

MICROCOPY RESOLUTION TEST CHART  
NATIONAL BUREAU OF STANDARDS-1963-A

Unclassified

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

12

ADA 124485

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER 16648.5-LS	2. GOVT ACCESSION NO. AD-A124485	3. RECIPIENT'S CATALOG NUMBER
TITLE (and Subtitle) Purification and Properties of <u>Clostridium</u> <u>Perfringens</u> Spore Lytic Enzymes		5. TYPE OF REPORT & PERIOD COVERED Final: 16 Oct 79 - 15 Oct 82
AUTHOR(s) Ronald G. Labbe		6. PERFORMING ORG. REPORT NUMBER
PERFORMING ORGANIZATION NAME AND ADDRESS University of Massachusetts Amherst, MA 01003		8. CONTRACT OR GRANT NUMBER(s) DAAG29 79 C 0191
CONTROLLING OFFICE NAME AND ADDRESS U. S. Army Research Office Post Office Box 12211 Research Triangle Park, NC 27709		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS
MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)		12. REPORT DATE Jan 83
		13. NUMBER OF PAGES 23
		15. SECURITY CLASS. (of this report) Unclassified
		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE
16. DISTRIBUTION STATEMENT (of this Report) Approved for public release; distribution unlimited.		
17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)		
18. SUPPLEMENTARY NOTES The view, opinions, and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy, or decision, unless so designated by other documentation		
19. KEY WORDS (Continue on reverse side if necessary and identify by block number) germination clostridium perfringens spores enzymes		
20. ABSTRACT (Continue on reverse side if necessary and identify by block number) The project has dealt with the mechanism of germination of <u>Clostridium</u> <u>perfringens</u> spores, specifically enzymes involved in this process. The object was to purify two lytic enzymes which cause germination of these spores. One is an extracellular enzyme (Initiation Protein) produced during growth; the other is part of the spore itself and is somehow activated during germination.		

DTIC  
FEB 16 1983  
H

DTIC FILE COPY

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

83 02 014 228

**PURIFICATION AND PROPERTIES OF CLOSTRIDIUM PERFRINGENS**

**SPORE LYTIC ENZYMES**

**Ronald G. Labbe**

**7 January 1983**

**U.S. Army Research Office**

**Contract DAAG29-79-C-0191**

**For Period 16 October 1979 - 15 October 1982**

**University of Massachusetts**

**Approved for Public Release:**

**Distribution Unlimited**

The project has dealt with the mechanism of germination of Clostridium perfringens spores, specifically enzymes involved in this process. The object was to purify two lytic enzymes which cause germination of these spores. One is an extracellular enzyme (Initiation Protein) produced during growth; the other is part of the spore itself and is somehow activated during germination. The following were achieved during the contract period:

1. We developed an assay for the Initiation Protein (IP) and for the spore-associated lytic enzyme. This involved the use of "bald" spores. The spores were stripped of their exterior protein coat. As a result they were very sensitive to the enzymes in question. The most effective method for obtaining such spores was treating them with dithiothreitol and sodium dodecyl sulfate.
2. The IP was partially purified from 7 hr culture supernatant fluid of cells of C. perfringens. A 3100-fold purification was achieved after cellulose-phosphate and Sephadex G-100 chromatography. The isolated IP had an apparent molecular weight of 100,000 and an isoelectric point of 7.9; it was reversibly inhibited by HgCl<sub>2</sub> and lysed isolated cortical fragments from C. perfringens spores. The partially purified material produced 3 bands following disc gel electrophoresis. Activity was associated with one of the three bands. A broad pH optimum of between 7 and 9 was found. The IP was sensitive to heat and pronase, indicating its protein nature.
3. Several alternate methods for purifying or concentrating the IP were explored. These included isoelectric focusing, and hydroxylapatite chromatography for purification and polyethylene glycol, rotary evaporation, ethanol precipitation and others for concentration. Only ethanol precipitation was judged to be a useful addition to the original protocol.
4. In an attempt to increase the concentration of IP in the starting material we modified the growth medium to include additional sodium phosphate and glucose. This increased IP levels about 30%.
5. The other enzyme we have studied is spore-associated and more difficult to obtain. Our final protocol involved treating sodium-nitrite germinated spores with urea-mercaptoethanol at pH 3. More activity was extracted by this method than any other. The crude, isolated enzyme had a pH optimum of 5.5 and was sensitive to proteolytic enzyme. Partial purification was obtained by treating the crude enzyme with sodium phosphate to remove contaminating proteins. To concentrate this enzyme several techniques were explored. Only flowing air dehydration in dialysis sacs was effective. Further purification was obtained using carboxymethylcellulose and Sephadex G-100 chromatography. At this point the purified produce contained one major and one minor protein, with a final fold-purification of 1800.



Dist	Special	codes
A		

**List of Publications - copies attached**

1. Franceschini, T. and Labbe, R. 1979. Sensitivity of chemically treated spores of Clostridium perfringens type A to an initiation protein. Microbios 25:85-91.
2. Gombas, D. and Labbe, R. 1981. Extraction of spore lytic enzyme from Clostridium perfringens spores. J. General Microbiology 126:37-44.
3. Labbe, R., Tang, S. and T. Franceschini. 1981. Partial purification and characterization of an initiation protein for germination from Clostridium perfringens spores. Biochimica et Biophysica Acta 678:329-333.

**Scientific Personnel**

Dr. Ronald G. Labbe

Dr. Shirley Tang

David Gombas - earned Ph.D. while working on project

## Sensitivity of chemically treated spores of *Clostridium perfringens* type A to an initiation protein

Thomas J. Franceschini and Ronald G. Labbe

Department of Food Science and Nutrition, University of Massachusetts, Amherst, Massachusetts 01003, U.S.A.

### Abstract

Extraction of *Clostridium perfringens* type A spores with dithiothreitol (DTT), DTT plus sodium dodecyl sulphate (DTT-SDS), urea-mercaptoethanol (UME), or alkali, solubilized from 18.6 to 46.5 of the total dry weight of spores. The initiation of germination and lysis of such treated spores with lysozyme and an initiation protein (IP) from the culture supernatant fluid of sporulating cells of *C. perfringens* was studied under various conditions. The ability of lysozyme and the crude IP to induce germination and lysis of extracted spores was concentration dependent up to 0.5 µg/ml and 5.6 mg/ml respectively. IP showed an optimum of activity between pH 7 and 8 for DTT-SDS and DTT extracted spores, and between pH 6 and 9 for UME extracted spores. The optimum temperature of activity for IP was 55°C. Dissimilarities in the extent to which lysozyme and the IP initiated germination and lysis of spores extracted by various methods may have been a reflection of the differences in amounts of protein solubilized by each treatment.

