The Effect of Chlorine and Heat on Spores of *Clostridium bifermentans*

By W. M. WAITES, N. R. KING AND CATHERINE E. BAYLISS

Agricultural Research Council, Food Research Institute, Colney Lane, Norwich NR4 7UA

(Received 5 May 1977)

INTRODUCTION

Waites *et al.* (1976) showed that spores of *Clostridium bifermentans*, which had lost peripheral phase brightness after being killed by incubation with high concentrations of hydrogen peroxide, had also lost most of their cortex, calcium and dipicolinic acid (DPA). Addition of monovalent cations to such spores resulted in a fall in turbidity, loss of the remaining phase brightness and swelling of the protoplasts although these changes could be prevented by the presence of calcium ions. Since monovalent cations are required for the germination of untreated spores and since the loss of calcium, DPA and cortex together with protoplast swelling also occur during germination (for a review see Gould, 1969), we suggested that replacing the calcium within the spore by monovalent cations could be one of the initiating events in germination. It is possible, however, that the changes in spores treated with peroxide might be specific to the action of the peroxide. We have therefore investigated whether other agents including chlorine and heat can produce spores which undergo similar changes. This communication reports the effect of these agents in combination.

METHODS

Organism, spore preparation and maintenance of cultures. The strain of *Clostridium bifermentans* used and the preparation and storage of spores were as described previously (Waites & Wyatt, 1971) except that spores were produced on a trypticase agar as described by Bayliss & Waites (1976). The organism was maintained in the reinforced clostridial medium of Hirsch & Grinsted (1954).

Treatment of spores with chlorine. Spores (about 0.7 mg dry wt ml⁻¹) were added to 250 mM-sodium phosphate buffer pH 7.0 containing sodium hypochlorite (BDH) to give 200 µg free chlorine ml⁻¹, incubated for 10 min at 0 °C, and then washed with glass-distilled water as described previously (Wyatt & Waites, 1973). Free chlorine was measured by the Palin method (Palin, 1957) with *N,N*-diethyl-*p*-phenylene diamine (DPD) tablets (BDH) and using the extinction coefficient at 553 nm.

Heat treatment of spores. Spores treated with 200 µg free chlorine ml⁻¹ were heated at 80 °C for 0, 10, 20, 30, 45, 60 or 90 min at about 7 mg dry wt ml⁻¹ in glass-distilled water in screw-capped glass bottles before centrifuging, resuspending in glass-distilled water and storing at 1 °C.

Measurement of dipicolinic acid. This was measured in the supernatants from heated spores both spectrophotometrically at 271 nm and colorimetrically by the method of Janssen, Lund & Anderson (1958) using a solution of DPA (Koch-Light) as a standard. The same results were obtained by either method.

Spore dry weight. Dry weights were estimated spectrophotometrically; E_{600} values were converted to spore dry weight using a standard calibration curve.

Measurement of decrease in turbidity and loss of phase brightness. Spore suspensions (about 40 µg dry wt ml⁻¹) were incubated at 37 °C in 2.5 ml glass-distilled water in a cuvette of 1 cm light path. Sodium chloride, sodium phosphate buffer pH 8.0 or Tris/HCl buffer pH 8.0 (0.3 ml) were added to a final concentration of 100 mM and the decrease in turbidity at 600 nm was recorded spectrophotometrically. Loss of central or peripheral brightness was measured under similar conditions by counting 100 spores using a phase contrast microscope.

Preparation of samples for electron microscopy. For electron microscopy, spore suspensions were fixed and embedded as described previously (Waites, Wyatt & Arthur, 1972).

Fig. 1. Effect of chlorine and heat on the ultrastructure of spores of *Clostridium bifermentans*. Electron micrographs show thin sections of spores (a) treated with 200 µg free chlorine ml⁻¹, (b) treated spore heated at 80 °C for 30 min. C, cortex; P, protoplast. Bar markers represent 0.25 µm.

RESULTS

Spores of *Clostridium bifermentans* killed by 200 µg free chlorine ml⁻¹ appeared bright on examination by phase contrast microscopy, and similar to untreated spores on examination by electron microscopy (Fig. 1a). Addition of 100 mM-sodium phosphate or Tris/HCl buffers at pH 8.0 or 100 mM-sodium chloride resulted in less than a 10 % fall in the turbidity of the spore suspension. On heating for 10 min or longer at 80 °C, at least 80 % of these spores lost phase brightness from their periphery and appeared to be identical to those treated with high concentrations of hydrogen peroxide (Waites *et al.*, 1976). When chlorine-treated spores were heated for 10 min, about 50 % of the DPA of the spore was lost into the supernatant; in most experiments little extra was lost on further heating, the maximum observed being a further 10 % after another 50 min. Electron microscope examination of thin sections of spores that had been heated for 30 min showed that the majority had lost much of their cortex and had swollen protoplasts (Fig. 1b). On adding 100 mM-sodium phosphate or Tris/HCl buffers at pH 8.0 or 100 mM-sodium chloride, the heated spores lost their remaining phase brightness and also 80, 63 or 56 % of their initial turbidity, respectively, within 10 min. As with peroxide-treated spores (Waites *et al.*, 1976), the presence of 5 mM-CaCl₂ prevented the heated spores losing central phase brightness and also turbidity on addition of Tris/HCl buffer.

DISCUSSION

We have shown that treatment of spores of *Clostridium bifermentans* with chlorine and heat produce similar changes to those produced by peroxide. However, although the spores treated with peroxide had lost all their cortex, the protoplasts remained much more contracted than those in the present study, probably because of the low pH of the treatment with peroxide (Hitchins & Gould, 1964).

