The Activation of Spores of *Clostridium bifermentans*

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SUMMARY

The activation of spores of *Clostridium bifermentans* by various treatments was investigated. Mercaptoacetate did not activate the spores but inhibited the spontaneous activation which occurred in buffer alone. Sodium borohydride effected activation of spores by increasing the pH value above pH 10.0 rather than by its reducing action. The pH value of a suspension had a marked effect on activation; at 37° spores held at less than pH 3.0 or at pH 10.0 or more were activated within 1 hr. Continued incubation at an alkaline pH value led to a decrease in the specific requirements for germination, an effect not produced by acid pH values. Heat-activated spore suspensions became partially de-activated on storage unless they were continuously aerated. Spores activated at 37° and pH 2.0 or pH 7.4 readily became de-activated on storage, whereas spores activated at pH 10.5 remained activated. A specific requirement of heat-activated spores for sodium ions for germination was also found.

INTRODUCTION

The transition of dormant bacterial spores to fully active vegetative forms can be divided into three distinct phases: activation, germination, outgrowth. Activation of spores or the breaking of dormancy is commonly achieved by heating spores in aqueous suspension, a process first described by Evans & Curran (1943). It was reported, however, that reducing agents or exposure to acid pH values activated spores of an aerobic organism, *Bacillus cereus* (Keynan, Evenchik, Halvorson & Hastings, 1964). The effect of these agents as activators of spores of anaerobic bacteria has apparently not been investigated, although it has been reported that spores of *Clostridium roseum* require reducing conditions for germination (Hitzman, Halvorson & Ukita, 1957). In a previous publication (Gibbs, 1964), some of the factors which affect the germination of heat-activated spores of *C. bifermentans* were described. The present paper describes experiments on the effect of reducing agents and pH value as activating agents for spores of this organism and also investigations of the inorganic ion requirement for germination.

METHODS

The technique for the production of suspensions of spores of *Clostridium bifermentans* (CN1617, Wellcome Research Laboratories' Culture Collection), activation by heat and estimation of germination were as previously described (Gibbs, 1964). A decrease in extinction of 55–60% was found to correlate closely with phase-darkening of 95–100% of the spores and, for speed and convenience, the decrease in extinction was used as a measure of germination. Since spores which were not activated did not

germinate within 1 hr at 37° on the addition of germinants, the degree of germination (% decrease in extinction) was taken as a direct measure of the degree of activation. All work with spore suspensions was done in 0.1 M-[Na/K] phosphate buffer (pH 7.4) unless otherwise stated.

Treatment of spores with reducing agents. For studies on the effect of mercaptoacetic acid on spores, the free acid was titrated to pH 7.4 with 10 N-NaOH, and added to spores suspended in phosphate buffer. Sodium borohydride and lithium aluminium hydride (L. Light and Co., Colnbrook, England) were added as solids (0.5–1.0 mg./ml.) to spores suspended in phosphate buffer (O.D. 680 m μ , ca. 1.5; Hilger 810 Biochem. Absorptiometer, 15 mm. diam. tube). Treated spores were washed twice with phosphate buffer before testing for germination.

Treatment of spores with acid or alkali. Spores were suspended in phosphate buffer, titrated to the required pH value with NaOH or HCl, and incubated at 37°. Samples were removed and the spores washed twice with phosphate buffer and tested for germination.

De-activation of spores. After activation by heating or exposure to acid or alkali, spores were washed with phosphate buffer and stored as suspensions in this buffer at 4° and at room temperature (16–20°) in screw-capped bottles three-quarters full, tightly screwed down to minimize aeration. A heat-activation spore suspension was also stored at room temperature with a current of sterile air bubbled through it. Two samples were removed at intervals from the stored suspensions, one was heat-shocked (at 85° for 10 min.) and both were tested for germination.

Extraction of phosphate buffer with diphenyl thiosemicarbazone (dithizone). Phosphate buffer (200 ml.) was extracted ten times with 10 ml. portions of dithizone dissolved in chloroform (5 mg./100 ml.) Dithizone remaining in the buffer was extracted with chloroform and excess chloroform removed by warming and aerating.

