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The viability of spores of some *Bacillus* species

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The percentage of spores of *Bacillus subtilis* capable of giving rise to macro-colonies ("viability") has been determined. Under optimum conditions (heat-activation and inclusion of dextrose in the counting medium) about 30% of spores formed colonies. This figure appeared to increase to about 70% on prolonged cold storage (0–10°). The viability of spores decreased with increase in the manganese content of the sporulation medium. Spores of four other species of *Bacillus* examined were not heat-activatable and were unaffected by the dextrose content of the counting medium. They showed viabilities of 40–75%.

ATTENTION has recently been drawn by Cook & Brown (1964) to the fact that suspensions of spores prepared by conventional methods do not consist entirely of viable spore-forms. The percentage of viable spores depends on the method of preparation of the suspension and on the conditions under which viability is determined. Of particular importance in assessing viability is the role of any stimulus, especially heat-activation, which may be necessary to induce maximum germination. Two of the factors necessary for maximum viability of *B. subtilis* have been examined; these are the amount of heat-activation and the concentration of dextrose in the counting medium. The viability of some other *Bacillus* species has been determined under the same conditions.

Experimental

METHODS

The percentage viability of spore suspensions was calculated from the ratio of viable count (determined by a roll-tube method) to total count (determined using a counting chamber). Heat-activation factors were calculated from the ratio of viable count after heat treatment, to that before. Determination of viability was made on spore suspensions no more than 2 or 3 weeks old. (The viability of suspensions stored for longer periods in the refrigerator increased).

Test organisms and preparation of suspensions. Organisms were the following NCTC strains: *Bacillus subtilis* 8236, *B. subtilis* 3610, *B. pumilus* 8241, *B. cereus* 9946, *B. megaterium* 9848 and a laboratory isolate of *B. licheniformis.*

Spore suspensions were normally prepared by washing off the growth from 10 day cultures on nutrient agar slopes (Oxoid CM3) to which had been added 1 mg of $MnSO_4.5H_2O$ per litre. The initial concentration of manganese in the nutrient agar was <1 ppm (Oxoid Ltd., analysis of current material). For *B. megaterium* 200 mg per litre $MnSO_4$ was necessary to obtain a good spore yield.

Vegetative cell-free suspensions of *B. subtilis* and *B. licheniformis* spores were prepared by centrifuging and resuspending in water five times. For *B. cereus* spores, separation in a two-phase system of poly-

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ethylene glycol and phosphate buffer was used (Sachs & Alderton, 1961). *B. megaterium* and *B. pumilus* spore suspensions were subjected to ultrasonic irradiation (20 kc/sec: 30 min) using an M.S.E. ultrasonic disintegrator. After this treatment spores were readily separated from vegetative cell debris by centrifuging (cf. Mol, 1957; Grecz, Anellis & Schneider, 1962).

The purity of suspensions was examined by plating on nutrient agar, by staining with 1% methylene blue and also by the carbol fuchsin–methylene blue technique (Powell, 1950).

Effect of manganese. To investigate the effect of the manganese content of the medium on spore viability of *B. subtilis*, the concentration of manganese sulphate was varied between 1 mg and 1 g per litre.

Total counts. The counting method incorporated the recommendations of Cook & Lund (1962). Two 0·1 mm slides were used for each count which was made using normal microscopy of all spores in each of three fields of sixteen squares selected at random on each slide. Normal microscopy was preferred to phase-contrast microscopy since the object was to determine the total count of all spore forms.

The Index of Dispersion, χ^2, was calculated for the counts from 50 slides. Since $\sqrt{2\chi^2} - \sqrt{2n-1} < 1{\cdot}645$, χ^2 did not significantly exceed expectation (Fisher, 1948) and thus the counts did not differ significantly from a Poisson distribution.

Using 25 pairs of counts, the average coefficient of variation between duplicate slide counts was 5·9%; when duplicate counts showed a difference $> 10\%$ the count was repeated. The same two counting slides were used throughout the work and there was no indication of bias between the slides.

Additionally, ten duplicate counts were made on the same suspension on different days and the coefficient of variation for these was 8·3%. The major source of variation in total counts is likely to be the fit between cover-slip and slide. Results quoted show that the total count estimates are not subject to undue variation from this source and are reproducible.