### Introduction

Gould and Hitchins (1963) first reported that bacterial spores were sensitive to lysozyme after treatment with various physical and chemical agents which ruptured the disulphide bonds of spore coat protein. Since then lysozyme-initiated germination of heat-injured *Clostridium perfringens* spores has been demonstrated (Cassier and Sebald 1969; Duncan *et al.*, 1972; Adams, 1973). Treatment of *C. perfringens* spores with reducing agents that removed spore coat layers (Cassier and Ryter, 1971; Labbe *et al.*, 1978) also rendered these spores sensitive to lysozyme. The removal or alteration of spore coat protein presumably allowed lysozyme to penetrate and attack the mucopeptide of the spore cortex (Gould and Hitchins, 1963).

The action of lysozyme on heat injured *C. perfringens* spores was mimicked by a factor, termed 'initiation protein', which was produced by sporulating *C. perfringens* cells (Cassier and Sebald, 1969, Duncan *et al.*, 1972). This factor was not produced by vegetative cells. In this report, we describe the effects of this initiation protein on chemically extracted spores of *C. perfringens* and demonstrate its similarity to lysozyme in this regard. In addition, several factors influencing the activity of initiation protein are presented.

### Materials and methods

#### Culture methods

*C. perfringens* type A strain, National Collection of Type Cultures (NCTC) 8798 (Hobbs serotype 9), was inoculated (2 drops) from cooked meat medium (Difco) into 20 ml of fluid thioglycollate medium. The latter was heated for 10 min at 75°C, cooled with tap water and incubated overnight (16 h) at 37°C. The entire culture was then inoculated into 2,000 ml of Duncan and Strong (DS) sporulation medium (Duncan and Strong, 1968), modified by replacing starch with 0.4% raffinose and

incubated at 37°C. After 24 h the culture (containing spores free of their sporangia) was cooled to approximately 4°C to minimize spore germination during harvesting. The culture was then harvested by centrifugation at 16,000 × g for 15 min at 4°C. The spores were washed repeatedly (10 to 12 times) with cold distilled water until a spore suspension containing a total of less than 5% germinated spores and vegetative cells was obtained, as confirmed by phase contrast microscopy. The spores were frozen as a pellet until further use.

#### Preparation of initiation protein

Concentrated culture supernatant fluid (CSF) containing the initiation protein (IP) was prepared from 7 h cultures of *C. perfringens* NCTC 8798 grown in DS sporulation media as described above. Cultures were centrifuged as described above and the supernatant fluid was concentrated by overnight dialysis against polyethylene glycol (Carbowax 20,000, Sigma) at 4°C. A ten-fold concentrate was obtained and was stored frozen until used.

#### Extraction procedure

Clean spores were suspended in 25 ml of cold distilled water and sonified briefly to disrupt clumps. The suspension was adjusted to an optical density (OD: Bausch and Lomb Spectronic 20) at 600 nm of 1.0 (dry weight: 7.0 mg/ml). After centrifugation at 12,000 × g for 10 min, the spores were resuspended in 25 ml of the various reagents described below. Following extraction with gentle stirring for 2 h at 37°C (1 h for UME, and 30 min at 4°C for NaOH treated spores) the mixture was centrifuged at 12,000 × g for 10 min. The spores were washed three to five times with cold distilled water and then frozen until used. The supernatant fluid was dialysed overnight at 2°C in distilled water (except in the case of dithiothreitol-sodium dodecyl sulphate DTT-SDS which was dialysed at room temperature), and protein content was determined by the method of Lowry *et al.* (1951).

The spores were extracted by four different methods (a) 0.05 M DTT, (b) 0.05 M DTT plus 0.5% (w/v) SDS, both prepared in 0.05 M glycine-NaOH buffer, with the pH adjusted to 10.0 with NaOH, (c) 8 M urea plus 1.0% (v/v) mercaptoethanol (UME), pH 8.5, and (d) 0.1 N NaOH.

Table 1 Extraction of protein from spores of *C. perfringens*

Extraction method	Dry weight of spores (%)
NaOH	18.8
UME	31.0
DTT	38.8
DTT-SDS	46.5

Spores were extracted as described in Methods.

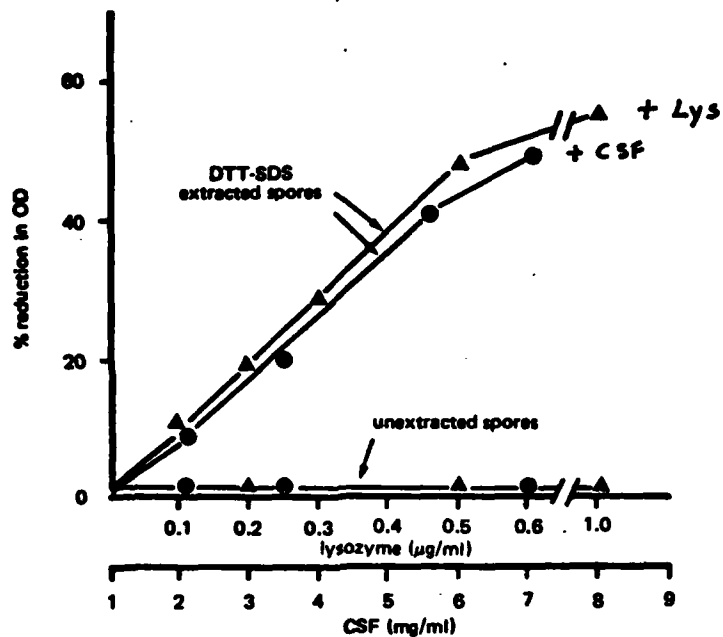


Figure 1 Effect of the concentration of CSF and lysozyme on the initiation of germination and lysis of DTT-SDS extracted spores of *C. perfringens*. Assay conditions: pH 6.0, 42 mM citrate buffer, 5 min at 45°C.

#### Assessment of sensitivity of extracted spores

Germination and lysis of the extracted spores were measured as the decrease in OD (600 nm) using a Spectronic 20. To 2.5 ml of the appropriate buffer, a sufficient amount of extracted spores was added to give an initial OD of 0.40 to 0.45. After pre-incubation of the spore suspension at the appropriate temperature for 2 to 3 min, 0.5 ml of CSF or lysozyme was added. The mixture was incubated at the selected temperature, and the decrease in OD was recorded during a 5 min period. The percent reduction in OD was determined from the linear portion of the curve, after subtraction of the appropriate blanks.

