The Effect of Sporulation Medium on Spores of *Clostridium bifermentans*

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

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

(Received 16 July 1979)

Spores of *Clostridium bifermentans* produced on a Trypticase medium (TRY) and on reinforced clostridial medium (RCM) were compared. RCM-spores contained less dipicolinic acid, outgrew more slowly, were less resistant to hydrogen peroxide and to heat and lost more dipicolinic acid on heating. After treatment with urea/mercaptoethanol both TRY- and RCM-spores germinated with lysozyme, although heating RCM- but not TRY-spores before urea/mercaptoethanol treatment decreased their subsequent germination rate. In thin-section electron micrographs, TRY-spores showed thicker cortices and smaller protoplasts with less visible ribosomes than RCM-spores. The sporulation medium evidently has a marked effect on spore properties. Resistance to heat and to hydrogen peroxide appears to be related to spore structure.

INTRODUCTION

Changes in the composition of the medium on which spores are produced may alter their heat resistance (Anema & Geers, 1973) and chemical resistance (Waites & Bayliss, 1980). The effects of different media on spore resistance and structure have rarely been examined although we have found a correlation between protoplast and cortex volumes and heat resistance (Waites *et al.*, 1979). In this communication we compare the properties (including resistance to heat and to hydrogen peroxide) and the structure of spores of *Clostridium bifermentans* produced on two different media.

METHODS

Organism, spore preparation and maintenance of cultures. The parent strain of *Clostridium bifermentans* and the slow germination mutant 1, derived from the parent, were as described by Wyatt & Waites (1971). Spores were prepared either on the Trypticase medium of Bayliss & Waites (1976) (TRY-spores) or on reinforced clostridial medium agar (Hirsch & Grinsted, 1954) (RCM-spores). Both organisms were maintained in reinforced clostridial medium.

Treatment of spores with heat, hydrogen peroxide and urea/mercaptoethanol. For all treatments, suspensions contained 700 µg dry wt spores ml⁻¹. Heating was at 70 or 85 °C in glass-distilled water. Treatment with hydrogen peroxide was at 25 °C for 30 min and samples were removed and diluted as described by Bayliss & Waites (1976). For the urea/mercaptoethanol treatment, spores were treated for 90 min at 37 °C with 4 M-urea in 10% (v/v) mercaptoethanol adjusted to pH 10.3 with 6 M-NaOH, and then washed twice by centrifugation with glass-distilled water at 4 °C (Wyatt & Waites, 1975).

Germination, outgrowth and colony formation. Germination was measured at 37 °C either microscopically, using a phase-contrast microscope, or spectrophotometrically. Microscopic counts were used for spores germinating in growth medium; for each count, 100 spores were scored as either phase-dark (germinated) or phase-bright (ungerminated). Spectrophotometric measurements were used for spores germinating with the 'Ala+' system of germinants (Bayliss & Waites, 1976). Spores treated with urea/mercaptoethanol were

Fig. 1. Effect of hydrogen peroxide and heat on the colony-forming ability of spores of *Clostridium bifermentans*. TRY-spores (○) and RCM-spores (●) were incubated with hydrogen peroxide at 25 °C for 30 min (a) or heated at 85 °C for up to 180 min (b). Samples were diluted and plated as described in Methods.

germinated in the presence of lysozyme (100 µg ml⁻¹) and sodium phosphate buffer (0.1 M, pH 7.0) at 37 °C and spectrophotometric measurements were made at intervals. The medium and conditions for outgrowth were those described by Waites & Wyatt (1974). Colony forming ability was tested on the reinforced clostridial medium agar of Hirsch & Grinsted (1954) under H₂/CO₂ (9:1, v/v) for 2 d at 30 °C.

Spore dry weight. Dry weights were estimated turbidimetrically at 600 nm and converted to spore dry weight using an appropriate calibration curve.

Dipicolinic acid (DPA) measurement. DPA was measured colorimetrically by the method of Janssen *et al.* (1958).

Preparation of samples for electron microscopy. For electron microscopy, spore suspensions were fixed and embedded as described by Waites *et al.* (1972).