Viable counts. Viable counts were determined by the roll-tube method (coefficient of variation 6%: Richardson, 1959). The effect of dextrose and soluble starch on viability was investigated by varying the concentrations of these substances in the nutrient agar counting medium.

Reproducibility of viability. Six suspensions of *B. subtilis* 8236 were prepared on different occasions and the viability of each crop determined immediately after preparation of the suspension. The six values had a mean value of $16{\cdot}2\% \pm 1{\cdot}7$ with a coefficient of variation of 10·5%. The variation in viability between spore crops prepared on different occasions is of the same order as the combined errors of total and viable count.

Heat-activation. The effect of different temperatures for varying periods of time was determined on the viable counts of suspensions of *B. subtilis* 8236 spores. Suspensions were sealed in 10×75 mm tubes and heated in a water-bath.

Attempted separation of heat-activatable fraction. An attempt was made to separate the heat-activatable fraction of the spore population so that its properties could be investigated. *B. subtilis* spores were allowed to germinate in a medium containing 1% peptone and 0·5% dextrose, and later in a medium identical to the counting medium except for the omission of agar, for periods from 5–16 hr. After incubation the cells were centrifuged and the ungerminated spores separated from the vegetative cells using the two-phase system. The heat-activation factor of the recovered spores was then compared with that of a normal population which had also been passed through the two-phase system.

Results and discussion

EFFECT OF MANGANESE IN THE SPORULATION MEDIUM

Manganese has been claimed to be an essential requirement for spore production in several species of *Bacillus* and work with these species has been summarised by Murrell (1961). The number of cells of *B. megaterium* giving rise to spores has been shown by Weinberg (1964) to be dependent on the manganese concentration in the medium. It therefore seemed possible that viability of spores might be affected by the manganese content of the sporulation medium. Fig. 1 shows that as the concentration of manganese sulphate in the medium increases, the

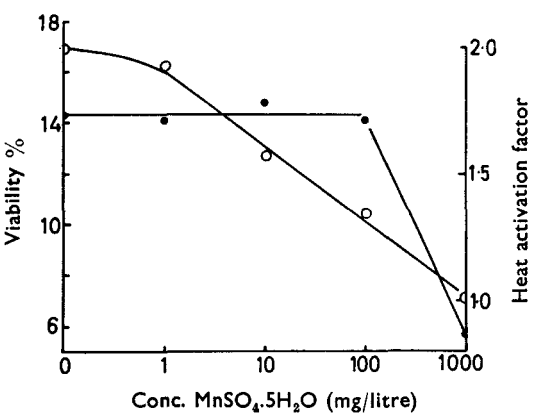

FIG. 1. The relationship of heat-activation and viability of *B. subtilis* spores to the manganese content of the sporulation medium. ○ = viability. ● = heat-activation.

percentage viability of *B. subtilis* falls significantly. At the highest manganese concentration growth was extremely variable. A suspension of spores was not obtained on every occasion from this medium, growth sometimes being restricted to the production of filamentous forms, with no tendency to sporulate. When spores were formed they were atypical. Growth and sporulation of *B. megaterium* and *B. licheniformis* were not inhibited by high concentrations of manganese.

These results suggest that it is not necessary to add manganese to the medium to induce the formation of viable spores of *B. subtilis*. But there is a marked variation of manganese tolerance with species and it seems that, even where manganese may be a requirement for sporulation, excessive manganese concentration may lead to a higher proportion of non-viable forms.

COMPOSITION OF COUNTING MEDIUM

The composition of the counting medium can markedly affect the viable count. The importance of dextrose and soluble starch in the medium, particularly for heated spores of *B. stearothermophilus*, has been demonstrated by Cook & Brown (1964). Fig. 2 shows that for both normal and pre-heated suspensions of *B. subtilis*, the count is much increased by the addition of dextrose. The highest increase in count is given by 0·5% dextrose. Higher concentrations do not result in further increases. Soluble starch in concentrations up to 1% did not affect the count and in media containing both dextrose and soluble starch the increase in count was no more than that attributable to dextrose alone. Thus for *B. subtilis* a medium containing 0·5% dextrose is recommended for viable counts.

