The Outgrowth of Spores of *Clostridium bifermentans*

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SUMMARY

Germination and outgrowth of spores of *Clostridium bifermentans* occurred rapidly and synchronously in a medium containing casein hydrolysate, vitamins and metal salts. Germination began immediately after inoculation and within 10 min more than 90 % of the spores were phase dark. Swelling of the spores began immediately thereafter and by 25 min after inoculation more than 70 % of the spores were swollen. Elongation of the swollen spores was first detected at about 35 min; by 65 min 80 % of the spores had become cells, and by 90 min cell division had started. Increases in turbidity, RNA and protein were first detected during swelling and an increase in DNA was first observed at the beginning of elongation. By the time cell division began DNA had doubled. When spores were heated at 80 °C for 10 min, 20 % survived; the survivors germinated very much more slowly than unheated spores and only formed cells if anaerobic conditions were maintained. The increases in RNA, protein and DNA occurred much later during outgrowth than with unheated spores.

INTRODUCTION

Many detailed studies have been made of the events which occur during the outgrowth of spores of bacilli (for a review, see Strange & Hunter, 1969). Outgrowth often occurs very rapidly, e.g. with *Bacillus cereus* net synthesis of RNA occurred 10 min and cell division 60 min after inoculation into growth medium (Fitz-James, 1955). With clostridial spores no comparable studies have been made. Germination, which precedes outgrowth, is often slower and less synchronous than with spores of bacilli. With *Clostridium botulinum* 15 % of spores were ungerminated and 45 % elongated when cell division began at 180 min, while an increase in RNA occurred after 60 min (Rowley, El-Bisi, Anellis & Snyder, 1970; Church, Germaine & Rowley, 1971). With such spores it is difficult to relate chemical changes to morphological development. *Clostridium bifermentans* has been found to germinate rapidly and synchronously (Waites & Wyatt, 1971). It was of interest, therefore, to study the morphological and chemical changes which occur during outgrowth in this strain and to compare the germination and outgrowth of heat damaged spores.

METHODS

Organism, spore preparation and maintenance of culture. The strain of *C. 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 & Grinsted (1954).

Preparation of heat damaged spores. About 13 mg dry weight of spores suspended in 5 ml glass-distilled water were heated at 75 or 80 °C for 10 min in screw-capped glass bottles,

and centrifuged and washed four times to remove any soluble materials released during the heat treatment, before storing overnight at 0 °C. Spores heated and used immediately without washing germinated and outgrew at similar rates to spores which had been washed and stored.

Spore outgrowth. The medium for spore outgrowth was based on that of Mead (1971) and contained (mg/l glass distilled water): Oxoid casein hydrolysate (acid), 41 000; tryptophan, 40; cysteine, 30; thiamine, 0.05; pyridoxal phosphate, 0.025; nicotinic acid, 0.5; pantothenate, 0.1; nitrilotriacetic acid, 300; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 20; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.48; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 2.0; CaCl_2 , 4.0; CoCl_2 , 0.02; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.2; Na_2MoO_4 0.002; KH_2PO_4 , 900, with NaOH added to adjust the pH to 7.2. In one experiment glucose (5 g/l) was also added. Medium (60 ml in a 100 ml conical flask, or, where samples for chemical estimation were required, 300 ml in a 500 ml conical flask) was sterilized by autoclaving and stored at 4 °C until required. The flask was then placed in a water bath at the required temperature, and $\text{H}_2 + \text{CO}_2$ (9:1, v/v) was passed first through a Deoxo tube (Engelhard Industries Ltd) to remove O_2 and then through the medium (via a gassing head). After at least 30 min the flask was sealed and the spores were added (2.6 or 13 mg dry wt, to give a final concentration of about 40 μg dry wt/ml). Samples (10 ml) withdrawn at intervals, with a minimum of disturbance, for measurement of turbidity, RNA, DNA and protein and for microscopic examination.

Measurement of turbidity. Turbidity was measured at 600 nm with a Unicam SP600 spectrophotometer using the blue photocell and cuvettes of 10 mm light path.

Measurement of RNA, protein and DNA. Samples (7 ml) were added to trichloroacetic acid (final concentration 6.25 %, w/v), cooled in ice-water and centrifuged. The pellets were washed once by centrifuging in trichloroacetic acid and then resuspended in 5 % (w/v) trichloroacetic acid (2 ml) before heating at 90 °C for 10 min, cooling in ice-water and centrifuging. The pellets were dissolved in 0.1 M-NaOH and protein estimated by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine plasma albumin fraction V (Armour Pharmaceuticals Ltd, Eastbourne) as a standard. RNA and DNA were measured in the supernatants by the methods of Schneider (1957) and Burton (1956) using D-ribose and 2-deoxy-D-ribose (Koch-Light Ltd, Colnbrook, Buckinghamshire), respectively, as standards.