RESULTS

Effect of heat and hydrogen peroxide on spores

RCM-spores were killed more rapidly than TRY-spores by incubation with hydrogen peroxide (Fig. 1a) or by heating at 85 °C (Fig. 1b). To produce colonies, spores must first germinate and then outgrow to form viable vegetative cells. We therefore examined the effect of heat on germination and outgrowth. The rates of germination of TRY- and RCM-spores were reduced to the same extent by heating at 85 °C, but heating at 70 °C reduced the germination rate of RCM-spores much more than that of TRY-spores (Fig. 2). Measurement of DPA in the supernatants of heated spores showed that RCM-spores lost 4% of their dry weight as DPA after heating at 70 °C for 120 min but TRY-spores lost only about 1%. Unheated TRY-spores contained more DPA (10.6% of dry wt) than RCM-spores (7.3% of dry wt) so that heating caused RCM-spores to lose about 50% of their DPA while TRY-spores lost only about 10%.

Unheated TRY- and RCM-spores of mutant 1 germinated at 4% of the germination rate of the parent strain. Heating at 70 °C for 60 min increased the germination rate of TRY-spores of the mutant about eightfold but decreased that of RCM-spores (Fig. 2).

Unheated RCM-spores of the parent strain outgrew more slowly than TRY-spores. Germination (measured by loss of turbidity or phase brightness) and swelling occurred at about the same rate, but elongation was much slower. Heating at 70 °C for 60 min increased the time taken for 50% of RCM-spores to outgrow from 90 to 300 min but both heated and unheated TRY-spores outgrew within 50 min. Outgrowth of RCM-spores was

Fig. 2. Effect of heat on the germination of spores of *Clostridium bifermentans*. TRY-spores ($\circ$, Δ) and RCM-spores ($\bullet$, $\blacktriangle$) of the parent strain ($\circ$, $\bullet$) and mutant 1 (Δ , $\blacktriangle$) were heated at 70 °C for up to 90 min before germination with the 'Ala+' germination system. Germination rates for unheated TRY- and RCM-spores of the parent strain were 64 and 52 % decrease in $A_{600} \text{ min}^{-1}$, respectively.

Fig. 3. Effect of heat before treatment with urea/mercaptoethanol on subsequent lysozyme-initiated germination. TRY-spores (a) and RCM-spores (b) were heated for 0 ($\circ$), 15 (Δ) or 30 ($\square$) min at 70 °C before treatment with urea/mercaptoethanol and germination with lysozyme as described in Methods.

delayed by 145 min by addition of chloramphenicol ($10 \mu\text{g ml}^{-1}$) but outgrowth of TRY-spores was only delayed by 90 min. TRY-spores of mutant 1 germinated and outgrew more rapidly after heating at 70 °C for 30 min but RCM-spores outgrew more slowly. Such results suggest that RCM-spores are less fitted to outgrow and are more easily damaged by heat.

Effect of heat on germination with lysozyme

Lysozyme will degrade the cortex and initiate germination of spores in which disulphide bonds in the coats have been reduced (Gould & Hitchins, 1963). Such germination may not involve the triggering mechanism used when germination is initiated by metabolites. As treatment with urea/mercaptoethanol will allow lysozyme to initiate germination of spores of *C. bifermentans* (Wyatt & Waites, 1974), we compared the effect of pre-heating RCM- and TRY-spores before treatment with urea/mercaptoethanol and germination with lysozyme (Fig. 3). Heating did not reduce the subsequent germination rate of TRY-spores but reduced both the rate and extent of turbidity decrease during the germination of RCM-spores, suggesting that heat either made the coat of RCM-spores more resistant to urea/mercaptoethanol and lysozyme or reduced the rate of the germination changes themselves.

Keynan *et al.* (1964) reported that heating spores of *Bacillus cereus* after treatment with urea/mercaptoethanol markedly reduced their subsequent lysozyme-initiated germination.

Fig. 4. Effect of two treatments with urea/mercaptoethanol on the heat-induced inhibition of lysozyme-initiated germination. TRY-spores were treated once (○, Δ) or twice (●, ▲) with urea/mercaptoethanol before heating at 70 °C for 0 (○, ●) or 60 (Δ, ▲) min and incubating with lysozyme as described in Methods.

