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Germination of Heat- and Alkali-Altered Spores of *Clostridium perfringens* Type A by Lysozyme and an Initiation Protein

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The normal system functioning in the utilization of metabolizable germinants by both heat-sensitive and heat-resistant spores of *Clostridium perfringens* was inactivated by heat or by treatment of the spores with alkali to remove a soluble coat protein layer. Altered spores were incapable of germination (less than 1%) and outgrowth (less than 0.0005%) in complex media without the addition of either lysozyme or an initiation protein produced by *C. perfringens*. The addition of either of these agents permitted, in the case of alkali-treated spores, both 90 to 95% germination and outgrowth, as measured by colony formation. In the case of heat-damaged spores, only 50% germination and 2% outgrowth resulted from addition of the initiation protein, whereas lysozyme permitted 85% germination and 8% outgrowth. Alteration of the spores by heat or alkali apparently inactivated the normal lytic system responsible for cortical degradation during germination. Kinetics of production of the initiation protein and conditions affecting both its activity and that of lysozyme on altered spores are described.

Among the multitude of germinative agents of bacterial spores, there are two which are lytic in nature. These are a lytic enzyme which can be extracted from spores of *Bacillus cereus* (4) and lysozyme (3). Among the known compounds excreted by germinating spores are amino acids, peptides, amino sugars, calcium, and dipicolinic acid (12). Lysozyme-like enzymes associated with spores are believed to cause the excretion of at least one of these types of compounds, the amino sugars or hexosamines. The structural location of the hexosamines is believed to be the cortical layer of the spore which is composed of murein, a mucopeptide polymer similar to that found in vegetative cell walls (11). Although lysozyme and spore lytic enzyme attack the murein substrate and release hexosamine peptides, the latter does so at fewer sites (6). Thus, although both enzymes produce hexosamine peptides as end products, spore-related enzymes and lysozyme are quite likely two distinct proteins.

Generally, for lysozyme or the lytic enzyme to act as germinants, spores must first be pretreated with agents such as urea and mercaptoethanol or thioglycolic acid which rupture disulfide bonds in the spore coat, allowing lysozyme to penetrate and attack the underlying mucopeptide of the spore cortex (3, 4). Exposure of mercaptoethanol- and urea-treated spores to alkali has been shown to remove an additional coat component, resulting in greater sensitization of the spore to the lytic agent (7).

B. megaterium ATCC 9855 has been shown to be sensitive to lysozyme without pretreatment with bond-breaking reagents (14). Presumably, the mucopeptide was directly accessible to lysozyme, perhaps owing to a defective spore coat. No other strain or *Bacillus* species tested has this sensitivity.

Strains of *Clostridium perfringens* type A are known to vary greatly in their spore heat resistance (16). Recently, it was shown that a portion of a population of heat-damaged spores of *C. perfringens* type A were rendered nonviable unless plated in a medium containing lysozyme, which initiated germination of the damaged spores (1). Only heat-sensitive strains had such a requirement. The same recovery phenomenon occurred when sterile culture filtrates of heat-sensitive strains replaced lysozyme in the recovery medium. This was the first report of a specific requirement for lysozyme or a lytic-like protein from culture fil-

trates to initiate spore germination. This initiation protein, as it was called, was presumably a lytic enzyme which could initiate germination by replacing the inactivated lytic enzyme normally functioning in germination of spores that were not heat-damaged. Proteolytic enzymes such as trypsin and Pronase had no effect. However, in that study a suspension containing a mixture of free spores, spores in their sporangia, and vegetative cells was utilized; the possibility of indirect action via release of metabolizable germinants from the sporangia or vegetative cells by lysozyme and the initiation protein was a distinct possibility.

In the present report, a clean spore preparation was used to show that lysozyme and the initiation protein act directly on heat-injured, alkali-treated, or, to a certain extent, unaltered spores of both heat-sensitive and heat-resistant strains at the level of germination. Conditions affecting the activity of the two proteins also are reported.

MATERIALS AND METHODS

Cultures. Strains of *C. perfringens* type A used included ATCC 3624, NCTC 8238, NCTC 8798, and FD1. Strain FD1 was obtained from the Food and Drug Administration. Strains ATCC 3624 and FD1 produced heat-sensitive spores ($D_{100} < 0.5$), and NCTC 8238 and NCTC 8798 produced heat-resistant spores ($D_{100} > 10.0$). The D_{100} value is the time in minutes for inactivation of 90% of the spores at 100 C. Stock cultures were maintained in Cooked Meat Medium (Difco) at room temperature.

Preparation of spore suspensions. Spores were produced in D-S sporulation medium (2) and cleaned by methods described previously (9). The spores were free from sporangial remnants as determined by electron microscopy. A working stock spore suspension of about 10^8 spores/ml of the heat-sensitive strains or about 5×10^7 spores/ml of the heat-resistant strains was prepared in 0.05 M phosphate buffer, pH 7.0. The remainder of the spores of each strain was lyophilized and stored desiccated at −20 C. Lyophilization did not alter heat resistance or apparent germinability of the spores.