RESULTS

Effect of reducing agents on spores of Clostridium bifermentans

Incubation of spores of *Clostridium bifermentans* at 16–20° in the presence of 0.01–0.5% mercaptoacetate for 2–3 days did not activate the spores to any greater degree than did incubation in buffer alone. However, after incubation for 7 days spores suspended in buffer alone had become markedly activated, whereas in the presence of mercaptoacetate little or no activation had occurred (Fig. 1 A, B). At all times heating was fully effective in activating the washed spores (Fig. 1, A', B').

Incubation of spores for 6 hr at 16–20° in the presence of the reducing agent sodium borohydride achieved full activation of the spores, but lithium aluminium hydride was without effect. However, it was found that the addition of sodium borohydride caused an increase of pH value to 9–10.5, depending on the amount added. It seemed possible, in the light of the results of Keynan *et al.* (1964), that either the pH value attained or the reducing conditions were responsible for the activation observed. The effect of pH value was therefore investigated.

Effect of pH value of the suspension on the activation of spores

Since it appeared possible that alkaline pH values could activate spores of *Clostridium bifermentans* and it had been reported that acid pH values activated spores of

Bacillus cereus (Keynan *et al.* 1964), the effect of a range of pH values was examined; the results are summarized in Fig. 2. Marked activation occurred at pH 10.0 and above, an effect not noted by Keynan *et al.* (1964), and also at pH 2.0; but in the range pH 3.0–9.0 the activation was much less marked. It was shown that the high concentrations of Na^+ or Cl^- ions, added when adjusting the pH values, were not responsible for the activation observed. It appeared therefore that the activating effect of NaBH_4 could be entirely accounted for by the alkaline pH value attained on addition of this compound.

Fig. 1. Effect of mercaptoacetate on spores of *Clostridium bifermentans* during storage at 16–20°. Spores were washed twice before testing for germination. A, spores suspended in 0.1 M-phosphate (pH 7.4); B, spores suspended in 0.1 M-phosphate (pH 7.4)+0.01 % mercaptoacetate; A' and B', as A and B but the spores were heat shocked (85° for 10 min.) before testing for germination.

Samples removed at intervals from a spore suspension incubated at pH 10.5 and 37° showed an increasing degree of activation during incubation, rising to complete activation after 30 min.; the rate of activation appeared to be almost linear up to 20 min. (Fig. 3). Little activation occurred in spore suspensions incubated at pH 7.4 and 37° for periods of up to 1 hr., although continued incubation of these spores led to 60–70 % activation in 2 hr.

Annulment of activation ('de-activation') on storage

Although activated spores of aerobic bacteria have been reported to undergo de-activation on storage (Curran & Evans, 1947; Desrosier & Heiligman, 1956; Keynan *et al.* 1964), there appear to be no reports of the phenomenon in activated

spores of anaerobic bacteria. It was therefore decided to investigate whether spores of *Clostridium bifermentans* previously activated by the treatments outlined above, would undergo de-activation on storage.

Spores which were activated by incubation at 37° and pH 7·4 for 2 hr. when stored at 16–20° or at 4° in tightly capped bottles (conditions of minimal aeration) became fully de-activated in 6–7 days. Heating at 85° for 10 min. fully re-activated these spore suspensions at all times during storage.

Fig. 2

Fig. 2. Activation of spores of *Clostridium bifermentans* by treatment at various pH values. Spores were suspended in 0·1 M-phosphate titrated to various pH values with NaOH or HCl, incubated for 2 hr. at 37°, washed twice with 0·1 M-phosphate (pH 7·4) and tested for germination.

Fig. 3

Fig. 3. Activation of spores of *Clostridium bifermentans* at pH 10·5, 37°. A, spores suspended in 0·1 M-phosphate (pH 7·4), 37°, B, spores suspended in 0·1 M-phosphate (pH 10·5), 37°. Samples were removed at intervals, washed twice with 0·1 M-phosphate (pH 7·4) and tested for germination.