Table 1. *Effect of chemical treatment on the heat-induced inhibition of lysozyme-initiated germination of spores of Clostridium bifermentans*

TRY-spores were treated with urea/mercaptoethanol and then heated at 70 °C for 30 min or left unheated; both heated and unheated spores were subsequently treated as indicated before incubating with lysozyme as described in Methods. The percentage decrease in turbidity was measured after 30 min at 37 °C.

Subsequent treatment	Decrease in turbidity (%)	
	Unheated spores	Heated spores
None	23	7
NaOH (0.1 M, 10 min, 0 °C)	26	22
Urea/mercaptoethanol	41	35
Chlorine (200 µg Cl ₂ ml ⁻¹ , 10 min, 0 °C)	42	41

We found that heating after pre-treatment with urea/mercaptoethanol also reduced the subsequent germination of TRY-spores, but a second pre-treatment with urea/mercaptoethanol (Fig. 4) or subsequent treatment with urea/mercaptoethanol, NaOH or chlorine (Table 1) removed this inhibition, indicating that chemicals with different activities were able to increase the permeability of spore coats to lysozyme.

Spore structure

Electron micrographs of thin sections of spores (Fig. 5) showed that the cortices of TRY-spores were thicker and the protoplasts smaller than those of RCM-spores. Calculation of the mean percentage protoplast and cortex volumes has confirmed that RCM-spores have larger protoplasts and smaller cortices than TRY-spores (Waites *et al.*, 1979). In addition, the ribosomes within TRY-spores were less visible than those in RCM-spores, although TRY-spores (especially the coats) stained about five times as quickly as RCM-spores.

DISCUSSION

RCM-spores germinated at about the same rate as TRY-spores but elongation to form vegetative cells was slower. This suggests that the concentrations of metabolites and/or enzymes required for outgrowth may be higher in TRY- than in RCM-spores. RCM-spores were more rapidly destroyed by heat and by hydrogen peroxide. Heat also caused TRY-

Fig. 5. Electron micrographs of thin sections of a TRY-spore (a) and an RCM-spore (b). Bar markers represent 1 μ m.

spores of mutant 1 to germinate and outgrow more rapidly but decreased the rate of germination and outgrowth of RCM-spores of the mutant. Such results indicate either that the enzymes within TRY-spores are more resistant to heat and to hydrogen peroxide, or that destruction of enzymes which already limit the rate of outgrowth of unheated RCM-spores results in a more rapid loss of viability.

Gould & Hitchins (1963) suggested that treatment with urea/mercaptoethanol, by reducing disulphide bonds in the spore coat, enabled lysozyme to initiate germination and that dialysis against distilled water decreased the effect of lysozyme by allowing the disulphide bonds to reform. In our work, heating spores after treatment with urea/mercaptoethanol decreased the subsequent lysozyme-initiated germination, but a second treatment with urea/mercaptoethanol removed this inhibition. In addition, heating RCM- but not TRY-spores before treatment with urea/mercaptoethanol decreased subsequent lysozyme-initiated germination, suggesting that only the coats of RCM-spores were altered by pre-heating. Germination of untreated TRY-spores of both mutant and parent strain by metabolites was also more resistant to pre-heating than that of RCM-spores. Heating may change the permeability of spore coats to germinants (Keynan *et al.*, 1964) and the coats of untreated TRY-spores may be less rapidly altered by heat.

The more resistant TRY-spores had thicker cortices, smaller protoplasts, less visible ribosomes, more DPA and, on heating, less DPA was lost. This is consistent with the more resistant spores having a more expanded cortex and more contracted protoplast as suggested by Gould & Dring (1975) and Warth (1978). Although spores with depressed DPA levels have been found to have lower heat resistances (Church & Halvorson, 1959; Black *et al.*, 1960) and decreased germination rates (Keynan *et al.*, 1962), spores of a mutant lacking the ability to synthesize DPA were heat-resistant but had a reduced germination rate (Zytkevich & Halvorson, 1972). However, Balassa *et al.* (1979) have described a mutant of *Bacillus subtilis* which only formed heat-resistant spores when DPA was added to the sporulation medium: this increase in heat resistance was coupled with a reduction in the visibility of ribosomes within the spore core. The differences in DPA concentrations in the present study may explain the differences in ribosome visibility and heat resistance, although an

alternative explanation would be that RCM-spores have defective coats and cortices and lose DPA (and metabolites) during maturation and storage as well as during heating.