Enumeration of spores and vegetative cells. Control viable counts of vegetative cells and heat-injured, heat-shocked, or alkali-treated spores were made by means of pour plates with the use of TSN agar base (10) without antibiotics. Deionized water and 0.01% peptone were used as dilution blanks for enumeration of spores and vegetative cells, respectively. Unless otherwise stated, incubation was at 37 C for 48 hr under a gas mixture of 90% N_2 and 10% CO_2.

Preparation of heat-shocked or heat-injured spores. Heat-shocked spores were prepared by heating 5 ml of a stock suspension in screw-cap tubes (16 by 125 mm) at 75 C for 20 min. For heat injury, 1.0 ml of the stock spore suspensions were heated in sealed Pyrex tubes (9 mm in outside diameter by 15 cm long) immersed in a constant-temperature propylene glycol bath. The contents of each tube were brought to a constant temperature by holding at 37 C for 5 min immediately before heat injury. Heat-sensitive spores were heated for 5 min at 90 C, and heat-resistant spores were heated for 5 min at 106 C. The tubes were then immediately cooled in ice water. Survivors were enumerated as described above. These times and temperatures were fairly critical for determining lysozyme dependence. They resulted in about a 5 and 6 log decrease in viable count for the heat-resistant and heat-sensitive strains, respectively, when compared with the counts obtained with heat-shocked spores.

Preparation of alkali-treated spores. Spores treated to remove an alkali-soluble coat component were prepared by suspending clean lyophilized spores in 0.1 N NaOH to a final concentration of about 10^{10}/ml, and incubating them for 15 min at 4 C. The spores were subsequently washed once with cold distilled water, and twice with 0.05 M phosphate buffer, pH 7.0. Spores were then diluted to a working concentration in buffer and stored not more than 1 week at 4 C.

Studies on recovery of heat-injured or alkali-treated spores. In certain experiments, the effect of lysozyme and of an initiation protein found in culture supernatant fluid on the recovery of heat-injured or alkali-treated spores was determined. With the initiation protein, a specific amount of lyophilized culture supernatant fluid (see below) was reconstituted by the addition of 50 ml of distilled water and was sterilized by filtration through a Seitz filter. The sterile fluid was then added to 500 ml of TSN agar (without antibiotics) at 46 C. With lysozyme, 10 ml of an appropriately diluted stock solution was added to 500 ml of the TSN plating medium. These modified TSN media were used to determine the viable spore count of the heat-injured or alkali-treated spore populations.

The results are presented as per cent recovery with the total viable count of heat-shocked spores plated with plain TSN (without antibiotics) taken as 100% recovery. At the concentrations used in this study, neither lysozyme nor the initiation protein affected the total viable count of heat-shocked spores.

Assessment of germination. Germination was measured as a decrease in optical density (OD) at 660 nm determined with a Bausch & Lomb Spectronic 20 colorimeter. Germination was confirmed by a loss of spore phase brightness as seen with a Zeiss phase-contrast microscope. The data are presented as changes in OD, initial OD minus final OD (Δ OD). The germination reagents were prepared as concentrated stock solutions. Final reaction volumes of 3.0 ml were contained in screw-cap tubes (13 by 100 mm). Initial OD values of the spore germination mixtures ranged from 0.38 to 0.49. In most cases, chloramphenicol (final concentration, 30 μg/ml) was added to prevent outgrowth. This antibiotic had no effect on germination.

Alcian blue (Fisher Scientific Co.)-treated spores were prepared as follows: 5 ml of a stock suspension of *C. perfringens* strain FD1 spores was heat-injured

as described above; alcian blue (final concentration, 5%) was added to the spore suspension which was then incubated at room temperature for 3 hr. The treated spores were centrifuged at 4 C and repeatedly washed with 0.05 M phosphate buffer, pH 7.0, until excess dye no longer appeared in the supernatant fluid. The sedimented spores were then resuspended in the phosphate buffer.

Preparation of concentrated culture supernatant fluids containing an initiation protein. A 10-ml volume of fluid thioglycolate medium (FTG; BBL) was inoculated with 0.25 ml of a given strain from a stock culture of Cooked Meat Medium. After 16 hr at 37 C, the entire FTG culture was inoculated into 1,000 ml of D-S sporulation medium. In one experiment, a similar 1,000-ml volume of FTG medium was used instead of sporulation medium. After selected time intervals, the cultures were centrifuged at 4 C and 5,000 $\times$ *g* for 30 min. Known volumes of supernatant fluid were concentrated by overnight dialysis against Carbowax 20,000 (polyethylene glycol) at 4 C. The concentrated dialyzed material was then lyophilized and stored at -20 C. With strain NCTC 8238, after dialysis against Carbowax, the concentrated fluid was held at 4 C for 24 hr to allow the settling out of nonutilized growth medium constituents. The supernatant fluid was removed, lyophilized, and subsequently used as a source of the initiation protein.

