Factors Affecting the Germination of Spores of *Clostridium bifermentans*

By P. A. GIBBS

The Wellcome Research Laboratories (Biological Division), Beckenham, Kent

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SUMMARY

The germination of spores of *Clostridium bifermentans* in media based on acid hydrolysates of casein has been studied. Germination of spores was estimated by observing the decrease in turbidity of a spore suspension and confirmed by observation with the phase contrast microscope and by the increase in permeability to stains. The optimum conditions for germination of spores suspended in phosphate buffer were incubation at 37° within the range pH 6.0–7.8 following heat shock at 85° for 10 min. Anaerobic conditions were not necessary for germination, and addition of mercaptoacetate decreased the rate of germination. The minimum requirements of compounds needed for the germination of spores of *C. bifermentans* was the presence of L- α -alanine, L-phenylalanine and lactate. L-phenylalanine could be replaced by L-leucine but the rate of germination was slower. L- α -Alanine was essential for germination and was not replaced by any other amino acid examined.

INTRODUCTION

Though there is now a considerable volume of published work on the optimum conditions for and specific stimulants of germination of spores of the aerobic genus *Bacillus*, there is comparatively little information of this type about the spores of the anaerobic genus *Clostridium*. Such information as is available appears to be largely confined to those obligate anaerobes which have economic and pathological importance. Much of the work is also restricted in that the major emphasis has been on the destruction of these spores or the prevention of germination. *Clostridium bifermentans*, a non-pathogenic obligate anaerobe, has apparently not been studied with respect to the germination of its spores and this dearth of information directly prompted the present investigation. The work reported here is in two sections: (1) the determination of the optimum conditions for germination; (2) the elucidation of the compounds specifically responsible for breaking of dormancy.

METHODS

Organism. *Clostridium bifermentans* CN 1617 (Wellcome Research Laboratories Culture Collection Catalogue number) was used throughout. This organism has been clearly differentiated from the closely related pathogenic species *C. sordellii* by biochemical (Brooks & Epps, 1958) and immunological methods (Walker, 1963).

Preparation of spore suspensions. The contents of an ampoule of dried culture were suspended in 20 ml. Brewer's thioglycollate (mercaptoacetate) medium (Brewer, 1940) and incubated at 37°. After 18 hr, subcultures (1 ml.) were transferred to

nutrient broth containing 0.25 % glucose, and incubated at 37° under anaerobic conditions for 72 hr. The resulting growth, consisting of 85–90 % phase-bright spores, was harvested by centrifugation and washed with sterile 0.1 M-phosphate buffer (pH 7.4). During the washing procedure the upper white layer of the deposit, consisting largely of cellular debris and vegetative organisms, was suspended in the buffer solution and discarded (Long & Williams, 1958). After washing five times with buffer, the suspension was aerated by vigorous shaking and stored overnight at 4° to allow any remaining vegetative forms to lyse. The spores were then washed five more times with phosphate buffer and the volume of the final suspension adjusted to have an extinction value of 0.35–0.4 at 680 m μ (15 mm. diam. tube; Hilger 810 Biochem. Absorptiometer), corresponding to approximately 10⁷ spores/ml.

Media. A medium (CMB) based on that described by Smith & Douglas (1950) was used and had the following composition: acid-hydrolysed casein, 2 % (w/v; Oxoid, London); KH₂PO₄, 0.5 % (w/v); biotin, nicotinamide, pyridoxal and calcium pantothenate, 10 μ g./ml. each; MgSO₄·7H₂O, 1.6 mg./ml.; MnSO₄·4H₂O, 80 μ g./ml.; FeSO₄·7H₂O, 80 μ g./ml. The medium was adjusted to pH 8.0, boiled, filtered, readjusted to pH 7.4, and sterilized by autoclaving at 121° for 20 min.

Preparation of vitamin-free acid hydrolysed casein. This was prepared in order to study a possible vitamin requirement for the germination of spores. Commercial casein (British Drug Houses Ltd., vitamin- and fat-free) was extracted with methanol and hydrolysed by the method described by Barton-Wright (1961), by using litharge to decrease the concentration of chloride in the final product. The final solution was freeze-dried and solutions of this hydrolysate were prepared as detailed above, with or without the salts of magnesium, manganese and iron.