#### Results and discussion

Table 1 presents the amounts of protein extracted from *C. perfringens* spores by four procedures. From 18.6% to 46.5% of the total spore dry weight was solubilized using NaOH and DTT-SDS, respectively. Cassier and Ryter (1971) reported that *C. perfringens* spores of this strain extracted with UME or DTT were devoid of spore coats. DTT-SDS

treated spores retained a thin layer of coat material. One would expect that such structural differences would be reflected in a chemical analysis (reported here) of the extracted coat material which is protein in nature. Yet our results show that the greatest amount of solubilization occurred using DTT-SDS. A possible explanation is that these authors used spores produced in a sporulation medium containing starch, while spores used in the present study were grown in the presence of raffinose. Electron microscopic observations of thin sections of sporulating cells grown in the presence of raffinose reveal that these spores often contain an additional, although fragmentary, spore coat layer, and are larger than starch-grown spores (Labbe and Rufner, unpublished results). Such differences may account for the unexpected results reported here.

Spores extracted with DTT-SDS were treated with different concentrations of lysozyme and CSF. Figure 1 shows that the percentage reduction in OD was proportional to the amount of lysozyme or CSF added up to a concentration of 0.5  $\mu\text{g/ml}$  and 5.6 mg/ml respectively. The reaction was extremely rapid. At a concentration of 7 mg of CSF per ml a 50% decrease in OD had occurred within 5 min. Reactions were equally rapid using lysozyme. Microscopic examination of reaction mixtures revealed that nearly all extracted spores treated with CSF (initiation protein) or lysozyme had lysed immediately following germination. The reduction in OD reached 90–95% after prolonged (10 min at 45°C) incubation. Lysis of chemically or heat injured *C. perfringens* spores following lysozyme treatment has not been reported previously

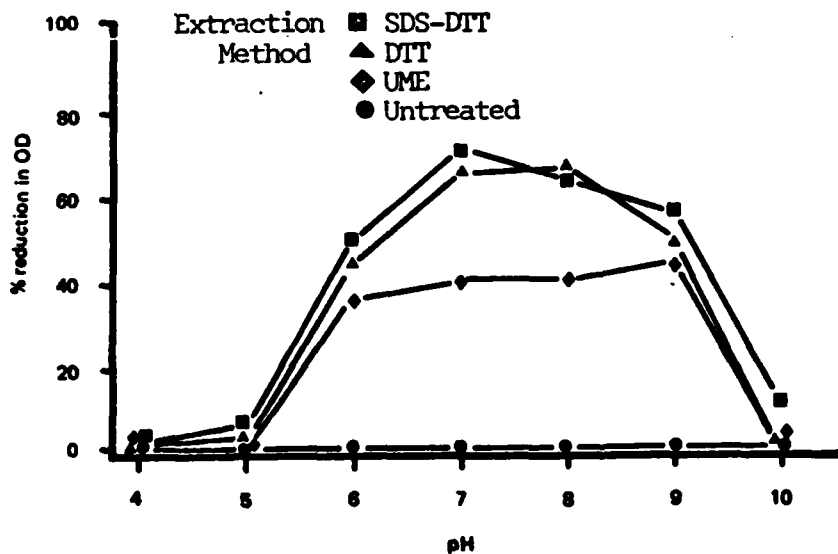


Figure 2 Effect of pH on initiation of germination and lysis of extracted spores of *C. perfringens* by CSF. Concentration of CSF was 7.0 mg/ml in each of the following buffers: citrate, pH 4.0, 5.0 and 6.0; phosphate 7.0; Tris (hydroxymethyl) aminomethane (Tris), pH 8.0; glycine-hydroxide, pH 9.0 and 10.0. Final buffer concentration, 42 mM. Incubated for 5 min at 45°C.

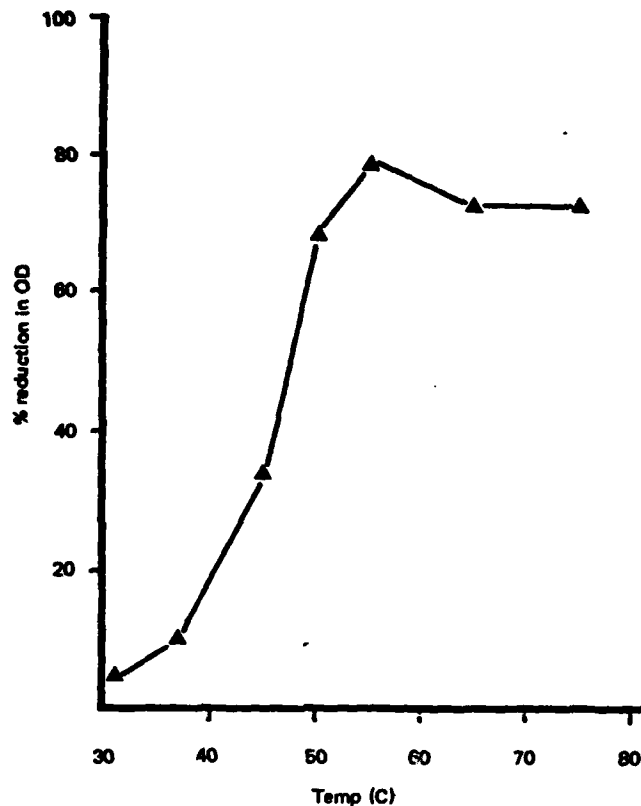


Figure 3 Effect of temperature on initiation of germination and lysis of DTT-SDS extracted spores of *C. perfringens* by CSF. Final CSF concentration, 7.0 mg/ml in 42 mM citrate buffer, pH 6.0. Incubated for 5 min, except at 65°C and 75°C which were incubated for 2 min.

(Cassier and Ryter, 1971; Duncan *et al.*, 1972). These workers had only reported on the effectiveness of lysozyme in promoting germination. This sensitivity to lytic agents following extraction of spore coat layer(s) emphasizes the protective role of this spore structure. Untreated spores were resistant to both lysozyme and IP (Figure 1). Heated (75°C, 15 min) preparations of CSF were ineffective in promoting germination and lysis.

IP was able to promote germination and lysis of chemically treated spores over a broad pH range (Figure 2). An optimum between pH 7 and 8 was observed for DTT and DTT-SDS extracted spores while UME extracted spores allowed a much broader optima between pH 6 and 9. The relative effectiveness of initiation protein on spores extracted by different methods, as reflected in the extent of germination and lysis, may have been a function of the amount of protein solubilized by each method (Table 1). Spores extracted by DTT or DTT-SDS, which exhibited the greatest percentage

reduction in OD, released larger amounts of coat protein than spores treated with UME NaOH. NaOH extracted the least amount of protein, and such treated spores were not affected by initiation protein at any pH. In a study of proteins solubilized following chemical treatment of *Bacillus thuringiensis* spores, Somerville *et al.* (1970) also found that the degree of lysozyme sensitivity (germination) of such extracted spores was dependent on the method of extraction.