RESULTS

Effect of lysozyme and an initiation protein on the recovery of heat-injured or alkali-treated spores. When clean spores of *C. perfringens* strain FD1 were heat-injured, only a small percentage (0.0001%) remained viable, as determined by pour plate counts. However, when lysozyme was added to the plating medium, a proportion (up to a maximum of about 6 to 8%) of the apparently dead spores in fact were viable (Fig. 1). The apparent reversibility of heat injury by lysozyme was concentration-dependent and was detected with a lysozyme concentration as low as 0.01 μg/ml. Recovery of heat-injured spores was a function not only of the concentration of lysozyme but also of the incubation time (Table 1). If per cent recovery was determined after only 24 hr, 0.06 μg of lysozyme/ml of TSN was required for increased recovery. However, a further 24-hr incubation period decreased this amount to 0.02 μg/ml. Thus, increasing the incubation time reduced the concentration of lysozyme required for maximal recovery. With 0.06 or 0.08 μg of lysozyme/ml of TSN, there was no difference in per cent recovery between 72 and 96 hr.

Alkali treatment of either heat-sensitive or heat-resistant spores (strains FD1 and NCTC 8798, respectively) reduced the apparent viability of the spores to a level (about 0.0005%), which was comparable to that of heat-injured spores. The addition of 0.1 μg of lysozyme/ml of TSN recovery medium permitted 90 to 95% recovery of the alkali-treated spores as compared with the nontreated spores.

The initiation protein found in D-S culture supernatant fluid of *C. perfringens* ATCC 3624 had an effect similar to that of lysozyme in producing an apparent reversal of heat injury of *C. perfringens* FD1 spores (Fig. 2). The effect of the initiation protein was concentration dependent, and, as in the case of lysozyme, there was an amount (about 4 mg of lyophilized supernatant fluid containing the initiation protein/ml of TSN plating medium) above which no further increase in per cent recovery of the injured spores was detectable. The effect of this initiation protein also was dependent upon incubation time of the spores in the recovery medium. For example, with 4 mg of lyophilized material/ml, there was an increase in the per cent recovery between 24 and 48 hr but there was no significant difference between 48 and 72 hr. The maximal recovery of heat-injured spores that could be obtained by the addition of the initiation protein to the plating medium was about 2% as compared to 8% with lysozyme. However, as was the case with lysozyme, almost total recovery of alkali-treated spores, 90 to 95%, was obtained when 4 mg of lyophilized culture supernatant material was added to the TSN recovery medium.

The apparent reversibility of heat injury of spores of strains other than FD1 by lysozyme and by the initiation protein is shown in Table 2.

Both lysozyme and the initiation protein mediated recovery of thermally damaged spores of both heat-sensitive (FD1 and ATCC 3624) and heat-resistant (NCTC 8238 and NCTC 8798) strains. Also, the initiation protein was present in the 11-hr-old culture supernatant fluids of both heat-sensitive and heat-resistant strains, which confirms the results previously reported by Cassier and Sebald (1). Regardless of the test spore strain, producer strain ATCC 3624, a heat-sensitive strain, appeared to be the most active producer of the initiation protein. Up to a 4 log increase in per cent recovery of heat-injured spores was obtained with the initiation protein of this strain as compared to recovery in the absence of the initiation protein.

Although the initiation protein could be detected in D-S sporulation medium, when strain ATCC 3624 was grown in FTG, a common growth medium in which sporulation

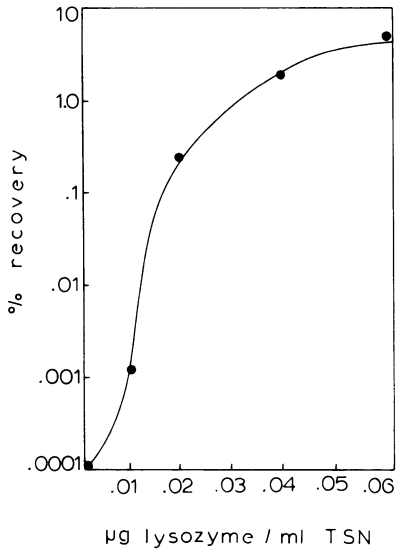

FIG. 1. *Effect of lysozyme concentration on the recovery of heat-injured C. perfringens FD1 spores in TSN plating medium. Incubation was at 37 C for 72 hr.*

TABLE 1. *Effect of incubation time on recovery of heat-injured spores of C. perfringens strain FD1 by lysozyme*

Incubation time (hr)	Percent recovery[a]				
	No lysozyme	0.02 μg[b]	0.04 μg	0.06 μg	0.08 μg
24	0.0001	0.0001	0.0001	0.014	0.28
48	0.0001	0.026	0.47	1.4	4.3
72	0.0001	0.26	2.0	5.3	6.9
96	0.0001	0.30	5.6	5.3	6.9

[a] Per cent recovery determined from viable counts obtained after incubation at 37 C. The number of viable spores obtained after heat shocking at 75 C for 20 min was considered 100% recovery.