We are grateful to Dr J. L. Peel for useful discussions and Mrs L. J. Wallwork and Mrs H. A. Gravenall for skilled technical assistance.

REFERENCES

- ANEMA, P. J. & GEERS, J. M. (1973). Controlling the sporulation of *Clostridium sporogenes* and the heat resistance of the spores. *Journal of Applied Bacteriology* **36**, 553-558.
- BALASSA, G., MILHAUD, P., RAULET, E., SILVA, M. T. & SOUSA, J. C. F. (1979). A *Bacillus subtilis* mutant requiring dipicolinic acid for the development of heat-resistant spores. *Journal of General Microbiology* **110**, 365-379.
- BAYLISS, C. E. & WAITES, W. M. (1976). The effect of hydrogen peroxide on spores of *Clostridium bifermentans*. *Journal of General Microbiology* **96**, 401-407.
- BLACK, S. H., HASHIMOTO, T. & GERHARDT, P. (1960). Calcium reversal of the heat susceptibility and dipicolinate deficiency of spores formed 'endotrophically' in water. *Canadian Journal of Microbiology* **6**, 213-224.
- CHURCH, B. D. & HALVORSON, H. (1959). Dependence of the heat resistance of bacterial endospores on their dipicolinic acid content. *Nature, London* **183**, 124-125.
- GOULD, G. W. & DRING, G. J. (1975). Role of an expanded cortex in resistance of bacterial endospores. In *Spores VI*, pp. 541-546. Edited by P. Gerhardt, R. N. Costilow & H. L. Sadoff. Washington, D.C.: American Society for Microbiology.
- GOULD, G. W. & HITCHINS, A. D. (1963). Sensitization of bacterial spores to lysozyme and to hydrogen peroxide with agents which rupture disulphide bonds. *Journal of General Microbiology* **33**, 413-423.
- 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.
- JANSSEN, F. W., LUND, A. J. & ANDERSON, L. E. (1958). Colorimetric assay for dipicolinic acid in bacterial spores. *Science* **127**, 26-27.
- KEYNAN, A., MURRELL, W. G. & HALVORSON, H. O. (1962). Germination properties of spores with low dipicolinic acid content. *Journal of Bacteriology* **83**, 395-399.
- KEYNAN, A., EVENCHIK, Z., HALVORSON, H. O. & HASTINGS, J. W. (1964). Activation of bacterial endospores. *Journal of Bacteriology* **88**, 313-318.
- WAITES, W. M. & BAYLISS, C. E. (1980). *The Preparation of Bacterial Spores for Evaluation of the Sporicidal Activity of Chemicals*, Society for Applied Bacteriology Technical Series (in the Press). London: Academic Press.
- WAITES, W. M. & WYATT, L. R. (1974). 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 & L. L. Campbell. Washington, D.C.: American Society for Microbiology.
- WAITES, W. M., STANSFIELD, R. & BAYLISS, C. E. (1979). The effect of sporulation medium on the structure and heat resistance of spores of *Clostridium bifermentans*. *FEMS Microbiology Letters* **5**, 365-368.
- WARTH, A. D. (1978). Molecular structure of the bacterial spore. *Advances in Microbial Physiology* **17**, 1-45.
- WYATT, L. R. & WAITES, W. M. (1971). Studies with spores of *Clostridium bifermentans*; comparison of germination mutants. In *Spore Research-1971*, pp. 123-131. Edited by A. N. Barker, G. W. Gould & J. Wolf. London: Academic Press.
- WYATT, L. R. & WAITES, W. M. (1974). The effect of sodium hydroxide or dithiothreitol-urea on spores of germination mutants of *Clostridium bifermentans*. *Journal of General Microbiology* **84**, 391-394.
- 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.
- ZYTKOVICZ, T. H. & HALVORSON, H. O. (1972). Some characteristics of dipicolinic acid-less mutant spores of *Bacillus cereus*, *B. megaterium* and *B. subtilis*. In *Spores V*, pp. 49-52. Edited by H. O. Halvorson, R. Hanson & L. L. Campbell. Washington, D.C.: American Society for Microbiology.