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EFFECT OF TEMPERATURE ON THE RATE OF GERMINATION IN *BACILLUS CEREUS*

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ABSTRACT

KNAYSI, GEORGES (Cornell University, Ithaca, N.Y.). Effect of temperature on the rate of germination in *Bacillus cereus*. J. Bacteriol. **87**:619–622. 1964.—By use of the change of the spores from bright to dark, when observed in dark contrast with a phase microscope, as a criterion for the incipience (first stage) of germination, and increase in the volume of the spore as a criterion for the second stage, it was found that the two stages differed in their cardinal temperatures. In the strain investigated, the first stage has a maximum of about 59, an optimum of 30, and a minimum estimated to be in the neighborhood of −1 C. The corresponding temperatures for the second stage are 44, 35, and 10 C. Spores exposed to temperatures above the maximum for the first stage have a tendency to turn dark at temperatures suitable for germination, and this tendency is not immediately arrested when the spores are exposed to pH 3.2 to 3.4 at 6 C. The potential value of the differences between the cardinal temperatures of the two stages for the control of sporeformers is discussed.

It is now generally agreed that germination of the bacterial endospore consists of two steps. The first, of relatively short duration, is exclusively characteristic of the spore. The second involves growth, and is terminated when the germ cell emerges from the spore coat with the appearance, structural organization, and enzymatic content typical of the vegetative cell of the particular organism; it would not usually include cell division. The first is often referred to as the stage of "incipient germination," or the "incipience of germination." Murrell (1961) suggested for this stage the term "initiation of germination," and Hermier (1962) called it the "initial phase." The second stage is often called "stage II" or, on purely etymological grounds, the stage of the germ cell. There is a precedent for this use in the term "germ tube" where the word germ is used in its etymological sense of sprout.

Mol (1957), working with strain 232 of *Bacillus cereus*, observed no growth in 4 months when spores, washed repeatedly and exposed to sonic vibration, were inoculated into yeast extract-glucose-phosphate medium and incubated at 8 C, whereas 85% germinated (i.e., entered the first stage) in the same environment in 6 hr. Wolf and Mahmoud (1957) observed germination (first stage) in spore suspensions of *B. cereus* and *B. subtilis* held in nutrient media at 0 C. *B. subtilis* germinated more readily than did *B. cereus*. Thorley and Wolf (1961) reported that in the genus *Bacillus* the optimum for the first stage generally lies between 30 and 37 C, but that in some members of the genus it may be at 41 or even 50 C. It is interesting that most workers employed heat-activated spores. The time and temperature of activation varied from 80 C for 20 min to 60 C for 10 min.

In the present work, both stages of germination were studied as the functions of temperature, on previously unheated spores, in the range of 1 to 80 C. The significance of preheating the spores is now being investigated and will be the subject of a subsequent report.

MATERIALS AND METHODS

In this study, we used microcultures on collodion of *B. cereus* C_3, a strain often employed in cytological investigations. These microcultures were prepared as previously described (Hillier, Knaysi, and Baker, 1948; Knaysi, 1959), and were incubated at 30 C for 2 or 3 days. Such microcultures consist mostly of spores (Knaysi, 1959), and may be stored for 1 or 2 weeks in a refrigerator, or at room temperature under conditions that would slow down the rate of drying (e.g., in a closed container), and used as needed.

The medium used for germination and growth was broth of pH 7.2 to 7.4, containing 0.3% beef extract and 0.5% each of Tryptone and glucose. A 250-ml beaker was filled with broth to about 1 in. from the top, covered with a petri dish cover,

and placed in a water bath of the desired temperature. When thermal equilibrium was reached, the petri dish cover was lifted, the microcolonies to be investigated were floated directly on the broth, and the beaker was recovered. At the end of the desired period, the microcolonies were refloated consecutively, with the aid of a glass slide covered with a film of 2% agar, on three beakers of distilled water at 56 to 60 C, remaining in the third beaker for 30 min. This allowed dialysis of the food material absorbed by the microcultures, while preventing germination. The microcultures were then picked with cover glasses and air-dried, and the proportion of germinating spores was determined. Floating the microcolonies at 6 C on distilled water, on 0.001 N HCl, on HCl-potassium hydrogen phthalate of about pH 3.6 (prepared by mixing 10 ml of 0.1 N HCl with 20 ml of 0.1 M potassium hydrogen phthalate and distilled water to make 1 liter), or on all three consecutively in the order of buffer, acid, and distilled water was found unsatisfactory, particularly when the microcolonies had been incubated on the broth at temperatures above 60 C (Table 1).