The effect of temperature on germination and lysis of spores extracted with DTT-SDS is shown in Figure 3. Incubation of the reaction mixture at 55°C gave the optimum response although the activity of initiation protein remained high at 65°C and 75°C. Denaturation of the CSF precluded assays above 75°C.

It is apparent that the actions of lysozyme and the initiation protein contained in CSF can affect altered spores of *C. perfringens* quite differently depending on the type of chemical treatment to which the spores have been subjected. In addition, both can promote recovery and, more specifically, promote germination of heat injured spores of this species (Cassier and Sebald, 1969; Duncan *et al.*, 1972; Adams, 1973). Yet, as reported here, when spores are treated with agents that remove coat layers, lysozyme and IP cause not only germination but also cell lysis. In this respect the IP behaves similarly to lysozyme-like products produced by other micro-organisms (Okada and Kitahata, 1973; Takahara *et al.*, 1974; Morita *et al.*, 1978; Ochi *et al.*, 1978). However, it is not a true lysozyme (N-acetylmuramidase) since it is ineffective against *Micrococcus lysodeikticus* cells (Duncan *et al.*, 1972). Furthermore, characterization of the biochemical nature of the IP awaits its purification.

#### Acknowledgements

This work constitutes paper no. 2258, Massachusetts Agricultural Station, University of Massachusetts at Amherst. The research was supported in part by Experiment Station Project no. 406, by a grant from the Research Council of the University of Massachusetts at Amherst, and by the Army Research Office.

#### References

- ADAMS D. M. 1973. Inactivation of *Clostridium perfringens* type A spores at ultra-high temperatures. *Appl. Microbiol.* 26 282-7.
- CASSIER M. and RYTER A. 1971. Sur un mutant de *Clostridium perfringens* donnant des spores sans tuniques à germination lysozyme-dépendante. *Ann. Inst. Pasteur* 121 717-32.
- CASSIER M. and SEBALD M. 1969. Germination lysozyme-dépendante des spores de *Clostridium perfringens* ATCC 3624 après traitement thermique. *Ann. Inst. Pasteur* 117 312-24.
- DUNCAN C. L., LABBE R. G. and REICH R. R. 1972. Germination of heat and alkali-altered spores of *Clostridium perfringens* type A by lysozyme and an initiation protein. *J. Bact.* 109 550-9.
- DUNCAN C. and STRONG D. 1968. Improved medium for sporulation of *Clostridium perfringens*. *Appl. Microbiol.* 16 82-9.
- GOULD G. W. and HITCHINS A. D. 1963. Sensitization of bacterial spores to lysozyme and to hydrogen peroxide with agents which rupture disulphide bonds. *J. gen. Microbiol.* 33 413-23.

- LABBE R., Reich R. and Duncan C. 1978. Alteration in ultrastructure and germination of *Clostridium perfringens* type A spores following extraction of spore coats. *Can. J. Microbiol.* 24 1526-36.
- LOWRY O. H., Rosebrough N. J., Farr A. L. and Randall R. J. 1951. Protein measurement with Folin phenol reagent. *J. biol. Chem.* 193 265-75.
- MORITA T., Saburo H. and Matsushima Y. 1978. Purification and characterization of lysozyme produced by *Streptomyces erythraeus*. *J. Biochem.* 83 893-903.
- OCHI N., Azegami M. and Ishii S. 1978. Bacteriolytic enzyme induced from pyocinogenic *Pseudomonas aeruginosa*. *J. Biochem.* 83 727-36.
- OKADA S. and Kitahata S. 1973. Purification and some properties of bacterial lysozyme. *J. Ferm. Tech.* 51 701-12.
- SOMERVILLE H., Delafield F. and Rittenberg S. 1970. Urea-mercaptoethanol-soluble protein from spores of *Bacillus thuringiensis* and other species. *J. Bact.* 101 551-60.
- TAKAHARA Y., Machigaki E. and Maruo S. 1974. General properties of endo-N-acetylmuramidase of *Bacillus subtilis* YT 25. *Agr. Biol. Chem.* 38 2357-65.

Accepted 15 June 1979

## Extraction of Spore-lytic Enzyme from *Clostridium perfringens* Spores

By DAVID E. GOMBAS AND RONALD G. LABBE\*

Food Microbiology Laboratory, Department of Food Science and Nutrition, University of Massachusetts, Amherst, Massachusetts 01003, U.S.A.

(Received 10 December 1980; revised 17 February 1981)

Various chemical reagents known to extract spore coat protein were used to extract spore-lytic enzyme (SLE) from intact and germinated spores of *Clostridium perfringens*. Of the reagents tested, 7.2 M-urea plus 10% (v/v) mercaptoethanol, pH 2.85, solubilized the most SLE activity per mg spores. The quantity of SLE extracted was dependent on the initial pH of the reagent, with a maximum between pH 2.7 and 3.0. Germinated spores yielded more SLE than non-germinated spores upon urea/mercaptoethanol extraction. SLE release during spore germination probably utilizes a trigger mechanism not satisfied by germination alone. Significant amounts of SLE were released during germination when spores were suspended in potassium chloride or a complex germinant mixture containing brain-heart infusion, yeast extract and chloramphenicol, but not during germination with sodium nitrite, which non-enzymically lysed the cortical peptidoglycan. Greater solubilization of SLE activity was obtained by urea/mercaptoethanol extraction of spores germinated with nitrite than of spores germinated with either potassium chloride or the complex germinant.

