Canners Association strain 2184 was used as a standard of comparison in determining whether the other strains were sensitive to the enzyme and whether similar cell granules were released upon lysis. Determinations of sensitivity were made by mixing a loopful of the cells with one drop of a solution of 1.0 per cent lysozyme (Armour Laboratories) in 0.9 per cent saline. A cover slip then was placed over the preparation and the lytic process observed under oil immersion using phase contrast equipment.

Anaerobic growth. Knight and Proom (1950) reported that *Bacillus coagulans* showed growth under anaerobic conditions. For this reason it was thought that anaerobic growth might be an additional means of separating the *B. stearothermophilus* group from the *B. coagulans* group. The cultures were streaked on plates of nutrient agar and placed in an ordinary 12 quart aluminum pressure cooker containing a paladinized asbestos catalyst. The vessel then was evacuated, filled with hydrogen gas, evacuated a second time, and finally nearly filled with a gas mixture of 90 per cent hydrogen and 10 per cent carbon dioxide. A small residual vacuum was left in order that the effectiveness of the seal on the vessel could be tested before opening it for observation. The cultures were incubated at 65 C for 1 to 3 days.

Growth temperatures. Cultures were inoculated on nutrient agar slants and incubated at the desired temperatures. At the higher temperatures (50 C to 75 C) incubations were carried out in both air and water baths. Actual media temperatures were verified in each experiment by the insertion of thermometers directly into agar slants which were placed in various parts of the

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[2] Present address: Department of Bacteriology, Brigham Young University, Provo, Utah.

incubator. Incubation periods at 45 C and higher were 1 to 3 days. At temperatures below 45 C cultures were incubated for 7 days.

pH of glucose broth cultures. The pH of the glucose broth cultures was determined after 7 days' incubation at 65 C by means of a glass electrode pH meter.

corded at each point where isolations were made. The material was transferred directly to nutrient agar slants by means of a pipette or wire loop, and the cultures, after incubation at 65 C for 16 to 18 hours, were streaked on nutrient agar plates which also were incubated at 65 C. Representative, isolated colonies were selected and

TABLE 1

Source and minimum and maximum growth temperatures of the thermophilic organisms studied

CULTURE NUMBER	SOURCE	TEMP OF POOL	pH OF POOL	MAXIMUM GROWTH TEMP	MINIMUM GROWTH TEMP
		C		C	C
1	Mammoth Terrace	68	7.5	75	35
4	Mammoth Terrace	68	7.5	70	50
7	Mammoth Terrace	68	7.5	70	35
10	Mammoth Terrace	69	7.5	75	35
12	Mammoth Terrace	69	7.5	70	35
12y	Mammoth Terrace	69	7.5	70	35
14	Mammoth Terrace	70	7.5	67	35
18	Fountain Paint Pots	82	7.5	67	45
23w	Near Firehole Lake	63	6.0	67	35
23y	Near Firehole Lake	63	6.0	67	45
24	Near Firehole Lake	70	6.0	67	35
27	Near Pump Geyser	73	6.0	75	35
29	Near Beach Spring	75	7.5	67	35
34	Near Giantess Geyser	70	5.5	67	35
35	Near Splendid Geyser	69	7.5	75	35
37	Near Punch Bowl Spring	73	7.5	70	45
38	Near Spouter Geyser	70	5.0	70	50
39	Near Spouter Geyser	90	4.5	75	50
41	Near Grand Geyser	73	7.0	75	35
42	Near Grand Geyser	68	6.5	70	45
44	Near Grand Geyser	73	5.5	75	35
47	Pool at Thumb	79	6.5	75	35
48Br	Spring at Thumb	71	4.5	67	35
48w	Spring at Thumb	71	4.5	67	35
National Canners Association strain 1373	Spoiled canned hominy	—	—	75	50
National Canners Association strain 1492	Spoiled canned pumpkin	—	—	75	50
National Canners Association strain 1493	Spoiled canned hominy	—	—	75	50
National Canners Association strain 1503	Exact source unknown	—	—	75	50
National Canners Association strain 1620	Spoiled canned hominy	—	—	75	50
National Canners Association strain 2184	Spoiled canned peas	—	—	70	50

Agar preparations. Agar concentrations of 2.5 per cent were used because such preparations were found to withstand incubation better at high temperatures.

