Changes in Spores of *Clostridium bifermentans* Caused by Treatment with Hydrogen Peroxide and Cations

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SUMMARY

Spores of *Clostridium bifermentans* were treated with hydrogen peroxide until their peripheries had lost refractility. The centres of such spores only retained refractility at acid pH. Adding monovalent cations or increasing the pH caused the treated spores to lose their remaining refractility and decreased the turbidity of spore suspensions. Divalent cations prevented or reversed this loss of central refractility and decreased the fall in turbidity. Calcium ions also prevented but did not reverse the loss of central refractility which occurred on drying or applying pressure. Electron micrographs of spores treated with hydrogen peroxide showed that the cortex was depleted or absent and that the loss of central refractility was accompanied by protoplast swelling. It is suggested that divalent cations make spores resistant to drying and pressure by cross-linking negatively charged groups within the protoplast, and that together with hydrogen ions they neutralize the negatively charged groups, thus preventing the swelling of the protoplast, loss of refractility and fall in extinction which occur when divalent cations are replaced by monovalent cations.

INTRODUCTION

Warth, Ohye & Murrell (1963*a, b*) suggested that divalent cations, such as calcium, help to maintain the structure of a bacterial endospore by cross-linking carboxyl groups in the spore cortex, and so give it the contractile properties proposed by Lewis, Snell & Burr (1960). Calcium ions may also stabilize the membranes of spore protoplasts (Fitz-James, 1971; Ellar, Eaton & Posgate, 1974). Monovalent cations such as sodium or potassium are generally required for the germination of spores, and an increase in the level of free ions within spores may be one of the first events in the initiation of germination (Gould & Dring, 1972). During germination, potassium ions are taken up by spores of *Bacillus subtilis* (Dring & Gould, 1971) and calcium ions are lost (Powell & Strange, 1953) but there is no direct evidence to indicate the site of these changes or their role in germination. During a study of the effect of hydrogen peroxide on spores of *Clostridium bifermentans* we noticed that the addition of sodium phosphate buffer pH 8.0 to peroxide-treated spores resulted in a loss of spore refractility and a decrease in turbidity of the spore suspension. Such changes usually occur during the germination of untreated spores, which requires organic media in addition to sodium ions. We have therefore studied the effect of changes in sodium and hydrogen ion concentrations on spores which have been treated with hydrogen peroxide.

Fig. 1. Effect of hydrogen peroxide on the refractility of spores of *C. bifermentans*. Phase-contrast photographs show (a) untreated, dormant spores; (b) spores after treatment with H_2O_2 and fixing in 25 % (v/v) glutaraldehyde to prevent loss of refractility during drying; (c) peroxide-treated spores after addition of 240 mM-NaCl.

METHODS

Organisms, spore preparation and maintenance of culture. The strain of *Clostridium bifermentans* used and the preparation and storage of spores have been described previously (Waites & Wyatt, 1971). The organism was maintained in the reinforced clostridial medium of Hirsch & Grinstead (1954).

Spore dry weight. Dry weights were measured spectrophotometrically using a calibration curve relating E_{600} to spore dry weight.

Treatment of spores with hydrogen peroxide. Spores (15 mg dry wt) were incubated in 1.0 ml of hydrogen peroxide solution (8.5 M) at 20 to 24 °C for 9 h, until the outer regions of the spores had lost refractility and the centres had become dull but still refractile when examined with a phase-contrast microscope (Fig. 1). Suspensions in which 40 % or more of the spores failed to lose this central refractility within 10 min of transferring into 50 mM-NaCl were again treated with H_2O_2 for 2 to 3 h. Treated spores were washed four times by centrifuging at 4 °C in glass-distilled water and adjusted to pH 3.8 with 0.1 M-HCl, before resuspension and storage in 2 ml acidified glass-distilled water in polypropylene centrifuge tubes. Such spores retained central refractility for at least 8 weeks when stored at 0 °C, but about 40 % of spores stored in glass-distilled water at pH 6.5 in glass vessels or at a concentration of less than 7 mg dry wt/ml lost central refractility in 8 weeks.