### INTRODUCTION

Powell & Strange (1953) postulated the presence of an enzymic lytic system in bacterial spores and its role during germination. Since then, the spore-lytic enzymes of *Bacillus cereus* have been isolated and extensively studied by Strange & Dark (1957), Gould *et al.* (1966), Warth (1972) and Brown *et al.* (1975, 1977, 1978). In most cases, this lytic system was found to hydrolyse the peptidoglycan of the spore cortex, resulting in germination-like changes in the spore. Warth (1972) found that *B. cereus* spores contain four separate cortex-degrading enzymes. Brown *et al.* (1977) reported that cortex-lytic activities were localized in both the core and in the coat fraction, and these could be isolated separately by particular extraction procedures. Further evidence for the association of cortex-lytic (germination) enzyme with the coat fraction follows from the observation that, in several cases, treatment of dormant spores with reagents which remove spore coat have resulted in a reduced ability of those spores to germinate. This has occurred with, for example, urea/mercaptoethanol (UME) treatment of *Bacillus megaterium* (Vary, 1973), alkaline dithioerythritol/sodium dodecyl sulphate treatment of *B. cereus* (Aronson & Fitz-James, 1976), dithiothreitol/urea or alkaline treatment of *Clostridium bifermentans* (Wyatt & Waites, 1974), and alkaline treatment (Duncan *et al.*, 1972) or alkaline dithiothreitol treatment of *Clostridium perfringens* (Labbe *et al.*, 1978). These observations could be the result of the cortex-lytic enzymes being either solubilized and removed or simply inactivated by the treatments. Indeed, Brown *et al.* (1978) have extracted and purified a cortex-lytic enzyme from *B. cereus* spores using urea/mercaptoethanol and guanidine/mercaptoethanol. However, it is not clear whether this enzyme plays the only or a major role in germination

since they were unable to observe germination-like changes in sensitized spores following treatment with this enzyme.

On the other hand, very little has been published on the lytic systems of other bacterial spores. In particular, a similar cortex-lytic enzyme was only recently reported in *C. perfringens* by Ando (1979), who found that spores germinated in the presence of potassium chloride at pH 7.0 exuded an enzyme capable of hydrolysing cortical fragments and causing germination of alkali-treated (coat-altered) spores.

In this study, several of the methods known to extract bacterial spore coats were tested for their ability to extract active spore-lytic enzyme from *C. perfringens* spores.

#### METHODS

**Organism.** *Clostridium perfringens* NCTC 8798, Hobbs serotype 9 (H9) was originally obtained from C. Duncan, University of Wisconsin.

**Media and sporulation.** Spores of *C. perfringens* H9 were grown and cleaned as described by Franceschini & Labbe (1979). Final spore suspensions contained less than 1% vegetative cells, as determined by phase-contrast microscopy.

**Preparation of germinated spores.** Cleaned spores were suspended in 25 mM-sodium phosphate buffer, pH 6.0, and heated at 75 °C for 15 min. For spores germinated with nitrite (N germinated spores), suspensions were brought to 0.1 M-sodium nitrite in the same buffer and a final spore concentration of about 5 mg (initial dry wt) ml<sup>-1</sup>, and held at 45 °C for 45 min; at this time microscopic examination showed greater than 95% phase-dark spores. For 'non-germinated' spores, suspensions were treated similarly but the nitrite was omitted; less than 10% of these spores became phase-dark. For potassium chloride-germinated spores (K-germinated spores), sodium phosphate buffer and sodium nitrite were replaced by 50 mM-potassium phosphate buffer, pH 7.0, and 50 mM-potassium chloride. Spores germinated in a complex medium (CG-germinated spores) were prepared by incubation in a final concentration of 5.0% (w/v) brain-heart infusion plus 0.5% (w/v) yeast extract plus 30 µg chloramphenicol ml<sup>-1</sup>. Greater than 95% of the spores germinated by these methods were sensitive to heating at 75 °C for 15 min.

**Enzyme extraction.** After germination, spores were pelleted by centrifugation at 3020 g for 10 min, washed once with cold sterile distilled water, and resuspended in one of the extraction reagents at 5.8 mg (initial dry wt) ml<sup>-1</sup>. The 'acidic' extraction reagents included 7.2 M-urea (U), 3.6 M-guanidine.HCl (G), 10% (v/v) mercaptoethanol (ME), 50 mM-dithiothreitol (DTT), 7.2 M-urea plus 10% (v/v) mercaptoethanol (UME) and 3.6 M-guanidine.HCl plus 10% (v/v) mercaptoethanol (GME). The 'alkaline' reagents included UME, DTT and ME. Spores were extracted at 45 °C for 90–120 min, except for spores in reagents containing guanidine which were extracted for 60 min only (see below). After extraction the spores were removed by centrifugation at 3020 g for 10 min and the supernatant fluid was dialysed overnight against distilled water at 4 °C before assaying for enzyme activity and protein concentration. In some experiments enzyme activity was assayed during germination. In these cases, samples were taken at specified intervals, the spores were removed by centrifugation as above, and the supernatant was assayed directly without prior dialysis.

In some cases, spore pellets were re-extracted with acidic UME for 120 min, centrifuged as above, and the re-extracted material was dialysed overnight before assaying for activity and protein.

**Enzyme assay.** Spore-lytic enzyme (SLE) activity was assayed using spores stripped of their coat protein, as described by Franceschini & Labbe (1979). These were prepared by treating clean spores with 50 mM-dithiothreitol plus 1% (w/v) sodium dodecyl sulphate, pH 10.0, at 37 °C for 2 h followed by extensive washing with distilled water. Stripped spores were suspended in either 25 mM-sodium phosphate, 25 mM-Tris/maleate or 25 mM-maleate buffer, pH 6.0, to a turbidity of 0.4 to 0.5 at 600 nm. Assays were performed at 37 °C in a Beckman 25 spectrophotometer equipped with a kinetic system. Activity was determined from the linear decrease in turbidity. One unit (U) of activity was defined as a decrease in  $A_{600}$  of 0.01 min<sup>-1</sup>.

In some experiments, cortical fragments, prepared from strain 8798 spores by the method of Hashimoto *et al.* (1972), were used in place of stripped spores, and the turbidity was monitored at 400 nm.

**Protein assay.** Protein was assayed by the method of Bradford (1976), using crystalline bovine serum albumin as a standard.

**Chemicals.** Urea, mercaptoethanol, dithiothreitol, chloramphenicol and bovine serum albumin were obtained from Sigma. Guanidine.HCl was from Eastman Kodak, sodium nitrite was from J. T. Baker Chemical Co. (Phillipsburg, N.J., U.S.A.), and potassium chloride and sodium thioglycollate were from Fisher Scientific (Fairlawn, N.J., U.S.A.). Brain-heart infusion and yeast extract were obtained from Difco. All chemicals were reagent grade.

Table 1. Extraction of spore-lytic enzyme from non-germinated and nitrite-germinated spores of *C. perfringens*

Clean spores were heated (75 °C, 15 min), then either incubated in sodium phosphate buffer (non-germinated spores) or germinated in sodium nitrite (N-germinated spores) as described in Methods. Spores were washed once in cold distilled water before extraction. Extracted spores were removed by centrifugation and the extracts were dialysed overnight before assaying for protein and SLE activity. Reagents: U, 7.2 M-urea; G, 3.6 M-guanidine.HCl; ME, 10% (v/v) mercaptoethanol; DTT, 50 mM-dithiothreitol; UME, 7.2 M urea plus 10% (v/v) mercaptoethanol; GME, 3.6 M-guanidine.HCl plus 10% (v/v) mercaptoethanol.