Yeast extract. A 25 % solution (YE) was prepared by the method of Phillips & Gibbs (1961).

Amino acids. For investigations of the amino acid requirements for germination of spores of *Clostridium bifermentans*, aqueous solutions of amino acids (5 mg./ml.; L. Light and Co.) were pasteurized by heating at 100° for 20 min., and stored at 4° until required for use; 0.5 ml. volumes of amino-acid solutions were added to 5 ml. spore suspension.

Carboxylic acids. For work on the specific stimulants of germination of spores of *Clostridium bifermentans* aqueous solutions of the following carboxylic acids were used: lactic acid (0.5 %, v/v; B.P., C₃H₆O 89.3 %); oxalosuccinic acid Ba salt, DL-isocitric acid lactone, *cis*-aconitic acid anhydride, oxaloacetic acid, α -oxoglutaric acid and L-malic acid (from L. Light and Co.); succinic acid, citric acid (British Drug Houses Ltd., Analar). Aqueous solutions of these acids (5 mg./ml.) were stored at 4°.

Heat shock of spore suspensions. Samples (5 ml.) of spore suspensions in sterile plugged tubes (150 × 15 mm.) were heated in water baths at selected temperatures ($\pm 1^\circ$) for 10 min., after which the tubes were cooled rapidly in chilled water.

Adjustment of pH value of spore suspensions. Amounts of acid or alkali to adjust spore suspension to required pH values were calculated by reference to a titration curve of the 0.1 M-phosphate buffer in which the spores were suspended.

Estimation of degree of germination. The germination of a spore suspension was followed by observing the decrease in extinction at 680 m μ . Samples of CMB

medium (1 ml.) or of YE solution (0.5 ml.) or both were added to 5 ml. of spore suspension, and measurements of extinction made at intervals of 10 min. (Hilger 810 Biochem. Absorptiometer; Powell, 1950). The extent of germination of a spore suspension was also determined by observation of films stained with 1% aqueous methylene blue for 30 min. (Powell, 1950; Levinson & Sevag, 1953) and by phase-contrast microscopy (Pulvertaft & Haynes, 1951). Those spores which were completely stained blue or were completely phase-dark were counted as having germinated.

RESULTS

Smith & Douglas (1950) described the growth requirements of *Clostridium bifermentans* in a medium based on acid-hydrolysed casein. Since these workers used a washed spore suspension for inoculation, the medium they described was apparently sufficient to stimulate germination and to support vegetative growth and was therefore used in the investigations of the optimum conditions for the germination of spores of *C. bifermentans*.

Fig. 1. The effect of temperature of heat shock for 10 min. on the germination of spores of *Clostridium bifermentans* in CMB medium + yeast extract.

Effect of glucose, tryptophan, cystine, thioglycollate and yeast extract on germination. Though glucose, tryptophan, cystine and thioglycollate are essential for vegetative growth, they were not required for germination in medium based on acid-hydrolysates of casein. Indeed the presence of thioglycollate, in concentrations between 0.005 and 0.1% (v/v), markedly decreased the rate of germination as compared with that observed in the absence of thioglycollate. However, outgrowth and formation of a vegetative culture of *Clostridium bifermentans* occurred only when thioglycollate was present. The addition of yeast extract to acid-hydrolysates of casein increased the rate of spore germination.

Effect of heat shock on spore germination. Maximum germination in CMB medium + yeast extract at 37° was attained following a heat shock for 10 min. at 80–85° (Fig. 1). Little germination of a spore suspension occurred when heated at 70° or below, and temperatures of 90° and above prevented subsequent germination. Spores heated at 95° for 10 min. did not lose phase-brightness but did take up

methylene blue in the spore coats to a limited extent. Viable counts in agar media showed that 95° for 10 min. was lethal for 99.99 % of the spores. In all subsequent work, spores were heated at 85° for 10 min., these conditions of heat shock being optimal for subsequent germination.

Effect of pH value on germination. There appeared to be no sharp optimal pH value for germination of heat shocked spores in CMB medium + yeast extract incubated at 37°. Germination occurred within the range pH 6.0–7.8 with little difference in rate. At pH 5.0 and pH 8.5 no germination occurred. Incubation at pH 10.0 or pH 3.0 had little effect on the ability of spores to germinate on subsequent re-adjustment to pH 7.4.