Isolation of cultures. Twenty-four of the cultures were isolated from the thermal areas of Yellowstone National Park in August of 1951. The temperature and approximate pH were re-

transferred to nutrient agar slants. Culture numbers are those assigned during the sampling process. The National Canners Association cultures, which were included for comparative purposes, were isolated from spoiled, canned foods in 1919, with the exception of strain 2184, which was isolated in 1922. The source, pH, and temperature at the point of isolation as well as the maxi-

mum and minimum growth temperatures of each of the strains studied are shown in table 1.

RESULTS

A comparison of the Yellowstone and National Canners Association strains with *B. stearothermophilus* is shown in table 2. The characteristics of

TABLE 2

Comparison of characteristics of Yellowstone and National Canners Association strains with those of Bacillus stearothermophilus

CHARACTERISTICS STUDIED	YELLOWSTONE STRAINS		NATIONAL CANNERS ASSOCIATION STRAINS	
	No. of strains similar	No. of strains differing	No. of strains similar	No. of strains differing
Glucose agar	24	0	6	0
Soybean agar	19	5	1	5
Stock culture agar	21	3	0	6
5% NaCl broth	24	0	6	0
Glucose asparagine agar	9	15	0	6
Tomato yeast milk	18	6	1	5
Citrate agar	24	0	6	0
Proteose peptone acid agar	24	0	6	0
Starch hydrolysis	20	4	5	1
Gelatin hydrolysis	20	4	5	1
Acetylmethylcarbinol	24	0	6	0
pH of glucose broth	24	0	6	0
Reduction of nitrate to nitrite	13	11	6	0
Gram reaction	20	4	1	5
Growth temperature	24	0	6	0
Macroscopic appearance	0	24	0	6
Vegetative cells	24	0	6	0
Sporangia and spores	24	0	6	0

21 of the 24 Yellowstone isolates conformed quite closely with the emended description of *B. stearothermophilus* as reported by Gordon and Smith (1949). However, 5 of the 6 National Canners Association strains and 3 of the Yellowstone strains were found to differ from *B. stearothermophilus* in several characteristics. These 8 anomalous strains, in contrast to *B. stearothermophilus*, showed excellent growth on soybean and stock culture agars, caused soft curd formation with reduction in tomato yeast milk, and were uniformly gram positive. Curd formation and reduction in the enriched milk were found to occur within 24 hours at either 55 C or 65 C. The 5 National Canners Association strains showed definite but not abundant anaerobic growth, while none of the Yellowstone strains exhibited significant growth under similar conditions.

The macroscopic appearance of the growth of the 5 National Canners Association strains differed consistently from the growth of all of the other strains. Growth of these organisms on nutrient agar slants was thin, spreading, and translucent. Isolated colonies exhibited entire to undulate edges. The growth of all the Yellowstone organisms, and of National Canners Association strain 2184, was abundant, spreading, and opaque, with a number of the cultures producing yellow-orange pigments. Isolated colonies possessed erose edges and were generally larger than the colonies of the National Canners Association strains.

The growth differences among these rapidly growing thermophilic organisms are most clearly apparent in 16 to 18 hour cultures, and it is important that young cultures be used as a source of inoculum.

Observations on lysozyme action showed that all strains were lysed by this enzyme; however, the nature of the cell-free preparations resulting from lysis appeared to differ from strain to strain. Some strains released granules similar to those obtained from National Canners Association strain 2184, while others appeared to break up completely.

DISCUSSION

Analysis of the results showed that there was a general conformity among the 30 strains with the characters ascribed to *B. stearothermophilus*. There were, however, marked differences exhibited by 8 of these strains, which related them more closely, in some respects, to *B. coagulans* than to *B. stearothermophilus*.

The principal means of separating *B. stearothermophilus* from *B. coagulans* as reported by Gordon and Smith (1949) were: *B. stearothermophilus* grew at 65 C and hydrolyzed gelatin moderately to strongly; its growth was inhibited on glucose, soybean, and stock culture agars. *B. coagulans* did not grow at 65 C, hydrolyzed

gelatin only slightly if at all, and grew well on the 3 selected agars. It grew abundantly on proteose peptone acid agar and curdled tomato yeast milk, while *B. stearothermophilus* was negative on these media. The 8 anomalous strains (4, 39, 44, National Canners Association 1373, National Canners Association 1492, National Canners Association 1493, National Canners Association 1503, and National Canners Association 1620) showed the following characteristics which were similar to those of *B. coagulans*: Formation of curd, with reduction, in tomato yeast milk, good growth on soybean and stock culture agars, positive gram reaction, and, in the case of the 5 National Canners Association cultures, definite anaerobic growth. In all other characteristics, with the exception of macroscopic appearance, the 8 strains were similar to *B. stearothermophilus*. The resemblance to *B. coagulans* in certain important characteristics, however, does not identify these strains with this species since all showed good growth at 65 C and 70 C and even some growth at 75 C.