[b] Amount of lysozyme per milliliter of TSN medium.

does not readily occur, activity of the protein could not be detected.

Kinetics of production of the initiation protein by strain ATCC 3624. Only supernatant fluids of cultures incubated 11 hr were used as sources of the initiation protein in the experiments described above. Therefore, a study of the time course of production of the initiation protein was made. The inoculation sequence described in Materials and Methods for preparation of the concentrated culture supernatant fluids was used, with the determination of spore and total cell populations after specific incubation times.

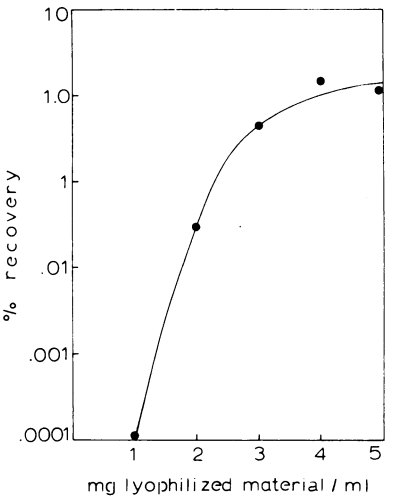

FIG. 2. *Effect of the concentration of C. perfringens ATCC 3624 lyophilized culture supernatant fluid containing an initiation protein on the recovery of heat-injured C. perfringens FD1 spores. Incubation of the TSN plating medium was at 37 C for 72 hr. See Materials and Methods for preparation of lyophilized culture supernatant fluid.*

Since older cultures had less recoverable lyophilized supernatant fluid on a weight basis than younger cultures, in assaying for the initiation protein, the percentage of the total lyophilized supernatant fluid (based on lyophilization of 800 ml) added to the plating medium was taken into account. Quantitation of the initiation protein as an equivalent amount of lysozyme was determined from a standard curve of the per cent recovery of heat-injured strain FD1 spores with known concentrations of lysozyme. Thus, micrograms of initiation protein per milliliter of culture supernatant fluid = equivalent amount of lysozyme ÷ (grams of lyophilized material used/grams of total recoverable lyophilized material) × 800 ml.

Figure 3 shows that the protein could be detected as early as 20 min after inoculation of vegetative cells from FTG growth medium into D-S sporulation medium. Concentration of the protein increased rapidly during the first hour of growth and then leveled off. About 3 hr separated the appearance of the protein and the detection of heat-resistant spores.

Studies on lysozyme-induced germination. The effect of lysozyme on the germination of unheated, heat-shocked, or heat-injured spores of strain FD1 in a liquid menstruum in the absence of metabolizable germinants is shown in Fig. 4. Slight germination was obtained after 48 hr with a lysozyme concentra-

TABLE 2. *Cross-stimulating action of the initiation protein from different C. perfringens strains on the recovery of heat-injured C. perfringens spores*

Addition to TSN recovery medium	Per cent recovery[a]			
	FD1	ATCC 3624	NCTC 8238	NCTC 8798
Lyophilized 11-hr culture supernatant fluid (4 mg/ml)				
FD1	0.48	0.06	0.12	0.58
ATCC 3624	1.0	0.16	1.0	1.4
NCTC 8238	0.03	0.003	0.001[b]	0.038
NCTC 8798	0.005	0.001	0.003[c]	0.01
No addition	0.0001	0.0001	0.0004	0.0007
Lysozyme (1 μg/ml)	8.0	1.3	1.1	1.5

[a] Per cent recovery of the test strains was determined from viable counts obtained after incubation at 37 C for 48 hr unless otherwise indicated. The number of viable spores obtained after heat shocking at 75 C for 20 min was considered 100% recovery.

[b] Per cent recovery after 72-hr incubation.

[c] Per cent recovery after 120-hr incubation.

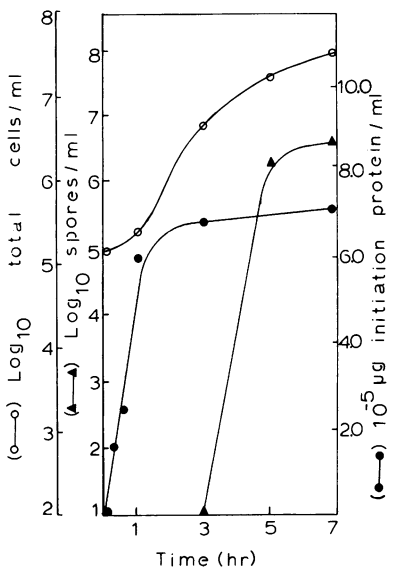

FIG. 3. *Kinetics of production of an initiation protein found in the culture supernatant fluid of C. perfringens ATCC 3624. See Results for method of assay for the protein. Heat-injured strain FD1 spores were used in assaying for the protein.*