Extraction procedure		Non-germinated spores			N-germinated spores		
Reagent	pH	Protein (mg ml <sup>-1</sup> )	Activity (U ml <sup>-1</sup> )	Specific activity (U mg <sup>-1</sup> )	Protein (mg ml <sup>-1</sup> )	Activity (U ml <sup>-1</sup> )	Specific activity (U mg <sup>-1</sup> )
NaPO <sub>4</sub>	6.0	0.05	0.1	NM			
NaNO <sub>2</sub>	6.0				0.05	0.2	NM
UME	8.3	0.12	0.4	2.9	0.28	0.1	NM
DTT	8.3	0.30	0.6	1.8	0.32	0.1	NM
U	3.0	0.15	1.7	11	0.17	2.8	16
G	3.0	0.05	0.4	NM	0.05	0.1	NM
ME	3.0	0.47	140	290	0.15	0.1	NM
UME	3.0	1.00	170	170	1.10	320	290
GME	3.0	0.06	0.9	15	0.67	68	100
DTT	3.0	0.16	4.3	27	0.19	0.1	NM

NM, Not measurable.

## RESULTS AND DISCUSSION

### Extraction of spore-lytic enzyme (SLE)

Non-germinated spores and nitrite-germinated spores (N-germinated spores) were extracted with sodium phosphate, sodium nitrite and reagents known to extract spore coat protein (Table 1). Detectable SLE activity was found in all extracts of non-germinated spores, with acidic urea/mercaptoethanol (UME) and mercaptoethanol (ME) extractions yielding the highest specific activity (170 and 290 U mg<sup>-1</sup>, respectively). Extraction of non-germinated spores with dithiothreitol/sodium dodecyl sulphate, pH 10.0, did not yield detectable activity (data not shown).

Extraction of N-germinated spores failed to yield significant enzyme activity except with acidic UME and guanidine/mercaptoethanol (GME). The specific activity obtained using acidic UME (290 U mg<sup>-1</sup>) was comparable with that obtained when non-germinated spores were extracted with acidic ME, but the SLE activity extracted from the N-germinated spores was more than twice that obtained from the non-germinated spores (320 versus 140 U ml<sup>-1</sup>). No enzyme activity was detected in the supernatant fluid of heated (75 °C, 15 min), intact spores or N-germinated spores.

The surprisingly high SLE activity obtained from N-germinated spores prompted an investigation into the extraction of spores germinated by means other than nitrite. These germinants were KCl (Ando, 1979) and a 'complex germinant' (CG). Like nitrite, both of these germinants led to rapid germination without observable outgrowth. However, unlike the supernatant fluid from N-germinated or non-germinated spores, the germination exudates with these germinants had significant levels of SLE activity (94 and 11 U ml<sup>-1</sup> for CG- and K-germinated spores, respectively; Table 2). Spores germinated in these media yielded significant SLE activity only when extracted with acidic ME, U and UME. The latter extracted the highest concentration of SLE regardless of the method of germination. As in the case of N-germinated spores, extraction of CG- or K-germinated spores with alkaline reagents did not yield significant levels of active SLE, nor did extraction with DTT, pH 3.0.

Table 2. Extraction of spore-lytic enzyme from CG- and K-germinated spores of *C. perfringens*

Clean spores were heated (75 °C, 15 min) and germinated in either complex germinant (CG) or potassium chloride (K) as described in Methods. Extraction conditions and reagents were as in Table 1. Four K germinated spore pellets were collected after extraction and each was re-extracted in UME, pH 3.0, for 120 min. Both the re-extracted material and the initial extracts were treated identically.

Extraction procedure	Reagent	pH	CG-germinated spores			K-germinated spores			Re-extracted K-germinated spores		
			Protein (mg ml <sup>-1</sup> )	Activity (U ml <sup>-1</sup> )	Specific activity (U mg <sup>-1</sup> )	Protein (mg ml <sup>-1</sup> )	Activity (U ml <sup>-1</sup> )	Specific activity (U mg <sup>-1</sup> )	Protein (mg ml <sup>-1</sup> )	Activity (U ml <sup>-1</sup> )	Specific activity (U mg <sup>-1</sup> )
CG		7.0	0.08	94	1200						
K		7.0				NM	11	NM			
ME		8.5	0.38	0.2	<1	0.41	0.2	0.5			
UME		8.5	0.63	<0.1	<1	0.98	<0.1	<1	0.08	47	600
DTT		8.5	0.49	1.0	2	0.68	0.5	1			
U		3.0	0.06	22	370	0.10	58	580			
G		3.0	0.12	2.0	17	0.11	NT	NM			
ME		3.0	0.13	72	550	0.10	130	1300	0.11	300	2700
UME		3.0	0.23	110	470	0.16	300	1900	0.41	68	170
GME		3.0	0.11	1.0	9	0.09	0.5	6	0.32	200	630
DTT		3.0	NM	0.2	NM	0.04	0.9	20			

NM, Not measurable; NT, not tested.

Alkaline UME treatment of K-germinated spores produced about fivefold more protein than acidic UME treatment (0.98 versus 0.16 mg ml<sup>-1</sup>). When both such treated spores were re-extracted with acidic UME, those spores which had originally been extracted with acidic UME continued to release both protein and active SLE, although at a much lower total activity than obtained during the first extraction (68 versus 300 U ml<sup>-1</sup>). Those spores which had previously been extracted with alkaline UME did not release much more protein upon re-extraction (0.08 versus 0.98 mg ml<sup>-1</sup>) but did release more enzyme (47 versus <0.1 U ml<sup>-1</sup>), i.e. about the same as that released by re-extraction of spores originally extracted with acidic UME. If alkaline UME had been unable to extract SLE specifically, then high levels of SLE might have been expected upon re-extraction with acidic UME. As this was not the case, it is likely that, although effective in solubilizing spore proteins, alkali solubilizes SLE in an irreversibly inactive form. Acidic UME re-extraction of spores previously extracted with acidic ME and GME indicated that a substantial amount of active enzyme remained in the spore after the initial extraction with these reagents. Consequently, as with non-germinated or N-germinated spores, acidic UME was the best of the reagents tested for extraction of active SLE. SLE extracted by UME hydrolysed isolated cortical fragments of strain 8798 (data not shown).