Determination of morphological stages. Samples of 100 cells were counted under a phase contrast microscope and scored as phase-bright, phase-dark, swollen or elongated spores.

Spore dry weights. Dry weights/ml of spore suspensions were measured spectrophotometrically using a calibration curve to relate E_{600} to dry weight/ml.

Measurement of glucose. Glucose was measured enzymically by the Boehringer blood sugar GOD-Perid method and the blue colour read at 430 nm.

Measurement of germination rate. Germination was followed at 37 °C either spectrophotometrically, by measuring the decrease in extinction at 600 nm (E_{600}), or microscopically, by counting phase-bright and phase-dark spores, as described by Waites & Wyatt (1971). The microscopic method was used in one case when the germination rate was less than 0.3 %/min and the results compared with those obtained spectrophotometrically by calculating a theoretical germination rate as described previously (Wyatt & Waites, 1971). Germinants were (mm): 'Ala' system, L-alanine (50), NaCl (100), and sodium phosphate (83) pH 8.0; 'Ala + ' system, L-alanine (50), L-arginine (5), L-phenylalanine (5), L-lactate (25), NaCl (100) and sodium phosphate (83) pH 7.5; 'Gly + ' system as for 'Ala + ' but with sodium phosphate at pH 6.25 and glycine (25) replacing L-alanine; casein hydrolysate (8.3 %, w/v); H_2O_2 (100), CuSO_4 (0.2) and tris-HCl (83) pH 8.0.

Fig. 1. Effect of glucose on outgrowth. Spores were incubated at 37 °C in the casein hydrolysate medium either with (solid symbols) or without (open symbols) glucose. Turbidity (○ or ●) and glucose (▲) measurements were made at intervals as described in Methods.

Viable counts. To determine the number of spores surviving heat treatment, the heated spores were diluted in distilled water and 0.1 ml samples spread on three plates containing reinforced clostridial medium (Hirsch & Grinsted, 1954) solidified with 1.5 % agar. The plates were then incubated in $H_2 + CO_2$ (9:1, v/v) for at least 6 days at 30 °C and the number of colonies counted.

RESULTS

Determination of conditions for outgrowth

In preliminary experiments with unheated spores at 37 °C, where medium plus added glucose was stirred after flushing with $H_2 + CO_2$ (9:1, v/v), no increase in turbidity was observed. Microscopic examination showed that 97 % of the spores became phase dark and that 30 % then became swollen, but no elongation was observed even after 7 h. By contrast, in unstirred cultures there was a rapid turbidity increase and cell formation. This was attributed to the adverse effect of aeration on the stirred culture. Omission of glucose from unstirred cultures did not affect the initial turbidity changes and no glucose was utilized before cell division began at about 90 min (Fig. 1). Subsequent experiments were carried out in the absence of glucose and without stirring.

Germination of this strain of *C. bifermentans* will occur at up to 61 °C (Waites & Wyatt, 1974) and it was of interest to determine whether outgrowth would occur at similar temperatures. Turbidity increases were measured at temperatures of up to 47 °C but microscopic examination showed that spore swelling and elongation were slower at 47 °C than at 37 °C,

Table 1. *Effect of temperature on outgrowth of spores of C. bifermentans*

Spores were added to the casein hydrolysate medium which had been equilibrated at the stated temperature and through which $H_2 + CO_2$ (9:1, v/v) had been flushed for 30 min before inoculation. Flushing was then stopped and samples taken at 15 min intervals or flushing was continued for a further 30 min, the flask sealed and a sample taken after 5 h incubation.

Temperature (°C)	Percentage of each cell type after 5 h			
	Phase-bright spores	Phase-dark spores	Swollen spores	Vegetative cells
With no additional flushing				
37	3	6	7	84
47	4	9	37	50
48	0	36	64	0
With additional flushing				
48	1	36	27	36
49	0	72	28	0
50	5	38	57	0

and that fewer spores were able to elongate (Table 1). At 48 °C no elongation or turbidity increase occurred unless $H_2 + CO_2$ (9:1, v/v) were bubbled through the culture for 30 min after inoculation and no samples were removed for 5 h. At 49 °C even this extra flushing failed to induce elongation although swelling occurred at 49 or 50 °C. Subsequent experiments were carried out at 37 °C.

Microscopic changes during outgrowth

Microscopic examination of samples taken at 5 min intervals showed that spores changed from phase-bright through phase-dark to swollen spores and then by elongation to rod-shaped vegetative cells (Fig. 2). Germination was rapid and by the time the 0 min sample was counted, 83 % of the spores had changed from phase bright to phase dark. After 10 min incubation, less than 10 % of the spores were phase bright and 60 % were swollen. Elongation of swollen spores began at about 35 min and most of the spores which became cells within 4 h had done so by 65 min. However, a few spores remained phase bright (3 %), phase dark (about 7 %) or swollen (about 8 %) even after 4 h incubation. Cell division became obvious between 75 and 90 min, and by 105 min 28 % of the developing spores had divided once and 2 % twice, while 53 % had elongated but not divided and 17 % had failed to form cells.