Spores heat-activated at pH 7·4 and stored under the same conditions, showed a rather slower rate of de-activation, a loss of 50% of activation being observed over a period of 10–14 days. Re-activation of these spores by heating at 85° for 10 min. did not occur. When heat-activated spores were stored with continuous aeration at 16–20° and pH 7·4, no de-activation was observed over a period of 10–14 days. A second heat treatment of these spores immediately before the addition of germinants, led to a much smaller degree of germination.

Spores activated at pH 2·0 showed a steady decrease in the degree of activation up to 10 days of storage, when approximately 30% of spores remained in the activated state. Heating of samples of these spore suspensions (85° for 10 min.) during the first 48 hr of storage showed a decrease in the degree of germination, but later heated samples showed an increased degree of germination on the addition of germinants as compared with unheated samples. It is possible that spores activated at pH 2·0 become sensitive to heat, but on de-activation regain their heat resistance, and also show a requirement for re-activation.

*Germination of spores of Clostridium bifermentans:
the minimum requirements*

In a previous paper (Gibbs, 1964) it was reported that lactate, L-phenylalanine and L- α -alanine were all essential for the germination of heat-activated spores of *Clostridium bifermentans* suspended in phosphate buffer. During the present work it was found that germination of heat-activated spores occurred only when all three compounds were present simultaneously; addition and removal of these three compounds in all possible sequences did not lead to germination of spores. When similar experiments were made with spores activated at pH 10.5 and 37°, rapid and complete germination of the washed spores occurred in the presence of L- α -alanine+lactate or L- α -alanine+L-phenylalanine when the activation time was 2 hr or longer. All three compounds were required however for the germination of spores activated at pH 3.0 and 37° for 2 hr. Spores activated at pH 10.5 for 2 hr at 37° were therefore able to dispense with either L-phenylalanine or lactate, in the presence of L- α -alanine, for germination. The rates at which spores lost the requirement for lactate or L-phenylalanine during activation at pH 10.8 and 37° are shown in Table 1. It is seen that the requirement for L-phenylalanine was lost steadily during the activation period, but the requirement for lactate was not lost until after at least 60 min. exposure to pH 10.8 at 37°. The presence of L- α -alanine was essential for germination at all times.

Table 1. *Variations in the minimum requirements for germination*

Spore suspensions were incubated at 37° at pH 7.4 or pH 10.8. Samples were removed at intervals, the spores washed with phosphate buffer (pH 7.4) and tested for germination with L- α -alanine+lactate and with L- α -alanine+L-phenylalanine. Extinction values 680 m μ were measured over 60 min. and the decrease in extinction over 60 min. calculated (%), $\left(\frac{E_0 - E_{60}}{E_0} \times 100\right)$.

Activation time at 37° (min.)	L- α -alanine+lactate		L- α -alanine+L-phenylalanine	
	pH 7.4	pH 10.8	pH 7.4	pH 10.8
	Decrease in extinction (E_{60}) over 60 min. (%)			
0	3.2	0	5	0
5	0	0	0	0
10	3.4	7.3	0	0
15	0	10.2	2.0	0
30	0	16.2	3.2	0
45	0	20.0	3.0	0
60	0	37	0	5
120	0	47	0	35

Inorganic ions required for germination. To investigate the requirements for inorganic ions for germination, spores were centrifuged from phosphate buffer suspension, washed five times with distilled water or with tris-HCl buffer, and resuspended in distilled water or tris-HCl. The suspensions were heat-activated (85° for 10 min.) and L- α -alanine, L-phenylalanine and lactate added. Germination of these suspensions was not observed during a period of 2 hr. When 0.1 M-phosphate buffer was added to these suspensions after 2 hr incubation, rapid and complete germination occurred. Further work showed that the degree of germination was proportional to the concentration of phosphate buffer, rising to a maximum at approximately 0.1 M. Heat treatment there-