Measurement of loss of refractility and decrease in turbidity. Spore suspensions, at a final concentration equivalent to about 40 µg dry wt/ml, were incubated at 37 °C in 2.5 ml glass-distilled water acidified to pH 3.8 in a cuvette of 1 cm light path. Appropriate reagents, in a volume of 0.3 ml, were added without shaking and the decrease in turbidity at 600 nm recorded spectrophotometrically. Loss of the central refractility was measured under similar conditions by counting 100 spores using a phase-contrast microscope, and scoring semi-refractile (i.e. refractile in the centre but with a non-refractile periphery) or non-refractile spores. Spores which remained wholly refractile after the peroxide treatment (less than 10 % of the population) were scored as semi-refractile. The standard deviation between

duplicate counts was less than 5 % and in separate experiments with the same spore preparation was less than 10 %. After each incubation the pH value was measured with a glass electrode.

Preparation of samples for microscopy. For phase-contrast photographs spore suspensions were dried on to coverslips which were placed on slides coated with agar. Spores treated with hydrogen peroxide were fixed with 25 % glutaraldehyde to prevent any loss of their remaining refractility during drying. For electron microscopy spore suspensions were fixed and embedded as described previously (Waites, Wyatt & Arthur, 1972).

Measurement of germination rate. Germination of untreated spores was followed spectrophotometrically at 37 °C by measuring the decrease in E_{600} as described by Waites & Wyatt (1971). Germination media were (mM): the 'Ala +' system, containing L-alanine (50), L-arginine (5), L-phenylalanine (5), L-lactate (25), NaCl (100) and potassium phosphate (83), pH 7.5; or the 'Gly +' system which was the 'Ala +' system with potassium phosphate (83) at pH 6.25 and with glycine (25) replacing L-alanine. The potassium phosphate buffer was KH_2PO_4 , adjusted to pH 7.5 with potassium hydroxide, and adjusted to a final concentration of 83 mM-potassium in the medium.

Polyacrylamide gel electrophoresis. Hydrogen peroxide extracts were dialysed against glass-distilled water and then concentrated by vacuum dialysis. Extracts containing 100 µg protein in 0.2 ml were added to polyacrylamide gels (7.5 %, pH 8.5) and electrophoresis carried out as described previously (Wyatt & Waites, 1971).

Measurement of dipicolinic acid, calcium and glucosamine. Dipicolinic acid was measured by the method of Janssen, Lund & Anderson (1958) using a solution of dipicolinic acid (Koch-Light) as a standard. Calcium was extracted by the wet ashing procedure of Slepecky & Foster (1959) and estimated in the presence of lanthanum chloride by atomic absorption spectrophotometry at 422 nm with a Unicam SP 900A as described by Linehan (1966) and Haworth & Cleaver (1961). Glucosamine was measured by the method of Rondle & Morgan (1955), after hydrolysis in 6 M-HCl at 100 °C for 5 h (Vary, 1973), using D(+)-glucosamine hydrochloride (Sigma) as a standard.

RESULTS

Effect of cations and pH on the refractility and turbidity of spores after treatment with hydrogen peroxide

Spores treated with H_2O_2 were incubated at 37 °C and the effect of a number of additions on the turbidity and refractility determined as described in Methods. Addition of 0.3 ml water adjusted to pH 3.8 with HCl did not result in the appearance of any completely non-refractile spores and the 10 % immediate decrease in turbidity due to dilution, did not increase further by 20 min (Fig. 2). Addition of 2 M-NaCl or 2 M-tris-HCl buffer pH 8.0, to give a final concentration of 100 mM, increased the pH to 4.8 or 7.8 respectively and resulted in a rapid loss of refractility from the centre of the spores (Fig. 1). Loss of central refractility occurred in more spores after the addition of NaCl than after the addition of the same concentration of tris-HCl; in contrast, the fall in turbidity was always greater after the addition of tris-HCl than after the addition of NaCl (Fig. 2). Addition of sodium phosphate buffer pH 8.0, to a final concentration of 100 mM in both sodium and phosphate, resulted in a rapid fall in turbidity and loss of refractility. The presence of 0.5 M-sucrose, which prevents lysis of spheroplasts of *Clostridium butyricum* (Kawata & Inoue, 1963), failed to prevent either change, suggesting that they were not produced by lysis of the protoplast. It appeared that the fall in turbidity was produced by the pH change, while the rapid loss of

Fig. 2. Effect of NaCl or tris-HCl buffer on refractility and turbidity of spores treated with H₂O₂. Peroxide-treated spores were incubated at 37 °C as described in Methods, NaCl (○, ●) or tris-HCl buffer pH 8.0 (□, ■) was added and (a) the decrease in turbidity (closed symbols) and (b) the loss of central refractility (open symbols) were detected by spectrophotometry and phase-contrast microscopy respectively.