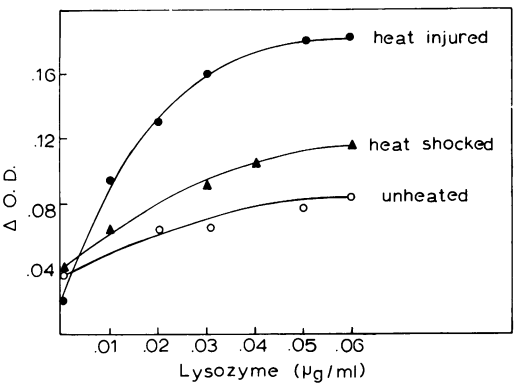

FIG. 4. *Effect of lysozyme on the germination of C. perfringens FD1 spores. Spores were incubated for 48 hr at 45 C in 47 mM phosphate buffer, pH 7.0, plus 30 μg of chloramphenicol/ml.*

tion as low as 0.02 μg/ml. Increases in the number of spores germinating were obtained by increasing the severity of heat treatment; with a lysozyme concentration of 0.06 μg/ml, a decrease in OD of 0.12 and 0.18 was obtained for heat-shocked and heat-injured spores, respectively. With heat-injured spores, this represented about 80 to 85% germination based on the decrease in OD of heat-shocked spores in a complex medium (2.5% Brain Heart Infusion plus 0.25% yeast extract) being taken as 100% germination. The 80 to 85% germination contrasts with the 6 to 8% recovery in the presence of lysozyme of heat-injured spores as determined by viable counts. It is evident that lysozyme can initiate germination of both viable and nonviable spores. In the solid plating medium, the action of lysozyme was reflected only by those spores which, after initiation by lysozyme, were viable and capable of outgrowth and colony formation.

Since even in the presence of lysozyme only a small percentage of heat-injured spores grew out, it is apparent that heat injuring the spores, in addition to rendering them lysozyme-dependent by blocking or inactivating the normal germination system, inactivated some other cellular function necessary for outgrowth. With alkali-treated spores, only the normal germination system was affected, since

the 90 to 95% germination and outgrowth obtained in the presence of lysozyme (see above) indicates almost complete viability of the spore populations.

The results on lysozyme-initiated germination are similar to those reported for *B. megaterium* ATCC 9885 (14), in which lysozyme (1.5 μg/ml) caused germination-like changes in untreated resting spores. In the present experiments, slight germination of heat-shocked spores was obtained after 48 hr with a lysozyme concentration as low as 0.02 μg/ml. Increasing the lysozyme concentration to 1.0 μg/ml resulted in germination of about 50% of the heat-shocked strain FD1 spores and about 40% of the heat-shocked NCTC 8798 spores within 120 min at 45 C and *p*H 7.0. Germination by lysozyme of spores of several other *Bacillus* and *Clostridium* species has been reported to occur only after a sensitization procedure with agents which ruptured spore coat disulfide bonds allowing access of lysozyme to the spore cortex (3). The cortex has been shown to be the site of the mucopeptide, which is thought to be the lysozyme substrate (15).

As evidence that lysozyme was acting directly (i.e., enzymatically) on spore cortical mucopeptide, the effect of lysozyme on heat-injured, alcian blue-treated spores was determined (Fig. 5). Alcian blue is known to complex with mucopeptide to inhibit lysozyme action by saturation of the lysozyme substrate (8). Lysozyme-induced germination was severely inhibited when the spores were pretreated with alcian blue, indicating the direct action of lysozyme on the spore mucopeptide.

Further proof of the direct action of lysozyme on the spore was obtained by comparing the ability of heat-injured or alkali-treated spores to germinate in the presence of lysozyme with their ability to germinate in the complex Brain Heart Infusion-yeast extract medium. In this medium, heat-shocked spores germinated quickly and nearly completely as observed by phase-contrast microscopy. Heat-injured or alkali-treated spores, on the other hand, exhibited very slight germination (less than 1%); yet, as shown above, heat-injured or alkali-treated spores were more susceptible to germination induced by low concentrations of lysozyme than unheated or heat-shocked spores. It is therefore unlikely that metabolizable germinants are involved in the lysozyme germination. Lysozyme probably acts directly on the cortical mucopeptide of the heat- or alkali-altered spores, resulting in spore germination by a not yet fully understood mechanism.

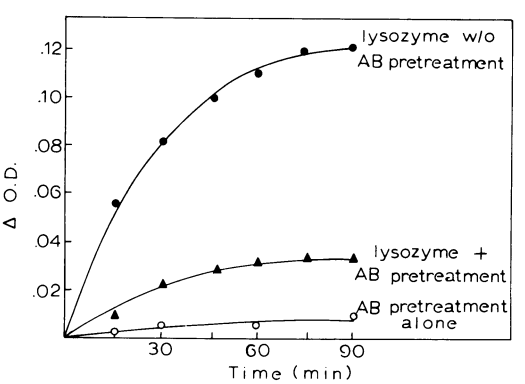

FIG. 5. *Inhibition of lysozyme-induced germination of heat-injured C. perfringens FD1 spores by pretreatment with alcian blue. Concentration of lysozyme was 2 μg/ml in 47 mM phosphate buffer, pH 7.0. See Materials and Methods for alcian blue treatment of spores. AB = alcian blue.*

The effect of *p*H and temperature on lysozyme-induced germination of heat-injured spores was investigated. Germination was greatest at alkaline *p*H values (Fig. 6). Maximal germination was at *p*H 10.0. These results are similar to those obtained with sensitized (by means of thioglycolic acid) spores of *B. cereus* (3).