Brown *et al.* (1978) reported that extraction of *B. cereus* with acidic UME produced inconsistent levels of SLE, while GME worked well. We found the contrary to be true for *C. perfringens*. Acidic UME extraction of N-germinated spores yielded a continuous increase in active SLE solubilized during 120 min, while GME extracts, first assayed at 60 min, showed a 40% loss of SLE activity by 120 min (data not shown). Unlike UME extractions, GME extractions were very dependent on the ratio of reagent to spores, losing activity more rapidly at greater dilutions. For example, N-germinated spores ( $7 \times 10^9$ ) extracted in 8.0 ml GME for 60 min yielded only 56% of the active SLE obtained when the same number of spores were extracted in 4.0 ml GME. Further, SLE activity in the more dilute system decreased 80% by 120 min.

The loss of activity upon prolonged extraction of spores in GME was not surprising, since this compound is an active protein denaturant. Urea acts in the same way yet, for some

reason did not irreversibly inactivate SLE. These reagents probably act by peeling away layers of spore coat after disulphide cross-links, which secure the coat structure, are broken by mercaptoethanol. It is not known if SLE remained active during extraction, or was denatured by UME and renatured during dialysis. UME extracts assayed before dialysis did not display any activity, but since SLE activity is sharply inhibited by high ionic strength (D. E. Gombas & R. G. Labbe, unpublished data) it was not possible to distinguish between these possibilities.

#### *Effect of pH on extraction of SLE*

It was apparent that, of the methods tested, acidic UME yielded the highest activity of SLE. This was true for both non-germinated and germinated spores. UME has been used by a number of researchers for extracting spore coats, and the importance of pH was demonstrated by Gould & Hitchins (1963). We therefore examined the effect of the initial pH on extraction of protein and enzyme activity from N-germinated spores. The pH of UME was found to be critical in the extraction of SLE. The highest SLE activities were extracted at pH values of 2.7 and 3.0 (data not shown). At pH values below 2.3 or above 3.5, both extractable activity and protein dropped off dramatically (10% and <0.5% of optimum activity, respectively). Gould & Hitchins (1963) have reported this sharp pH range for extraction of spore coat material in *B. cereus*. They suggested that it might be because spores are reported to have a net zero charge around pH 3, and that this could influence the accessibility or reactivity of spore coat disulphide bonds. Brown *et al.* (1977) have suggested that this pH range is effective because mercaptoethanol is most effective as a reducing agent around pH 3. A starting pH of 2.85 was selected for all further studies.

#### *Influence of the sulphhydryl reagent on extraction of SLE*

Urea (7.2 M) was included with 1.3 M-DTT or 1.3 M-sodium thioglycollate or 2.6 M (20%) ME, and each was compared with 7.2 M-urea plus 1.3 M (10%) ME (UME) for their ability to extract protein and SLE from N-germinated spores. Increasing the concentration of ME did not significantly affect the amount of protein or SLE extracted (data not shown). Similar levels of protein and enzyme activity were obtained when urea plus DTT was used in place of UME.

The importance of the sulphhydryl reagent is not yet clear. Although acidic ME alone was capable of extracting significant amounts of SLE, DTT was not. When coupled with urea, both sulphhydryl reagents responded synergistically, and with equal efficiency. However, urea plus thioglycollate, pH 2.85, was incapable of extracting active SLE, or significant amounts of protein.

#### *Release of SLE during germination*

As previously mentioned, after germination with KCl or CG, a significant level of SLE activity was detected in the exudate. Figure 1 shows the kinetics of release of this activity from spores germinated with KCl, NaNO<sub>2</sub> or CG. Microscopic examination at each sampling time showed that greater than 95% of KCl- and NaNO<sub>2</sub>-treated spores had germinated within 30 and 45 min, respectively. However, only 60 U ml<sup>-1</sup> was released by germination with KCl and none with NaNO<sub>2</sub>. No SLE activity was detected following incubation of heat-shocked or unheated spores (non-germinated) in sodium phosphate buffer (data not shown). Although the initial rate of SLE release and phase-darkening in CG occurred more slowly than in KCl, the final activity approached 40 times that of the K-germinated spores. The presence of chloramphenicol did not significantly affect the levels of SLE released by spores germinated in CG, but did prevent spore outgrowth (data not shown). As with UME extracts, the KCl exudate and the CG exudate were active in hydrolysing cortical fragments. These preparations were not characterized further.

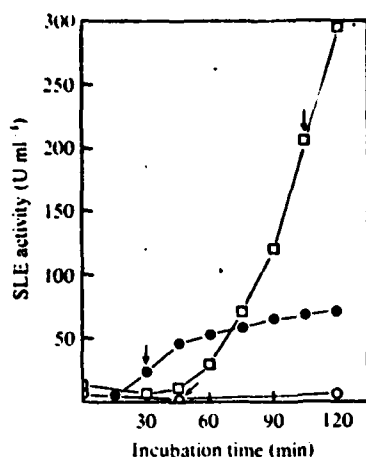


Fig. 1. Release of SLE in exudate during germination of *C. perfringens* spores. Clean spores were heated (75 °C, 15 min) and germinated at 45 °C with sodium nitrite (○), potassium chloride (●) or complex germinant (□) as described in Methods. At intervals, samples were centrifuged and the supernatants were assayed for SLE activity without prior dialysis. Arrows indicate the times at which at least 95% of spores were phase-dark.

Since SLE, present in the germination exudates of K- and CG-germinated spores, was absent from the supernatant of N-germinated spores, some relevant properties of NaNO<sub>2</sub> were explored. It was possible that NaNO<sub>2</sub> might inactivate SLE during germination. However, SLE did not lose any activity upon holding for up to 1 h in NaNO<sub>2</sub>, pH 6.0, at concentrations as high as 160 mM (final nitrite concentration during SLE assay was 32 mM). Ando (personal communication) has suggested that nitrite will non-enzymically hydrolyse spore peptidoglycan at pH 4.0. We tested this in our system by incubating cortical fragments in the presence of increasing concentrations of NaNO<sub>2</sub>, pH 6.0, and found that there was a significant increase in the rate of turbidity decrease with NaNO<sub>2</sub> at concentrations above 50 mM. In assay spore suspensions with 200 mM NaNO<sub>2</sub>, turbidity decreased at a rate of  $7.4 \times 10^{-3} A_{600} \text{ units min}^{-1}$ ; this was 16 times faster than in the absence of NaNO<sub>2</sub>. When incubated in 200 mM-KNO<sub>2</sub>, cortical fragments and assay spores both showed similar rates of decrease in turbidity to those observed with NaNO<sub>2</sub>, but neither cortical fragments nor assay spores showed a significant decrease in turbidity when incubated in 200 mM NaCl or KCl (data not shown). Further, NaNO<sub>2</sub> had a 10-fold higher spore-lytic activity at pH 6.0 than at pH 8.0 (data not shown), indicating the protonated form of nitrite was the active agent. Our results therefore support Ando's findings, i.e. that nitrite, or nitrous acid, will non-enzymically lyse spore cortex, and thereby cause spores to germinate.