Fig. 2. The effect of temperature of incubation on the germination of suspensions of spores of *Clostridium bifermentans*. a, 25°; b, 30°; c, 37°; d, 45°. A, ○—○, + CMB medium; B, △—△, + yeast-extract; C, ●—●, + CMB + yeast extract. % decrease in opacity = $(E_0 - E_x/E_0) \times 100$ where E_0 and E_x are extinctions immediately after the addition of germinating agents and at time x respectively.

Effect of temperature of incubation. The germination of spores of *Clostridium bifermentans* in CMB and yeast extract both separately and as a mixture was investigated between 12 and 45°. At temperatures below 20°, little or no germination

occurred with 60 min. The maximum rate of germination at each temperature was attained in CMB medium + yeast extract (Fig. 2). At 45° germination was markedly decreased, particularly in CMB medium and was absent in yeast extract. The optimum temperature of incubation was 37° and all subsequent work was therefore carried out at this temperature.

Investigation of the compounds specifically responsible for the germination of spores of Clostridium bifermentans

Effect of vitamin- and fat-free casein hydrolysate. Medium prepared with vitamin- and fat-free casein hydrolysate did not stimulate the germination of spores, whereas commercial casein hydrolysate did. Addition of yeast extract to the vitamin-free casein hydrolysate stimulated germination to a degree comparable with that obtained with CMB medium + yeast extract. The addition of the four vitamins biotin, nicotinamide, pyridoxal and pantothenate, salts of manganese, magnesium and iron to the vitamin-free hydrolysate had no effect. Amino acid analysis (Beckman-Spinco, Model 120 Amino Acid Analyzer) of the commercial and vitamin-free casein hydrolysates revealed no major differences in amino acid composition and addition of the commonly occurring L-amino acids to the vitamin-free casein hydrolysate did not stimulate germination in the absence of yeast extract. It seemed likely therefore that amino acids alone could not stimulate the germination of spores of *Clostridium bifermentans*, and that an unknown factor present in yeast extract was required.

Amino acid requirement for germination in the presence of yeast extract. Twenty-seven L-amino acids were examined for their effect on germination in the presence of yeast extract. Of these L-phenylalanine, L-leucine and L-cystine markedly increased the rate of germination; glycine, L-valine, L-methionine and L-cysteine inhibited germination; the other amino acids tested were without effect (Fig. 3).

Replacement of yeast extract in the presence of vitamin-free casein hydrolysate. Since nucleosides have been implicated in the germination process of spores of *Bacillus* spp. (see Halvorson & Church, 1957), the effect of some purine and pyrimidine bases and ribosides as replacements for yeast extract was examined. The addition of thymine, uracil, uric acid, guanine, adenine, xanthine, hypoxanthine, adenosine and guanosine did not stimulate spore germination in vitamin-free casein hydrolysate. Ultraviolet absorption spectra of solutions of commercial and vitamin-free casein hydrolysates showed no significant differences at 260 m μ , the region in which many purines and pyrimidines and their derivatives are strongly absorbing.

The other group of compounds of metabolic importance investigated included glucose and some of its degradation products, namely the carboxylic acids pyruvic, lactic, acetic, citric, *cis*-aconitic, isocitric, oxaloacetic, malic, succinic, α -oxoglutaric and oxalosuccinic acids. In the presence of commercial casein hydrolysate, lactate, pyruvate, malate, succinate and oxaloacetate stimulated germination, but in vitamin-free casein hydrolysate only lactate had a marked effect on germination, and pyruvate and oxaloacetate a slight effect; succinate and malate were inactive. In a mixture of amino acids which simulated vitamin-free casein hydrolysate, lactate stimulated rapid germination, but pyruvate and oxaloacetate were very much less active.