Changes in turbidity, RNA, protein and DNA during outgrowth

Increases in RNA and protein began at between 10 and 20 min and in DNA at about 30 min after inoculation of the medium (Fig. 3). During the first 5 min of incubation the turbidity decreased rapidly as the spores became phase dark, but after 15 min the turbidity increased at a constant rate until at 55 min it accelerated. The constant rate corresponded approximately to the time during which cell elongation took place. Protein and RNA showed similar but shorter periods of increase at constant rates. Net synthesis of DNA began at about the time when cell elongation started and by the time cell division was first detected DNA had doubled. By 85 min turbidity and protein had also doubled but RNA had quadrupled.

Fig. 2. Microscopical changes during outgrowth. Spores were incubated in the casein hydrolysate medium at 37 °C, samples removed, and 100 cells counted and scored as vegetative cells (Δ), divided cells (●), phase-bright (▲), phase-dark (□), or swollen spores (■), as described in Methods.

Fig. 3. Changes in turbidity, RNA, protein and DNA during outgrowth. Spores were incubated in the casein hydrolysate medium at 37 °C and samples removed at intervals to measure turbidity (Δ), RNA (□), protein (●) and DNA (○), as described in Methods.

Fig. 4. Changes in turbidity, RNA, protein and DNA during outgrowth of spores heated at 80 °C. Spores were heated at 80 °C for 10 min before incubation in the casein hydrolysate medium at 37 °C. Symbols as for Fig. 3.

Fig. 5. Microscopical changes during outgrowth of spores heated at 80 °C. Spores were heated at 80 °C for 10 min and then incubated in the casein hydrolysate medium. Symbols as for Fig. 2.

Behaviour of heat-damaged spores

Spores heated at 80 °C for 10 min produced about 20 % of the colonies formed by unheated spores. In the outgrowth medium, the turbidity increase was delayed and did not occur within 6 h. However, if the medium was constantly flushed with $\text{H}_2 + \text{CO}_2$ (9:1, v/v), in three different experiments an increase in turbidity occurred after 275 (Fig. 4), 150 or 130 min. Such flushing was also necessary before elongation occurred after 360 (Fig. 5) 150

Table 2. *Effect of heat on germination of spores of C. bifermentans*

Spore suspensions were incubated at 37 °C in the germinant systems described in Methods. Germination was measured spectrophotometrically or microscopically and the rate, calculated from the maximum rate of decrease in E_{600} per min, expressed as a percentage of the initial E_{600} value.

Germination system	Germination rate (%/min)		
	Unheated spores	Spores heated at 75 °C for 10 min	Spores heated at 80 °C for 10 min
'Ala'	13	0.3	0.03
'Ala +'	31	6.6	3.0
'Gly +'	18	2.7	0.3
Casein hydrolysate	21	3.1	0.4
H ₂ O ₂	9.3	4.0	7.2

or 130 min in different experiments. Swelling occurred without continuous flushing, although more slowly than with flushing. Increases in RNA, DNA and protein were delayed in such spores (Fig. 4) compared with unheated spores.

When spores were heated at 75 °C for 10 min, about 50 % formed colonies and these spores germinated and outgrew more slowly than unheated spores but without continuous flushing.

Further experiments were carried out to determine whether the number of viable spores in the inoculum determined the time taken for outgrowth. It was found that an unheated spore inoculum of 12 µg dry wt/ml outgrew without continuous flushing with H₂ + CO₂, but that an inoculum as low as 0.63 µg dry wt/ml would outgrow within 180 min if flushed continuously. Unheated spores thus behaved like a spore inoculum sixty times as large which had been heated at 80 °C, suggesting that the heat treatment had damaged some of the spores so that they were able to form colonies but were more sensitive to their environment during outgrowth.

Heat damaged spores of *C. botulinum* type E and some strains of *C. perfringens* are unable to produce colonies in the absence of lysozyme (Sebald & Ionesco, 1972; Cassier & Sebald, 1969; Duncan, Labbe & Reich, 1972). We therefore examined the germination rates of heated spores by a more sensitive method, using a recording spectrophotometer (Table 2). The germination rates of spores heated at 75 °C were decreased more than fourfold and those of spores heated at 80 °C more than tenfold, except when germination-like changes were initiated by hydrogen peroxide, which may by-pass physiological germination mechanisms (Gould & Hitchins, 1963). It is possible, therefore, that heated spores failed to form colonies because of delayed germination. However, addition of lysozyme (5 µg/ml) to the plating medium did not increase the number of colonies formed.

