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SHORT COMMUNICATION

Germination rate of spores of *Bacillus megaterium*

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THE most commonly accepted criteria of bacterial spore germination are loss of heat resistance, acquisition of stainability by simple stains and a change in absorbance (Wynne & Foster, 1948; Powell, 1951; Pulvertaft & Haynes, 1951; Levinson & Sevag, 1953). To these might be added a decrease in resistance to certain germicides. Although it has been suggested that some of these changes occur simultaneously (Campbell, 1957), most of the evidence for this is qualitative rather than quantitative.

This communication deals with a comparison of the rate of loss of refractility observable by phase contrast microscopy, with the rate of loss of heat resistance by a population of *Bacillus megaterium* spores.

METHODS

Spore suspensions of *B. megaterium* ATCC 8245 in dilutions appropriate to the experiment were made. Samples were heat shocked at 80° for 10 min before each experiment and the rate of loss of refractility during incubation was examined by two methods.

Method 1. A loopful of a spore suspension was placed on the surface of a dried MRVP agar (Difco Bacto) plate; the agar layer was uniform and not more than 1 mm thick. A spore coated disc of approximately 3 mm diameter was cut from the plate: it was mounted in a microscope stage designed for the purpose and heated to approximately 37°. A field of 100 or so spores was observed by phase contrast using a × 100 fluorite oil immersion objective. Photographs were taken at intervals during incubation at 37° and counts of the numbers of refractile and non-refractile spores were made from these.

Method 2. A spore suspension (1 ml) was added to double strength MRVP broth (Difco Bacto) (20 ml) and sterile water (19 ml). The whole was shaken at 37° in a water-bath. At the beginning of the experiment and every 5 min a loopful of the culture was removed and placed on the surface of a dried agar plate of Ionagar No. 2 (Oxoid) 1·5% w/v. Discs of approximately 3 mm diameter were cut, mounted on thin slides, covered with thin coverslips and photographed and counted as described above. This method has obvious advantages over the observation and photography of wet preparations.

The rate of loss of heat resistance was examined by incubating spores in liquid MRVP broth as described above (Method 2); a suspension containing about 800,000 viable spores per ml was used. Samples (1·0 ml) were withdrawn from the culture at the beginning of incubation and after 5, 10, 20, 30, 45, 60, 90 and 120 min. Each sample was added to sterile water (9·0 ml) and heated for 10 min at 80° to destroy any

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organisms which had lost this degree of heat resistance; samples were then cooled rapidly. A further dilution (10^{-1}) was made in sterile water, and each of 5 × 1·0 ml samples of this 10^{-2} dilution (containing originally about 200 viable organisms) was plated onto the surface of a dried nutrient agar counting medium. Plates were incubated for 24 hr at 37°.

The number of colonies growing on each plate was counted and by subtraction of the mean count at each time interval from the mean zero count, the mean of the number of spores which had lost heat resistance was calculated.

RESULTS AND DISCUSSION

In all experiments the numbers of viable spores originally present and the numbers which had lost either refractility or heat resistance, were used to calculate weighting coefficients. The figures for % "germination" at each sample time were used to calculate regression equations of probit of % spores germinated on log time. For these calculations, results with probit values lying outside the range 3·7 to 6·3 were rejected unless they lay close to the provisional probit line. Such results are derived from the bottom and from the top of the sigmoidal curve and carry little weight.

From the equations, the slope (b) of each line was calculated and also the log germination time corresponding to probit 5·0 (log G.T.50).

Variances between replicate counts were satisfactory and heterogeneity χ^2 values for the points on each of the lines were not significant.

From the results (Fig. 1) it may be inferred that, in method 2, loss of refractility by some spores occurs within 5 min and continues until about 95% have become phase dark after about 60 min (b = 2·4).

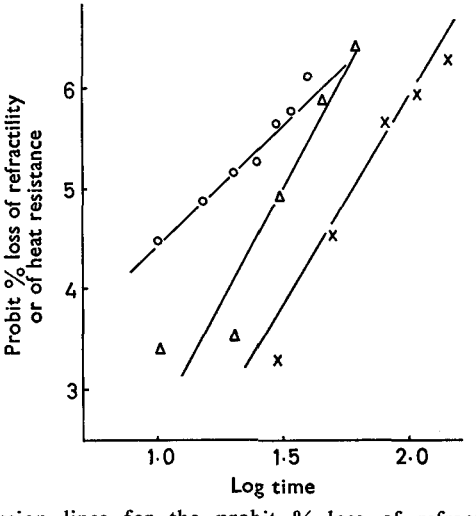

FIG. 1. Regression lines for the probit % loss of refractility on log time. X = method 1 (mean of 6 experiments). ○ = method 2 (mean of 3 experiments). Regression line for the probit % loss of heat resistance on time (△—△) for germinating spores of *B. megaterium* ATCC 8245. (Mean of 3 experiments.)

The results of method 1 suggest a lag of about 40 min during which a very small but increasing proportion of spores lose refractility. During the following 60–70 min the rate of loss of refractility is higher ($b = 4{\cdot}3$). The lag is attributed to slow heat transference from the heated stage through the agar disc to the spores on the surface.

With loss of heat resistance there appears to be a lag of 20–25 min during which the rate of loss of resistance is low. During the subsequent 40 min the rate is high ($b = 4{\cdot}7$).

The G.T.50 values of the two refractility probit lines (17 min and 60 min) differ due to the lag inherent in method 1. The G.T.50 for the heat resistance line is 31 min.

Since the incubation methods used are identical, a comparison can be made between the rate of loss of refractility by method 2 and the rate of loss of heat resistance. The slopes of the lines, 2·4 and 4·7 and the G.T.50 values, 17 min and 31 min differ significantly and suggest that loss of refractility and loss of heat resistance by these spores do not occur simultaneously.

The heated stage (method 1) is a useful method for following rate of loss of refractility. If the lag in heating could be determined with accuracy, so that an arbitrary zero time could be established, the slope of the line would be reduced and might well agree with the slope for method 2.

References

Campbell, L. L. (1957). *Spores*, Editor, Halvorson, H. O., Publication No. 5, pp. 33–44, Washington, D.C.: American Institute of Biological Sciences.
Levinson, H. S. & Sevag, M. G. (1953). *J. gen. Physiol.*, **36**, 617–629.
Powell, J. F. (1951). *J. gen. Microbiol.*, **5**, 281–294.
Pulvertaft, R. J. V. & Haynes, J. A. (1951). *Ibid.*, **5**, 657–663.
Wynne, E. S. & Foster, J. W. (1948). *J. Bact.*, **55**, 61–67.