Lysozyme-induced germination of the heat-injured spores was greatest at about 60 C (Fig. 7), which is in contrast to the results reported for *B. megaterium* ATCC 9885 spores where lysozyme-induced germination was not affected by a temperature as high at 70 C or a *p*H as low as 5.0 (14).

Non-heat-injured but heat-shocked spores of both strains FD1 and NCTC 8798 germinated rapidly, but not completely (45 and 75% germination, respectively, within 60 min) when the optimal conditions of *p*H 10.0, a temperature of 60 C, and a concentration of 1 μg of lysozyme/ml were used.

Germination of spores by the initiation protein found in C. perfringens culture supernatant fluid. The initiation protein acted similarly to lysozyme in the following respects: (i) it mediated recovery in solid agar of heat-injured spores of *C. perfringens*, its effect being concentration-dependent, and (ii) prolonged incubation was necessary for its action to be manifested completely. Further study revealed that this protein also acted at the level of germination, causing a decrease in OD of heat-injured FD1 spores in suspension, and that its activity was concentration-dependent (Fig. 8). However, at concentrations of the initiation protein yielding maximal germination,

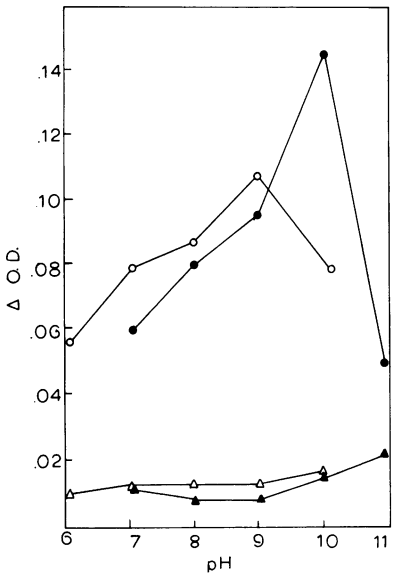

FIG. 6. *Effect of pH on lysozyme- or initiation protein-induced germination of heat-injured C. perfringens FD1 spores. The germination mixtures contained either 1 μg of lysozyme/ml or 15 mg of lyophilized culture supernatant fluid/ml in buffer plus 30 μg of chloramphenicol/ml. Buffers used were: phosphate, pH 6.0 and 7.0; tris(hydroxymethyl)aminomethane, pH 8.0; glycine-hydroxide, pH 9.0 and 10.0; and carbonate-hydroxide, pH 11.0. Final buffer concentration was 38 mM. Incubation times for lysozyme and the initiation protein were 60 min and 48 hr, respectively, at 45 C. Symbols: ●, with lysozyme; ○, with lyophilized 11-hr culture supernatant fluid; ▲, without lysozyme; △, with 0-hr lyophilized culture supernatant fluid.*

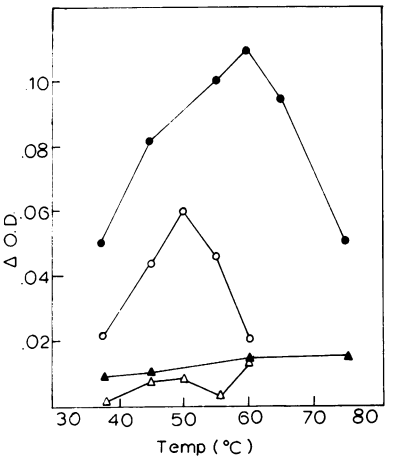

FIG. 7. *Effect of temperature on lysozyme- or initiation protein-induced germination of heat-injured C. perfringens FD1 spores. Lysozyme concentration was 1 μg/ml in 38 mM phosphate buffer, pH 7.0. Concentration of the lyophilized 11-hr culture supernatant fluid containing initiation protein was 15 mg/ml in pH 8.0, 38 mM tris(hydroxymethyl)aminomethane buffer plus 30 μg of chloramphenicol/ml. Incubation times were 60 min and 24 hr for lysozyme and the initiation protein, respectively. Symbols: ●, with lysozyme; ○, with lyophilized 11-hr culture supernatant fluid; ▲, without lysozyme; △, with 0-hr lyophilized culture supernatant fluid.*

the extent of germination of heat-injured spores was less than with lysozyme, i.e., 28% versus 85% germination. With alkali-treated spores, the extent of germination by the initiating protein was comparable to that obtained with lysozyme (90 to 95%). Essentially no germination occurred with the lyophilized 0-hr culture supernatant fluid.

Pretreatment of the heat-injured spores with alcian blue also blocked germination by the initiation protein (Fig. 8). The site of action of this protein, as in the case of lysozyme, is apparently the mucopeptide of the spore cortex.