DISCUSSION

In this study we have shown that the increases in RNA, protein and DNA associated with outgrowth of spores of *C. bifermentans* in a casein hydrolysate medium occurred rapidly in a sequence related to the morphological events. Stirring, possibly by increasing the concentration of oxygen in the medium (O'Brien & Morris, 1971), prevented elongation but allowed phase-darkening and swelling of spores to occur. Fujioka & Frank (1966) also found that

when germination and swelling of spores of *C. sporogenes* occurred aerobically, there was no elongation to form cells. In our study glucose was not necessary for outgrowth and was not utilized until after cell division had begun. It is possible that amino acids repressed glucose utilization, since Anthony & Guest (1968) have previously shown that they prevent glucose utilization by cells of *C. tetanomorphum*, possibly by inhibiting glycolytic enzymes.

Our results show that outgrowth takes place more rapidly and more synchronously in this strain than has been reported for other species of *Clostridium* (e.g. *C. botulinum*, Church *et al.* 1971; *C. pasteurianum*, Mackey & Morris, 1972) and that, for the first time, the beginning of net syntheses can be related to the morphological stages of clostridial outgrowth. Generally, the morphological and chemical changes occurred at a similar rate and in the same sequence as those found during outgrowth of spores of *Bacillus* species (Strange & Hunter, 1969). An exception is the timing of DNA synthesis. In the studies reported here, DNA began to increase simultaneously with elongation, so that by the time division septa were first observed DNA had doubled and most cells must have contained at least two genomes. As most cells divided later, they must have contained more than two genomes at the time of division. During outgrowth of *Bacillus* spores significant DNA synthesis begins at a later stage. In *Bacillus subtilis*, Donnellan, Nags & Levinson (1965) found no DNA synthesis until 50 % of the spores had elongated, while in *B. cereus* DNA synthesis was first detected colorimetrically just before the first cell division, although a low rate of DNA synthesis was detected by incorporation of radioactive thymidine as early as 5 min after germination commenced (Steinberg & Halvorson, 1968). The factors which limit DNA synthesis during outgrowth are unknown although it has been suggested that a protein essential for DNA replication is absent in spores and is synthesized during outgrowth (Steinberg, Idriss, Rodenberg & Halvorson, 1969). It is possible that such a protein is also absent from spores of our strain of *C. bifermentans* but that during outgrowth it is synthesized more rapidly than in bacilli.

The study of outgrowth at different temperatures, showed that spores of *C. bifermentans* were able to swell at 50 °C but that elongation only occurred below 49 °C. Previously, we have found that germination would occur up to 61 °C (Waites & Wyatt, 1974). In similar experiments, Levinson & Hyatt (1970) found that spores of *Bacillus megaterium* germinated at 50 °C but only outgrew below 47 °C. It is apparent that the later the event during germination and outgrowth the more sensitive it is to high temperatures, presumably because the metabolic requirements for the later stages of development are more heat sensitive.

With regard to the effect of heat on spores, we have shown that spores heated for 10 min at 75 °C germinate more slowly, and those heated for 10 min at 80 °C germinate much more slowly, than unheated spores. Heat damaged spores of *C. botulinum* type E and some strains of *C. perfringens* are only able to germinate and form colonies in the presence of lysozyme, and it has been suggested that heat damages the germination mechanism which is by-passed by the action of the lysozyme (Cassier & Sebald, 1969; Duncan *et al.* 1972; Sebald & Ionesco, 1972). Such an explanation could also be applied to our results. However, the response of spores heated at 75 °C was not uniform; some failed to become phase dark while others were unable to swell or elongate. The presence of lysozyme in the plating medium did not result in an increase in the number of colonies formed. Although after heating at 80 °C some spores became swollen under the usual experimental conditions, continuous flushing with H₂ + CO₂ was required before cell formation could occur and even then spore elongation was delayed. After heating at 80 °C about 20 % of the spores formed colonies but the outgrowth of an inoculum of 42 µg dry wt of heated spores/ml occurred at about the same time as an inoculum

of 0.63 µg dry wt of unheated spores/ml. Therefore even those spores heated at 80 °C which were able to form cells were heat-damaged and were more sensitive to environmental conditions than were undamaged spores. It was noticeable that spores heated at 80 °C were able to swell without accompanying increases in turbidity, RNA, protein and DNA, and such swelling may be associated with water uptake (Steinberg *et al.* 1969). The increases in turbidity, RNA, protein and DNA which occurred at specific morphological stages in unheated spores were delayed in spores heated at 80 °C, and it would be of interest to see if changes in substrate utilization and energy levels during germination and outgrowth are also delayed in heat damaged spores.

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