Table 1. *Stimulation of germination of spores of C. bifermentans by monovalent cations*

Spore suspensions were incubated at 37 °C with the 'Ala + ' or 'Gly + ' systems and 100 mM of sodium, potassium, lithium or ammonium chloride either with or without potassium phosphate buffer, as described in Methods. Germination was measured spectrophotometrically and the germination rate is calculated from the maximum rate of decrease in E_{600} (in min⁻¹), expressed as a percentage of the initial E_{600} value.

Added chloride	Germination rate (% min ⁻¹)			
	'Ala + '		'Gly + '	
	+ Buffer	- Buffer	+ Buffer	- Buffer
None	23	4.2	6.6	< 0.2
Sodium chloride	77	86	22	30
Potassium chloride	55	46	14	1.6
Lithium chloride	44	43	9.2	1.7
Ammonium chloride	44	21	7.5	< 0.2

refractility was initiated by sodium ions. However, other cations could replace sodium as 5 min incubation with sodium, potassium, lithium or ammonium chloride at 24 mM (the concentration at which the greatest differences between the different cations occurred) produced loss of refractility in 59, 36, 30 or 11 % of spores respectively.

Germination of untreated spores of *C. bifermentans* requires sodium ions, although with some germination media potassium ions can substitute with a reduced efficiency (Waites & Wyatt, 1971). The germination rates of untreated spores with sodium, potassium, lithium or ammonium chloride showed a similar descending order (Table 1) to the effects obtained with treated spores.

Hitchins & Gould (1964) isolated refractile 'cores' from spores of *Bacillus subtilis*; these 'cores' were stable at acid pH but lost refractility if the pH was increased to 7.0 unless calcium chloride was present. The presence of CaCl₂ markedly reduced the percentage of peroxide-treated spores which lost central refractility (Fig. 3), and at 90 mM also reduced the fall in turbidity from 55 to 35 % after the addition of 100 mM-tris-HCl. Calcium, barium

Fig. 3. Effect of calcium chloride on the loss of refractility initiated with NaCl in spores treated with H_2O_2 . Spores treated with H_2O_2 were incubated at $37^\circ C$ with the concentrations of $CaCl_2$ described, NaCl (186 mM) was added, and the loss of central refractility was determined by phase-contrast microscopy.

or magnesium chloride (50 mM) reduced the percentage of peroxide-treated spores which lost central refractility after the addition of 200 mM-NaCl from 58 % to 5, 18 or 46 % respectively. Addition of $CaCl_2$ to peroxide-treated spores which had lost central refractility in the presence of NaCl caused about half to regain refractility, but spores which had lost refractility on drying or applying thumb pressure to the coverslip were unable to regain refractility. The presence of $CaCl_2$ during drying or applying pressure prevented loss of refractility.

Structure of spores after treatment with hydrogen peroxide

In electron micrographs of thin sections of peroxide-treated spores the spore cortex was depleted or in some preparations removed completely (Fig. 4*a, b*). In addition, the electron density of the coat was decreased and the ribosomes in the protoplast appeared disordered compared with untreated spores. Addition of sodium and then calcium ions to peroxide-treated spores resulted in swelling (Fig. 4*c*) and then contraction of the protoplast (Fig. 4*d*).

Polyacrylamide gel electrophoresis of the supernatant from spores treated with H_2O_2 showed two bands, one of which had the same electrophoretic mobility as that removed from spore coats by NaOH (Wyatt & Wailes, 1971), while the other, more diffuse, band was less mobile. Calcium and dipicolinic acid were less than 0.1 % of the dry weight of spores treated with H_2O_2 , but were about 1.2 and 5 % respectively of the dry weight of untreated dormant spores. Glucosamine was reduced from 2.7 % to between 1.3 and 0.9 % of the dry weight by different treatments with H_2O_2 .

DISCUSSION

In this study a series of changes, collectively resembling germination, have been produced in three stages in spores of *C. bifermentans*. The first stage, loss of peripheral refractility, was the result of incubation with H_2O_2 . The second and third stages, loss of refractility in the remainder of the spore and a decrease in turbidity, were initiated by increasing the pH from 3.8 to 7.8 in the presence of sodium ions. These two stages could be partially separated by the addition of sodium ions at pH 4.8 or by increasing the pH to 7.8 with tris-HCl buffer. Other monovalent cations were less effective than sodium and were also less effective in stimulating the germination rate of untreated spores. Hitchins & Gould (1964) isolated 'cores' from spores of *B. subtilis* by shaking in a Mickle disintegrator below pH 4.2. Such 'cores', which consisted of the spore protoplast and part of the cortex, lost refractility when the pH was raised to 7.0, but no decrease in turbidity or requirement for monovalent cations