As a criterion of germination, the change of the spore from bright to gray or black, as observed in dark contrast with the phase microscope, was used. Spores which underwent that change without an apparent increase in volume were considered to be in the first stage. An increase in volume was taken to indicate the second stage.

To determine the proportion of germinating spores, the air-dried microcolony was usually inverted on a thin film of 2% agar, and the preparation was sealed with paraffin or Vaspar (a mixture of Vaseline and paraffin in equal proportions). Spores that had been exposed to temperatures above 60 C for more than a few minutes tended to be unstable on ordinary agar, and had to be mounted on agar dissolved in HCl-potassium hydrogen phthalate buffer (pH 3.2 to 3.4) on which they showed less instability. Observations were made with a phase microscope (American Optical Co. Buffalo, N.Y.) with a combination of an oil-immersion objective (97×; na = 1.25) and a 15× ocular. Every determination involved counting of more than 500 spores, and sometimes 3,000 to 4,000 spores were counted.

RESULTS

In Fig. 1, the percentage of dark spores (i.e., spores that have begun to germinate) is plotted

TABLE 1. *Effect of dialysis on the stability of endospores previously exposed to glucose broth near the limiting temperatures for stage I*

Expt no.	Broth treatment		Conditions of dialysis			Proportion of dark spores
	Time	Temp	Fluid	Temp	Time	
		C		*C*	*min*	%
1	60 min	3	Distilled water	55	30	0.0
	60 min	3	Distilled water	55	30	0.0
	60 min	3	Distilled water	3	60	0.7
2	6 hr	3	Distilled water	55	30	4.8
	6 hr	3	Distilled water	3	60	5.4
3	5 min	25.5	Distilled water	55	30	59.0
	5 min	25.5	Distilled water	5	30	54.6
4	6 hr	60	Distilled water	60	30	0.0
	6 hr	60	Buffer	6	20	
			+			
			0.001 N HCl	6	20	
			+			
			Distilled water	6	20	12.9
5	6 hr	58	Distilled water	58	30	0.8
	6 hr	58	Distilled water	6	60	16.4
	6 hr	58	Buffer	6	60	27.3
6	4 hr	58	Distilled water	58	30	0.0
	4 hr	58	Distilled water	6	60	29.0
	4 hr	58	Buffer	6	60	42.8

against the temperature for each of four incubation periods, namely, 5 min, and 1, 2, and 6 hr. Only the 5-min curve is complete. The other curves show discontinuity in a range around the optimum, caused by the fact that in that range many of the spores had gone beyond the second stage, so that the microculture contained many actively motile vegetative cells in pairs and chains of variable length. In the case of the 6-hr period, long fascicles with multiple loops were formed within that range, and determination of the rate of change became highly inaccurate and, indeed, was no longer practical. These curves served mostly to determine the temperature limits for both stages, as well as the optimum for the second. The optimum for the first stage was determined from the 5-min curve. To avoid confusion in the figure, the 2-hr curve was drawn only above 60 C.