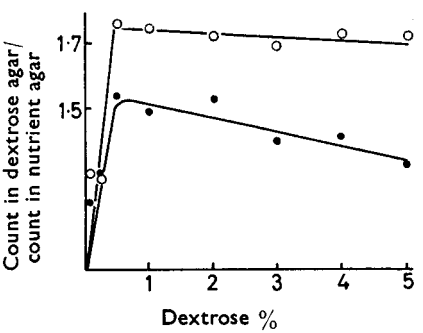

FIG. 2. The increase in viable count of *B. subtilis* spores caused by dextrose in the counting medium. ○ = heat-activated spores. ● = normal spores.

HEAT-ACTIVATION

Fig. 3 shows that as the temperature increased, activation occurred more rapidly and to a greater extent. Maximum activation occurred in 4–10 min at 85°. When suspensions were heated in capillary tubes instead of ignition tubes at this temperature, activation time was reduced to less than 30 sec. Since activation occurs at temperatures which are also rapidly sporicidal, the death of some viable spores may occur so that it is possible that the activation curve may represent less than the true number of potentially activatable spores.

Desrosier & Heiligman (1956) claimed that activation occurred with *B. thermoacidurans* and *B. globigii* only at temperatures which were subsequently lethal. This was not so in the present work with *B. subtilis* with which there was no decrease in count up to 5 hr at activating temperatures of 60° and 70°.

Powell & Hunter (1955) investigated the increase in the rate of germination brought about by heat-activation of *B. megaterium*. They plotted rate of germination against time of heating and claimed that the curve obtained was typical of the effect of temperature on a first order reaction. However it seems equally possible that the increase brought about by heat in numbers of spores forming colonies may be due to heterogeneous heat-activation characteristics existing among a spore population.

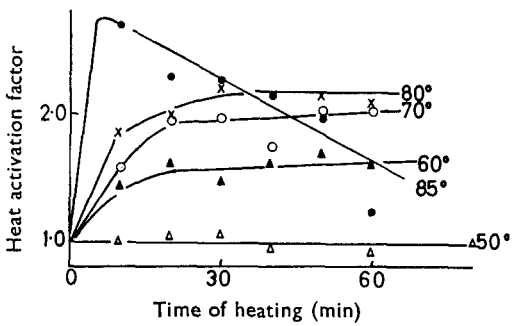

FIG. 3. Heat-activation curves for *B. subtilis* 8236.

Heat-activation and manganese content of sporulation medium. The spore suspensions prepared from media of varying manganese content were tested for heat-activation. The factors obtained remained constant except for spores from the medium of highest manganese content. Suspensions from the latter showed mortality rather than activation after heating at 85° for 10 min. This is compatible with the previous suggestion that spores produced on this medium are abnormal.

VIABILITY OF SPORES OF *Bacillus* SPECIES

The viability of several species was determined under conditions which were optimal for *B. subtilis* 8236. Viability was also determined after heating at 60° for 30 min and by counting on media with and without dextrose. The results are shown in Table 1. The heat-resistant *B. subtilis* even after activation had a lower viability than the other organisms. Under the optimum conditions *B. subtilis* showed a viability of about 30% (similar figures were obtained from both strains). Under the optimum conditions for *B. subtilis*, *B. licheniformis* showed a viability of 75%. The inclusion of dextrose in the counting medium did not result in an increase in count for organisms other than *B. subtilis*, although all are able to metabolise dextrose (Bergey, 1957). Dextrose may be a specific inducer of germination in some species (Powell, 1951).

Suspensions of *B. subtilis* which had been stored in the refrigerator for up to 8 months showed a marked increase in viable count although the total count remained constant. Results from the three suspensions available are presented in Table 2. It appears that concurrent with "storage

activation" there is a decrease in heat-activatability. The overall effect was that the viability rose from a comparatively low figure to a value at least equal to that for the other species. These results inevitably suggest that heat-activation is merely an acceleration of a process which occurs more slowly under normal environmental conditions.