fore was effective in producing activation of spores in distilled water or in tris-HCl buffer, but some component of the phosphate buffer appeared to be essential for the germination process. Trace metals have been shown to be active in promoting germination (Levinson & Sevag, 1953; Levinson & Hyatt, 1955). For this reason routine phosphate buffer was extracted with a solution of dithizone in chloroform. Spores suspended in dithizone-extracted buffer at first showed a decrease in the degree of germination, but this was traced to residual traces of chloroform in the buffer and when this effect was eliminated, the extracted buffer was found to be as effective as was non-extracted buffer in producing rapid and complete germination. The effect therefore appeared not to reside in the trace elements removed by dithizone but in the major ions present. The routine buffer was prepared from KH_2PO_4 titrated to pH 7.4 with NaOH. Heat-activated spores suspended in a buffer made by substituting KOH for NaOH did not germinate on addition of L- α -alanine + L-phenylalanine + lactate. From this it appeared that it was the sodium ions in the original buffer that were essential for germination. To test this, tris-HCl buffer was prepared in 0.1 M-NaCl. Heat-activated spores suspended in this buffer showed complete and rapid germination on the addition of the three specific germinants. However, when NaCl was replaced by KCl, germination did not occur. It would therefore seem that heat-activated spores of *Clostridium bifermentans* have an obligate requirement for sodium ions for germination.

DISCUSSION

It has long been known that many bacterial spores require some form of treatment before rapid germination in a nutrient medium will occur. This treatment has usually consisted of a short period of heating, so-called heat-shock, which was first described by Evans & Curran (1943) and has since been well-documented (Murrell, 1961). The term in use at present for this process is 'activation', describing an increase in metabolic activity of activated spores (Church & Halvorson, 1957) and also increased sensitivity to inimical reagents and heat (Halvorson & Church, 1957). Vinter (1960, 1961) showed that much of the inert nature of bacterial spores may be attributed to the presence of large numbers of cystine disulphide bonds present in the spore-coat protein, and that these are broken during germination. It was possible that reduction of these bonds would cause a modification of the structure of the spore-coat protein, thus activating spores either by increasing the permeability of the spore-coat to germination agents, or by exposure of the enzymes necessary for germination, or by a combination of these effects. The results obtained here with spores of *Clostridium bifermentans* treated with mercaptoacetate indicated that no activation occurred and indeed a marked inhibition of activation was observed. Such was not apparently the case with spores of *Bacillus cereus* since mercaptoacetate effected activation (Keynan *et al.* 1964). However, mercaptoacetate is not without some effect on spores of *C. bifermentans*, since it has been reported that spores so treated are lysed by hydrogen peroxide or lysozyme (Gould & Hitchins, 1963).

From our experiments on activation during incubation at various pH values, it would appear that activation occurs over a wide range of pH values, but that the rate of activation is greater at the extremes of the range. If activation is due to a reversible denaturation-like process of the spore-coat protein, as suggested by Keynan *et al.* (1964), then it is to be expected that the rate of activation would increase as extremes

of pH value or temperature are approached. However, it appears that activation of the spores of *Clostridium bifermentans* at pH 10.5 is more radical than activation at neutral or acid pH values or by heat-shock, since the germination requirements are then simplified and also de-activation does not occur on storage.

De-activation of heat-activated spores of *Clostridium bifermentans* has been shown to occur most readily in conditions of partial anaerobiosis and not in highly aerated conditions. This suggests that enzymic reactions are necessary for de-activation and not oxidation of sulphhydryl groups since the organism is a strict anaerobe.

Many workers have noted a requirement for inorganic ions for spore germination (e.g. Levinson & Sevag, 1953; Rode & Foster, 1962*a, b*; Fleming & Ordal, 1964) but most of these results have been obtained with spores of species of the genus *Bacillus*. There has apparently been no other report of an obligate requirement for Na⁺ ions for the germination of spores of an obligate anaerobe like *Clostridium bifermentans*.

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