The specific stimulants of germination of spores of Clostridium bifermentans

Since germination of spores was obtained in a mixture of amino acids + lactate, an examination of those specific amino acids which were needed for the breaking of dormancy in the presence of lactate was made. A mixture of L-phenylalanine, L-leucine and L-cystine in the presence of lactate did not stimulate germination, but on addition of L- α -alanine to this mixture, rapid germination occurred; L- α -alanine was not replaced by any other amino acid tested. Further investigations showed that L-phenylalanine + L- α -alanine + lactate constituted the specific requirements for germination and that L-phenylalanine could be replaced by L-leucine, although the rate of germination was then decreased (Fig. 4).

Fig. 3

Fig. 3. The effect of amino acids on the germination of suspensions of spores of *Clostridium bifermentans* in yeast extract (YE) solution. a, $\bigcirc$ — $\bigcirc$, +YE only; b, $\triangle$ — $\triangle$, +YE + L-phenylalanine (50 $\mu\text{g./ml.}$); c, ∇ — ∇ , +YE + L-leucine (50 $\mu\text{g./ml.}$); d, $\blacktriangle$ — $\blacktriangle$, +YE + L-cystine (50 $\mu\text{g./ml.}$); e, $\blacktriangledown$ — $\blacktriangledown$, +YE + L-methionine (50 $\mu\text{g./ml.}$); f, $\bullet$ — $\bullet$, +YE + glycine, or L-valine or L-cysteine (50 $\mu\text{g./ml.}$).

Fig. 4

Fig. 4. The minimum requirements for the germination of suspensions of spores of *Clostridium bifermentans*. a, $\bigcirc$ — $\bigcirc$, +CMB medium + yeast extract; b, ∇ — ∇ , +CMB medium; c, $\triangle$ — $\triangle$, +yeast extract; d, $\blacktriangle$ — $\blacktriangle$, +L-phenylalanine (50 $\mu\text{g./ml.}$) + L- α -alanine (50 $\mu\text{g./ml.}$) + lactic acid (0.5 $\mu\text{l./ml.}$); e, $\blacktriangledown$ — $\blacktriangledown$, +L-leucine (50 $\mu\text{g./ml.}$) + L- α -alanine (50 $\mu\text{g./ml.}$) + lactic acid (0.5 $\mu\text{l./ml.}$).

DISCUSSION

Although the heat resistance of spores is usually regarded as the feature that most clearly differentiates them from the vegetative forms, spores may be clearly recognized by other properties. Thus, the heat sensitivity of spores of some aerobic and anaerobic bacilli is paralleled by loss of phase-brightness, increased permeability to stains and decrease in turbidity of spore suspensions, so making it possible to use the latter group of properties as criteria of germination. The criteria of germination used in the present work were the microscopic appearance and change in turbidity of suspensions of spores. In common with spores of the aerobic bacilli (Powell & Hunter, 1955) spores of *Clostridium bifermentans* were found to require activation by heat shock before rapid and complete germination ensued. This observation appears to distinguish the spores of *C. bifermentans* from those of *C. roseum* studied by Hitzman, Halvorson & Ukita (1957).

Although strictly anaerobic conditions are required for the vegetative growth of *Clostridium bifermentans*, these conditions are not essential for the germination of its spores. Indeed the addition of thioglycollate markedly inhibited germination as previously reported for spores of *C. botulinum* (Treadwell, Jann & Salle, 1958). The germination of spores of other species of the genus *Clostridium* is apparently not inhibited by thioglycollate, and anaerobic conditions are apparently required for germination to occur (Wynne, Mehl & Schmieding, 1954; Hitzman *et al.* 1957). By making experiments under aerobic conditions therefore, it was possible in the present work to differentiate between germination and subsequent outgrowth since the latter (see above) will only take place under anaerobic conditions. Thus it was possible to observe separately the three major phases in the transition from dormant spores to vegetative forms, namely activation, germination and outgrowth.

The compounds found specifically responsible for stimulating the germination of spores of *Clostridium bifermentans* included L- α -alanine and L-phenylalanine, compounds also required by spores of *C. roseum* (Hitzman *et al.* 1957). L- α -Alanine is also required by the spores of many aerobic bacilli (Thorley & Wolf, 1960), and it is possible that L- α -alanine is essential for the germination of spores of aerobic and anaerobic bacilli. However, lactic acid has apparently not been reported previously to be required for the germination of spores, although some dicarboxylic acids have been reported to stimulate the germination of spores of *C. botulinum* (Wynne & Foster, 1948).

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