The effect of *p*H and temperature on germination of heat-injured FD1 spores by the initiation protein present in the culture supernatant fluid was investigated. As with lysozyme, alkaline *p*H values were more effective in promoting germination than neutral or acidic values (Fig. 6). Maximal germination (50%) was at *p*H 9.0.

A temperature of 50 C was found to be most effective in initiating germination (Fig. 7). The extent of germination decreased markedly when the incubation temperature was increased to 60 C, which was the optimum for lysozyme-induced germination. However, with alkali-treated spores of strains FD1 and NCTC

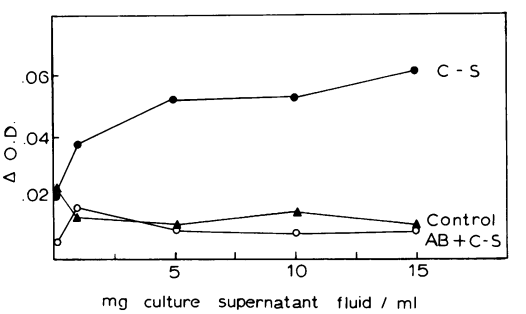

FIG. 8. *Effect of the concentration of C. perfringens ATCC 3624 initiation protein in lyophilized culture supernatant fluid on the germination of alcian blue-treated and untreated heat-injured C. perfringens FD1 spores. Incubation was at 45 C for 48 hr in 32 mM phosphate buffer, pH 7.0, and 30 μg of chloramphenicol/ml. Symbols: ●, C-S 11-hr lyophilized culture supernatant fluid; ▲, control, 0-hr lyophilized culture supernatant fluid; ○, AB + CS, alcian blue-treated spores plus 11-hr lyophilized culture supernatant fluid.*

8798, no difference in the extent of germination at the two different temperatures could be detected. The decreased activity at 60 C with heat-injured spores may have been due to some inactivation of the initiation protein, since its activity has been reported to be reduced about 50% by heating at 65 C for 30 min (1). With alkali-treated spores, lower concentrations of active protein would be required owing to a more effective breach in the coat permeability barrier.

Effect of lysozyme and an initiation protein from the culture supernatant fluid of C. perfringens ATCC 3624 on Micrococcus lysodeikticus cells. Cells of *M. lysodeikticus* are a widely used substrate for the assay of lysozyme activity. Experiments were conducted to compare the lytic activity of lysozyme and the initiation protein with *M. lysodeikticus* cells as substrate. Cells of *M. lysodeikticus* (Difco) were suspended at a final concentration of 0.17 mg/ml in 40 mM phosphate buffer (*p*H 7.0) at 45 C. The addition of lysozyme at a final concentration of 1 μg/ml resulted in rapid lysis of the cells as determined by a decrease in OD at 375 nm. Lyophilized 11-hr *C. perfringens* ATCC 3624 culture supernatant fluid at a final concentration of 15 mg/ml resulted in no decrease in OD after 2 hr.

With respect to their ability to induce lysis of *M. lysodeikticus*, lysozyme and the initiation protein are apparently different.

DISCUSSION

Cassier and Sebald first reported a lysozyme dependence for germination of heat-damaged spores of *C. perfringens* (1). However, the preparations used in their study contained a mixture of free spores, spores still in their sporangia, and vegetative cells. The possibility of indirect germination by products of the action of lysozyme on the sporangia could not be ruled out. Using a clean spore preparation, we have shown that lysozyme does act directly on the spore at the level of germination. In addition, the concentration of lysozyme required to reverse apparent thermal injury by inducing germination of the spores was one-tenth of that previously reported. The absence of vegetative cells or sporangial wall material presumably eliminated the competition of these mucopeptide-containing substrates for lysozyme and resulted in direct action on the spores by lower concentrations of lysozyme. Treatment of the spores with lysozyme prior to heat damage did not alter their lysozyme dependence (*unpublished results*).

Cassier and Sebald also reported that an initiation protein present in the culture supernatant fluid of *C. perfringens* ATCC 3624 was able to mimic the effect of lysozyme on heat-injured spores. Regardless of the heat treatment employed, however, they could not demonstrate reversal of heat injury if the spores of the test strain were heat-resistant. The present study has shown that thermal injury to a portion of a population of spores of both heat-resistant and heat-sensitive strains could be overcome by an initiation protein in the culture supernatant fluid of heat-resistant and heat-sensitive strains, as well as by lysozyme. The ability to demonstrate this effect was dependent on a critical time-temperature relationship for heat-injuring the spores. Treatment of the spores with alkali to remove an alkali-soluble coat layer mimicked the action of heat by rendering the spores dependent on lysozyme or the initiation protein for germination.