All four curves show a general similarity in shape and are fairly symmetrical. They rise rapidly to a maximum, vary slowly over a range of about 10 to 20 C, and then drop, more rapidly than they rose, to zero or near-zero values. The maximum shown by the 5-min curve at about 30 C corresponds to the optimum for the first stage. Mol (1957) found the same optimum for his strain. The points of intersection of a curve with the temperature axis represent the limits for a period of incubation represented by the curve. For the 5-min and 1-hr curves, these points are at about 10 and 56 C, and at 3 and 56 C, respectively. The intersection of the 2-hr curve was determined only for the upper limit; it falls between 58 and 60 C. The 6-hr curve did not intersect the temperature axis within the range used in the present investigation. The lowest temperature used was 1 C. At this temperature, the 6-hr curve still has an ordinate of about 2.5. However, the curve is rectilinear between 1 and 5 C, and it would be reasonable to expect that it would continue to be so below 1 C. Accordingly, one gets by interpolation an intersection point at about −1 C. This means that complete inhibition of germination between −1 and 0 C in a chemically suitable medium sould result only from a change in the physical state of the medium. The 6-hr curve does not intersect the temperature axis above the optimum; instead, it exhibits a minimum at about 58 C followed by a rise to about 20 at 80 C. The other three curves show a similar rise at a rate that is a function of the period of incubation and becomes noticeable only above 60 C. Significance of the rise in the proportion of dark spores above the maximal temperature has now been investigated, and the results will be reported in a subsequent communication. Unfortunately, this rise prevents intersection of the 6-hr curve with the temperature axis, and thus slightly interferes with exact determination of the maximal temperature for the first stage.

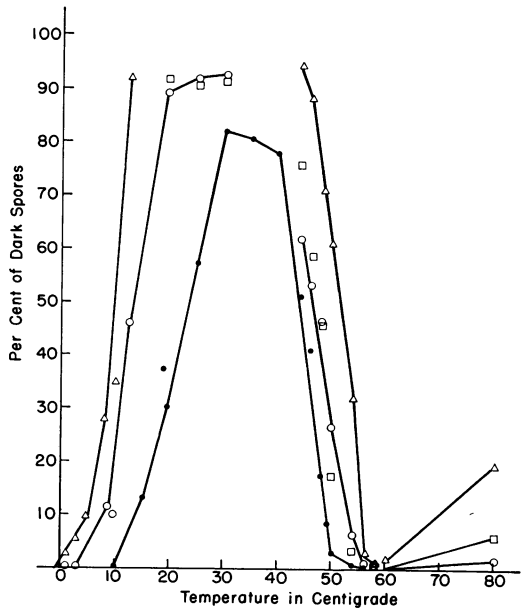

FIG. 1. *Germination as a function of temperature for four different periods of incubation:* ● = *5 min,* ○ = *1 hr,* □ = *2 hr, and* △ = *6 hr.*

It results from the observations reported in the preceding paragraph that the cardinal temperatures for the first stage of germination of the investigated organism are about −1, 30, and 59 C, for the minimum, optimum, and maximum, respectively.

Determination of the cardinal temperatures for the second stage of germination was based on the fact that the second stage involves growth and, consequently, an increase in the volume of the spore. Therefore, the minimal temperature for that stage would be the last, below the optimum, which would allow increase in the volume of the spore during a reasonably long incubation period, in the present case 6 hr. The optimum would be the temperature which shows, during a properly selected incubation period (in the present case, 1 hr), the largest proportion of germ cells and of dark spores showing definite enlargement.

Finally, the maximal temperature would be the last temperature, above the optimum, which would allow enlargement of the dark spores during a reasonably long period of incubation (in the present case, 6 hr). On the basis of these definitions, the cardinal temperatures for the second stage would be 10, 35, and 44 C, respectively, for the minimum, optimum, and maximum.

DISCUSSION

The 6-hr limit set for the observation of maxima and minima was dictated by practical considerations, and a longer incubation period would, probably, somewhat lengthen the range between the two limiting temperatures. Nevertheless, sensitivity of the method used allows the detection of very early changes in both stages, which lends special significance to the 6-hr period. It enabled us to detect a measurable rate for the first stage at 1 C, and indicated that the true minimum for this stage is in the neighborhood of −1 C. Mol (1957) reported 4 C as the minimum for the first stage in another strain of *B. cereus*.

Of potential promise in the control of sporeformers is the existence of a wide difference between the temperature maxima, and also between the minima, of the two stages of germination. In the organism presently investigated, these differences are, respectively, about 15 and 11 C. It would result, for instance, that a food product contaminated with spores of *B. cereus*, and suitable for the development of *B. cereus*, would be rid of more than 90% of these spores by holding for a few hours at 45 to 50 C. The relatively few remaining spores would be of the slowly germinating type. However, the practical value of such a procedure would depend on whether the wide difference observed here between the temperature maxima of the first and second stages is general among sporeformers.

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