TABLE 1. THE VARIATION IN % VIABILITY OF SPORES OF SOME *Bacillus* SPECIES WITH HEAT SHOCK AND DEXTROSE CONTENT OF MEDIUM

Organism	Conditions					
	No heat shock		60° for 30 min		85° for 10 min	
	NA	DA	NA	DA	NA	DA
B. subtilis 3610	10·5	13·1	11·2	15·8	17·2	28·5
B. subtilis 8236	12·1	16·2*			16·3	33·1*
B. cereus	40·0	41·7	29·6	32·3	19·2	22·5
B. pumilus..	45·1	45·1	47·9	50·0	47·5	54·4
B. megaterium	56·2	56·9	47·8	50·7	36·9	39·1
B. licheniformis	57·9	55·0	75·5	62·3	43·5	22·7

NA = nutrient agar DA = dextrose agar
* Average value from six crops

None of the organisms showed a viability of 100% although most published work supports the findings that virtually all the spores in a suspension are able to initiate the germination process as indicated by the criteria of loss of refractility and change in staining reactions. The germination of *B. subtilis* 8236 was followed using as the criterion the change in staining reaction to carbol fuchsin and methylene blue (see Powell, 1950). It appeared that after 5–6 hr about 90% of the spores had

TABLE 2. THE INFLUENCE OF STORAGE-ACTIVATION AND HEAT-ACTIVATION ON THE VIABILITY OF SPORES OF *B. subtilis* 8236

Crop	Storage (months)	Viability of non-activated spores (%)	Storage-activation factor	Heat-activation factor of spores after storage	Viability due to combined effects of storage and heat-activation %
2	0	13·6	1·0	2·3	31·2
	8	—	3·3	1·2	53·8
3	0	18·4	1·0	1·3	23·9
	3	—	1·4	1·2	30·9
	6	—	2·3	1·0	43·3
4	0	17·5	1·0	2·5	43·7
	3	—	2·3	1·5	60·4
	6	—	3·1	1·3	70·5

$$\text{Heat-activation factor} = \frac{\text{viable count after heating—85°, 10 min}}{\text{viable count before heating}}$$

$$\text{Storage-activation factor} = \frac{\text{viable count after storage in refrigerator}}{\text{initial viable count}}$$

germinated (ratio of blue staining forms to total cells; about 800 cells being counted). Counts after this time were unreliable due to the proliferation of vegetative forms. After 8 hr only 2% of spores were present but this low figure may have been due to an increase in the total count.

The absolute accuracy of total cell counts using 0·02 mm slides has been questioned by Norris & Powell (1961). From their results it can

be calculated that the depth of liquid entrapped by such slides was on average about 30% in excess of the normal depth of the slide. However, using 0·1 mm slides, Cook & Lund (1962) have obtained counts which were about 70% of those using 0·02 mm slides, which suggests that the magnitude of absolute error with the deeper slides is much less, if indeed it does occur. It seems likely, therefore, that in the present work the estimates of viability using 0·1 mm slides are reliable and that the low viability reported is not due to errors of total count. The discrepancy between the figures for viability determined as described above and those for germination may be reasonably ascribed to the fact that not all germinated forms grow to give macro-colonies, an observation which has previously been made by Pulvertaft & Haynes (1951). They suggested that the freshly formed vegetative cells were liable to lyse due to their more exacting growth requirements.

ATTEMPTED SEPARATION OF HEAT ACTIVATABLE SPORES

It was expected that under the conditions of germination the heat-activatable fraction would not germinate and that these potentially viable spores could then be recovered. The recovered spores should then fail to germinate without heat treatment and should show a heat-activation factor of infinity.

No significant difference between the heat-activation factors for normal and recovered spores was found among seven separations. Results from two separations, for which viability of the recovered spores was also determined, are presented in Table 3.

TABLE 3. THE VIABILITY AND HEAT-ACTIVATION FACTORS OF NORMAL SPORES AND OF RESIDUAL SPORES RECOVERED AFTER A PERIOD OF GERMINATION

	Total count $\times 10^6$/ml	Viable count $\times 10^6$/ml		Viability of unheated spores (%)	Heat-activation factor
		Unheated	Heat-activated		
Normal spores	101·5	200	476	19·7	2·4
Recovered spores, 7 hr germination	33·9	20·8	45·0	6·1	2·2
Recovered spores, 8 hr germination	37·5	22·8	52·4	6·1	2·3

The sterile filtrate obtained after germination was added to a normal suspension before counting and also included in the counting medium, without having any effect on the count. Also, in a second experiment, after the initial germination, the cells were separated from the liquid and a fresh spore suspension added to the liquid. On further incubation the fresh spores germinated normally. Thus it appeared that germination in the original stage was not brought to a standstill by the products of germination.

It is difficult to explain satisfactorily the failure to separate the heat-activatable fraction by this method. It is hoped that it may prove possible to separate the heat-activatable spores by utilising properties which may correlate with heat-activation.

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