The existence of a spore-associated lytic enzyme was first shown by Strange and Dark (13). Presumably in the normal germination process this enzyme is somehow activated, perhaps by release from a binding site. It is believed that the enzyme then disrupts the spore cortex, resulting in subsequent germination. It seems reasonable to assume that such a lytic enzyme system should also be present in spores of *C. perfringens*, thus being involved in normal physiological germination of the spores. When spores are heat-injured, the level of such an enzyme may be reduced by inactivation of the enzyme or inactivation of some mechanism functioning in its release from a binding site. When spores are alkali-treated, some component of the normal lytic system is either inactivated or removed from the spore as a part of the alkali-soluble coat protein. In such cases, growth from spores (as measured by colony formation) becomes dependent on an exogenous lytic system, i.e., lysozyme or an initiation protein, for spore germination.

In this study, lysozyme and the initiation protein from the culture supernatant fluid of *C. perfringens* acted similarly in several respects. Slight differences were observed with regard to temperature and *p*H optima. In addition, whereas lysozyme caused rapid and complete lysis of *M. lysodeikticus* cells, the initiation protein had no effect on the same cells. Therefore, it seems that the two proteins produced similar end results on the bacterial spore, but the production of these results may have been mechanistically different. Clearly, further comparison, as well as identification of the function of the initiation protein, must await its purification and further study.

Lysozyme-induced germination of intact spores was first reported only recently (14), and until the lysozyme dependence of heat-injured *C. perfringens* was discovered, only *B. megaterium* ATCC 9885 spores had been found to be directly susceptible to germination by this enzyme without the necessity of a mercaptoethanol-urea sensitization treatment. In the present study, a portion of the population of intact spores of *C. perfringens* was found to be likewise susceptible to germination by lysozyme. However, with *C. perfringens*, lysozyme germination was dependent on *p*H and temperature, whereas with *B. megaterium* low *p*H (5.0) and high temperatures (70 C) had little effect on lysozyme activity.

Both the disulfide-rich protein and the alkali-soluble protein that presumably are important in determining the resistance of spores to enzymes (7) are thought to be located in the outer coat layers of the spore. Treatment with mercaptoethanol and urea, which breaks disulfide bonds, caused spores of various bacteria to become sensitive to lysis by lysozyme, a spore enzyme, and hydrogen peroxide; further treatment with alkali caused greater sensitization to these lytic agents (7). In the results reported in this paper, increasing the heat treatment given *C. perfringens* spores increased the extent of germination by lysozyme and by an initiation protein. Heat, in addition to inactivating the normal lytic system functioning in germination, apparently eliminates or damages whatever peremability barrier exists, presumably a spore coat layer, allowing a more direct and efficient access to the mucopeptide of the spore cortex. Alkali was more effective in eliminating the permeability barrier as evidenced by the greater extent of germination obtained with alkali-treated versus heat-injured spores. The possibility also exists that an alternative or additional mechanism might involve partial heat or alkali denaturation of the substrate, making it more susceptible to enzyme action.

Contrary to the results obtained by Gould et al. (7), in which the alkali treatment of mercaptoethanol-sensitized spores of a variety of *Bacillus* species only increased their sensitivity to exogenous lytic enzymes without affecting viability or heat resistance, *C. perfringens* spores were rendered strictly dependent on lysozyme or the initiation protein for germination and hence viability. Heat resistance of the spores was not affected by alkali treatment if indeed recovery was measured by use of a lysozyme-containing plating medium.

A critical time-temperature relationship exists in heat-injuring spores of *C. perfringens* sufficiently to produce a dependence on lysozyme or the initiation protein for germination and, in the proper environment, outgrowth. If heat injury is too severe, the spores are not prevented from germinating in the presence of lysozyme or the initiation protein, but they will not undergo outgrowth and colony formation and neither will they germinate in a complex medium without lysozyme or the initiation protein. These results indicate that an early, if not the first, manifestation of heat injury to the bacterial spore is inactivation of the normal lytic enzyme, or its release mechanism, functioning in cortical degradation. The fact that this normal lytic system may also be inactivated or removed by alkali without altering spore heat resistance would indicate the close association or location of a critical component of the system with the alkali-soluble coat protein. Gould et al. (5) previously reported that the normal lytic enzyme of *B. cereus* was probably bound on or within the central core of the spore. It is doubtful that the alkali treatment of *C. perfringens* spores would alter a lytic enzyme located deep within the spore in or on the core without also altering the spore's heat resistance. Either the lytic enzyme in *C. perfringens* is not located at the core but is associated with the alkali-soluble coat layer or an enzyme release mechanism alone is associated with the soluble coat layer. The spacial separation of the spore core from the alkali-soluble coat by the cortex and perhaps other coat layers would seemingly make the coat region a more logical area in the spore for location of the normal lytic system functioning in germination. The fact that inactivation of the lytic system is an initial manifestation of heat injury would also argue for its location in a coat layer. If it were core-associated, it should be better protected from heat injury and would be heat-inactivated only along with the heat inactivation of enzymes concerned with outgrowth of new vegetative cells. At least with spores of *C. perfringens*, this does not appear to be the case.

Additional studies are in progress to investigate the specific nature of the heat- and alkali-induced alterations of the spore permeability barriers and of the normal lytic enzyme system functioning in germination.

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