Fig. 4. Effect of sodium and calcium chloride on the ultrastructure of spores treated with H₂O₂. Electron micrographs show: thin sections of spores (a) untreated and (b) after treatment with H₂O₂; (c) treated spore after the addition of 240 mM-NaCl; (d) treated spore after the addition of 240 mM-NaCl followed by 80 mM-CaCl₂. C, cortex; P, protoplast.

was reported. Neither the 'cores' nor peroxide-treated spores were able to form colonies on agar growth media. In spores, glucosamine is present only in the cortex (Murrell, 1969). Different peroxide treatments removed between 50 and 70 % of the glucosamine from spore populations, and examination of electron micrographs of thin sections of peroxide-treated spores showed that some appeared to have lost all their cortex. If the cortex was completely removed, a contractile cortex as suggested by Lewis *et al.* (1960) cannot be responsible for the stability of the spore protoplast at acid pH. Moreover, Pearce & Fitz-James (1971) studied a mutant of *Bacillus cereus* whose spores had no cortex but showed central refractility only.

Calcium ions reduced the loss of refractility and fall in turbidity of peroxide-treated spores and also prevented loss of refractility of spore 'cores' (Hitchins & Gould, 1964) but did not inhibit germination of untreated spores or cause untreated germinated spores to regain refractility except at higher concentrations (Vinter, Štastná & Čáslavská, 1969; Sacks, 1972; Hashimoto, Frieben & Conti, 1969). Other divalent cations also reduced the loss of refractility from peroxide-treated spores. Calcium ions prevented the irreversible loss of refractility produced by drying or pressure and caused contraction of protoplasts swollen after addition of NaCl. Hydrogen ions or cations inhibit the lysis of protoplasts made from vegetative bacterial cells (Edebo, 1961; Grula & Hartsell, 1957) and lysis of growing clostridia by monovalent ions has been reported (Ogata & Hongo, 1973), although in our experiments protoplast swelling, not lysis, occurred. Calcium accounts for about 2 % of the dry weight of dormant spores (Murrell, 1969) and it has been suggested that calcium ions may stabilize the cortical peptidoglycan (Warth *et al.*, 1963*a, b*) or the protoplast membrane of dormant spores (Fitz-James, 1971; Ellar *et al.*, 1974). Spores treated with H₂O₂ lost nearly all their calcium and were unstable at neutral pH in the absence of added calcium or other divalent ions, suggesting that such ions help to stabilize dormant spores. The cortex appeared to be absent in electron micrographs of some peroxide-treated spores, and in such spores the added divalent ions could not act by stabilizing cortical material but might affect the protoplast membrane or the protoplast itself. As suggested by Hitchins & Gould (1964), negatively charged groups in the dormant spore might be neutralized by hydrogen ions and neutralized and cross-linked by calcium ions. Removal of calcium ions would allow the spore to remain stable only at high concentrations of hydrogen ions. At higher pH there would be insufficient hydrogen ions to neutralize the negative charges and this would result in swelling of the spore protoplast, loss of refractility and spore material and a fall in turbidity. Addition of sufficient sodium ions to replace any remaining divalent ions would result in loss of cross-linkages and produce similar changes at pH values at which the negative groups are neutralized. The continued neutralization of negative groups might result in less severe changes in the spore so that a smaller loss of spore material and hence a smaller fall in turbidity would result. Calcium ions bound to membranes may be displaced by sodium or potassium ions (Rojas & Tobias, 1965) and, for example, Ehrström *et al.* (1973) have shown that calcium ions confer rigidity on membranes isolated from vegetative cells of *B. subtilis* while monovalent ions like sodium have a negligible ordering effect and at high concentrations (0.1 to 1.0 M) cause disorganization. Joos & Carr (1967) have also shown that in the physiological pH range, membranes containing phosphatidylserine, triphosphoinositide and phosphatidylethanolamine possess many calcium binding groups and can show large variations in bound ions with comparatively small pH changes, while Papahadjopoulos & Ohki (1969) found that phospholipid membranes with calcium ions present on one side are stable only if the other side is lowered to pH 3.0.

During germination of untreated spores monovalent ions, like sodium, may act by

replacing calcium ions at sensitive sites within the spore. Loss of divalent linkages and removal of the calcium ions by dipicolinic acid, as suggested by Gould & Dring (1972), may then initiate germination.

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