Among the strains more closely resembling *B. stearothermophilus*, Yellowstone strain 7 and National Canners Association strain 2184 were unique in that both failed to hydrolyze either starch or gelatin, even upon repeated, prolonged incubation. These results agree with the enzymatic studies of Militzer *et al.* (1949) in which they were unable to demonstrate the presence of amylases or proteinases in cell-free fractions from National Canners Association strain 2184. All strains of *B. stearothermophilus* studied by Gordon and Smith (1949) hydrolyzed starch, and 94 per cent of these strains hydrolyzed gelatin strongly. Stark and Tetrault (1951) have demonstrated the presence of starch saccharifying enzymes in cell-free preparations from thermophilic bacteria identified as *B. stearothermophilus*.

The observations on lysozyme action show that one cannot select at random any of the organisms capable of growth at 65 C and from it obtain a cell granule preparation corresponding to that from National Canners Association strain 2184.

As Smith, Gordon, and Clark (1946) have stated so aptly, the knowledge of the investigator is limited to his collection of strains. Accordingly, it should be emphasized that the present study is limited because only 30 strains were characterized. The 24 strains from Yellowstone National Park do not represent a true cross section of the bacterial flora of the thermal areas because cultures were not obtained from all of the springs, and the method of isolation excluded any organism not able to grow at 65 C. Furthermore, no studies have been made yet on the anaerobic cultures obtained from this region.

Because of the current interest in biochemical and cytological investigations of thermophilic organisms, and in view of the strain differences described above, it seems desirable that broader, more complete studies of the *B. stearothermophilus* group should be conducted, with the objective of further strain differentiation and possibly even new species designation.

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SUMMARY

Twenty-four cultures of aerobic, sporeforming bacteria, capable of growing at 65 C, were isolated from the hot springs of Yellowstone National Park and characterized along with 6 similar strains obtained from the National Canners Association. Twenty-one of the Yellowstone strains and one National Canners Association strain were found to conform quite closely to *Bacillus stearothermophilus*. The characteristics of the remaining 8 strains could not be well correlated with any previously described species. These 8 anomalous strains appeared to represent a group intermediate between *Bacillus stearothermophilus* and *Bacillus coagulans*.

The use of lysozyme action as an aid in characterizing thermophilic bacteria was introduced.

REFERENCES

DONK, P. J. 1920 A highly resistant thermophilic organism. J. Bact., **5**, 373–374.

GEORGI, C. E., MILITZER, W., BURNS, L., AND HEOTIS, J. P. 1951 On the existence of a cell granule in a thermophilic bacterium. Proc. Soc. Exptl. Biol. Med., **76**, 598–601.

GORDON, R. E., AND SMITH, N. R. 1949 Aerobic sporeforming bacteria capable of growth at high temperatures. J. Bact., **58**, 327–341.

KNIGHT, B. C. J. G., AND PROOM, H. 1950 Nutritional study of genus *Bacillus*. J. Gen. Microbiol., **4**, 508–538.

MARSH, C. L., AND MILITZER, W. 1952 Thermal enzymes. V. Properties of a malic dehydrogenase. Arch. Biochem. Biophys., **36**, 269–274.

MILITZER, W., AND TUTTLE, L. C. 1952 Thermal enzymes. IV. Partial separation of an ATPase from an Apyrase fraction. Arch. Biochem. Biophys., **39**, 379–386.

MILITZER, W., SONDEREGGER, T. B., TUTTLE, L. C., AND GEORGI, C. E. 1949 Thermal enzymes. Arch. Biochem., **24**, 75–82.

MILITZER, W., SONDEREGGER, T. B., TUTTLE, L. C., AND GEORGI, C. E. 1950 Thermal enzymes. II. Cytochromes. Arch. Biochem., **26**, 299–306.

MILITZER, W., TUTTLE, L. C., AND GEORGI, C. E. 1951 Thermal enzymes. III. Apyrase from a thermophilic bacterium. Arch. Biochem. Biophys., **31**, 416–423.

SMITH, N. R., GORDON, R. E., AND CLARK, F. E. 1946 Aerobic mesophilic sporeforming bacteria. U. S. Dept. Agr. Misc. Pub. 559.

STARK, E., AND TETRAULT, P. H. 1951 Isolation of bacterial, cell-free, starch saccharifying enzymes from the medium at 70 C. J. Bact., **62**, 247–249.