There are obvious similarities between heat activation of spores and the changes produced in the present work by heating chlorine-treated spores. After heat activation, spores germinate more rapidly and with a greater number of germinants (Keynan & Evenchik, 1969) and during heat activation up to 10 % of the spore DPA may be lost (Keynan, Murrell & Halvorson, 1961). We found that DPA is also lost on heating. The ability to undergo germination-like changes on adding sodium chloride alone could be considered an

extension of the relaxation of germination requirements since amino acids and sodium ions are required for germination of untreated spores of this strain (Waites & Wyatt, 1971). Spores damaged by chlorine lose heat resistance (Dye & Mead, 1972; Wyatt & Waites, 1975) and, on mild heating, lose spore constituents such as DPA (Alderton & Halbrook, 1971; Dye & Mead, 1972). However, structural changes accompanying the loss of these constituents and the subsequent effects of cations were not examined, although treatment of spores of *Bacillus cereus* with higher concentrations of chlorine degrades the spore coat and causes eventual loss of calcium ions and DPA (Kulikovskiy, Pankratz & Sadoff, 1975). Since there is no evidence that heating at 80 °C degrades the cortex of untreated spores, the chlorine treatment in our study must alter the cortex and make it susceptible to such heating.

Although the heated spores in our study retained much of their phase brightness, they had lost most of their cortex and the protoplast had expanded. Under phase contrast their appearance was similar to that of the heat-inactivated germinated spores of Hashimoto, Frieben & Conti (1972), but whether such spores had also lost cortical material and had expanded protoplasts is unknown. The remaining brightness under phase contrast is presumably due to the physical characteristics of the partially swollen protoplast. As DPA was lost during expansion of the protoplast, it would be of interest to determine if any protoplast swelling occurs when DPA is lost during activation of untreated spores.

We are grateful to Dr J. L. Peel for useful discussions and Miss L. J. Nockolds for skilled technical assistance.

REFERENCES

- ALDERTON, G. & HALBROOK, W. U. (1971). Action of chlorine on bacterial spores. *Bacteriological Proceedings*, p. 12.
- BAYLISS, C. E. & WAITES, W. M. (1976). The effect of hydrogen peroxide on spores of *Clostridium bifermentans*. *Journal of General Microbiology* **96**, 401–407.
- DYE, M. & MEAD, G. C. (1972). The effect of chlorine on the viability of clostridial spores. *Journal of Food Technology* **7**, 173–181.
- GOULD, G. W. (1969). Germination. In *The Bacterial Spore*. Edited by G. W. Gould and A. Hurst. London: Academic Press.
- HASHIMOTO, T., FRIEBEN, W. R. & CONTI, S. F. (1972). Kinetics of germination of heat-injured *Bacillus cereus* spores. In *Spores V*, pp. 409–415. Edited by H. O. Halvorson, R. Hanson and L. L. Campbell. Washington, D. C.: American Society for Microbiology.
- HIRSCH, A. & GRINSTED, E. (1954). Methods for the growth and enumeration of anaerobic spore-formers from cheese, with observations on the effect of nisin. *Journal of Dairy Research* **21**, 101–110.
- HITCHINS, A. D. & GOULD, G. W. (1964). Release of cores from bacterial spores by mechanical breakage in acidic media. *Nature, London* **203**, 895–896.
- JANSSEN, F. W., LUND, A. J. & ANDERSON, L. E. (1958). Colorimetric assay for dipicolinic acid in bacterial spores. *Science*, **127**, 26–27.
- KEYNAN, A. & EVENCHIK, Z. (1969). Activation. In *The Bacterial Spore*. Edited by G. W. Gould and A. Hurst. London: Academic Press.
- KEYNAN, A., MURRELL, W. G. & HALVORSON, H. O. (1961). Dipicolinic acid content, heat-activation and the concept of dormancy in the bacterial endospore. *Nature, London* **192**, 1211–1212.
- KULIKOVSKY, A., PANKRATZ, H. S. & SADOFF, H. L. (1975). Ultrastructural and chemical changes in spores of *Bacillus cereus* after action of disinfectants. *Journal of Applied Bacteriology* **38**, 39–46.
- PALIN, A. T. (1957). The determination of free and combined chlorine in water by the use of diethyl-*p*-phenylene diamine. *Journal of the American Water Works Association* **49**, 873–880.
- WAITES, W. M. & WYATT, L. R. (1971). Germination of spores of *Clostridium bifermentans* by certain amino acids, lactate and pyruvate in the presence of sodium or potassium ions. *Journal of General Microbiology* **67**, 215–222.
- WAITES, W. M., WYATT, L. R. & ARTHUR, B. (1972). Effect of alkali treatment on the germination and morphology of spores of *Clostridium bifermentans*. In *Spores V*, pp. 430–436. Edited by H. O. Halvorson, R. Hanson and L. L. Campbell. Washington, D. C.: American Society for Microbiology.
- WAITES, W. M., WYATT, L. R., KING, N. R. & BAYLISS, C. E. (1976). Changes in spores of *Clostridium bifermentans* caused by treatment with hydrogen peroxide and cations. *Journal of General Microbiology* **93**, 388–396.
- WYATT, L. R. & WAITES, W. M. (1973). The effect of hypochlorite on the germination of spores of *Clostridium bifermentans*. *Journal of General Microbiology* **78**, 383–385.
- WYATT, L. R. & WAITES, W. M. (1975). The effect of chlorine on spores of *Clostridium bifermentans*, *Bacillus subtilis* and *Bacillus cereus*. *Journal of General Microbiology* **89**, 337–344.