With this system, it is clear that germination, by itself, is necessary but not sufficient for release of SLE from the spore. The latter requires a release mechanism which seems to be satisfied by CG, only partially satisfied by KCl and not satisfied by NaNO<sub>2</sub> at all.

Germination with CG most closely approaches germination of spores in typical *C. perfringens* growth media. Therefore, gradual release of SLE during germination would be expected to aid in the germination of nearby spores which are deficient in this enzyme, or injured, e.g. by heat, but permeable enough to accept the enzyme. This could explain the presence of 'secondary' or satellite colonies which frequently appear on plates of heat-injured *C. perfringens* spores after prolonged incubation (Cassier & Ryter, 1971).

#### Kinetics of release of SLE by UME

As shown in Tables 1 and 2, the highest SLE extraction was obtained by extracting spores using acidic UME. However, it was not directly clear whether these conditions affected the

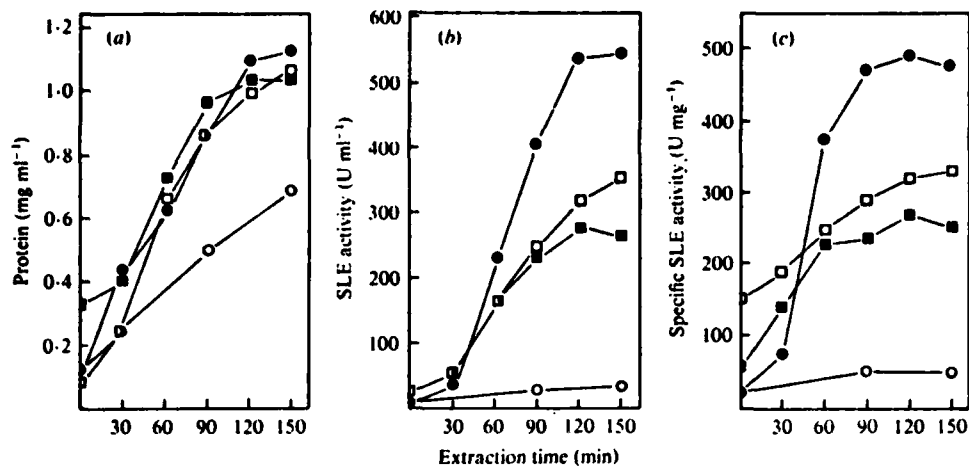


Fig. 2. Kinetics of release of SLE during UME extraction of *C. perfringens* spores. Clean spores were heated (75 °C, 15 min) and either 'non germinated' (○), or germinated with sodium nitrite (●), potassium chloride (□) or complex germinant (■), as described in Methods, until at least 95% of spores were phase-dark. Spores were washed once in cold distilled water, and extracted with UME, pH 2.85, at 45 °C. Samples were dialysed overnight before assaying for protein (a), SLE activity (b), or specific SLE activity (c).

quantity of active SLE extractable by UME. The kinetics of release of protein and SLE from non-germinated and N-, K- and CG-germinated spores are shown in Fig. 2. Germination was required for maximum protein extraction (Fig. 2a), but the germinant used did not significantly affect either the rate or extent of protein extraction. However, the germinant used did significantly affect the total activity extracted. The greatest amount of SLE, expressed as U ml<sup>-1</sup> or U mg<sup>-1</sup>, which could be extracted with UME was obtained from spores germinated with nitrite. K- and CG-germinated spores yielded less activity (Fig. 2b, c). While UME was capable of extracting protein from the non-germinated spores at about half the rate of germinated spores, extraction of active SLE was even more retarded, resulting in a maximum specific activity only 10% of that obtained from N-germinated spores in 2 h.

Both Gould *et al.* (1966) and Ando (1979) have suggested that germination is a necessary step for maximum release of SLE from spores. Results presented here support this observation, as extraction of non-germinated spores yielded far less protein and active enzyme. The reason for this is not known. Although Gould *et al.* (1966) could not obtain evidence that this is due to activation of zymogens, they suggested it might be due to release of the enzyme from a bound form. Release of SLE in the presence of chloramphenicol suggests that *de novo* synthesis during germination is not responsible for the disparity in active enzyme levels. This is further supported by the results of Cleveland & Gilvarg (1975), who found that spores of *B. megaterium lys* mutants were equally capable of germination in the presence or absence of exogenous lysine. Further, UME extraction of vegetative cells prepared in Fluid Thioglycollate Medium, a glucose-rich asporogenic growth medium, did not yield detectable activity (data not shown), indicating that extractable SLE is not a product of vegetative cell metabolism.

It is not yet possible to say that the SLE released from the spore during germination is the same protein as the SLE extracted by UME. Indeed, Warth (1972) identified four separate cortex-degrading activities in spores of *B. cereus*. However, it is interesting to note that, although the differences are not stoichiometric, the germinated spores which exude the least amount of SLE, i.e. N-germinated spores, yield the greatest concentration of SLE upon UME extraction, and that the converse is also true.

This work was supported by the U.S. Army Research Office and by Experiment Station Project no. 484.

## REFERENCES

- ANDO, Y. (1979). Spore lytic enzyme released from *Clostridium perfringens* spores during germination. *Journal of Bacteriology* **140**, 59-64.
- ARONSON, A. I. & FITZ-JAMES, P. C. (1976). Structure and morphogenesis of the bacterial spore coat. *Bacteriological Reviews* **40**, 360-402.
- BRADFORD, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248-254.
- BROWN, W. C. & CUHEL, R. L. (1975). Surface-localized cortex-lytic enzyme in spores of *Bacillus cereus* T. *Journal of General Microbiology* **91**, 429-432.
- BROWN, W. C., CUHEL, R. L. & GREER, C. (1977). Isolation and properties of a surface-bound cortex-lytic enzyme from spores of *Bacillus cereus* T. In *Spore Research 1976*, pp. 335-349. Edited by A. Barker, L. Wolf, D. J. Ellar, G. J. Dring & G. W. Gould. London: Academic Press.
- BROWN, W. C., VELLOM, D., SCHNEFF, E. & GREER, C. (1978). Purification of a surface-bound hexosaminidase from spores of *Bacillus cereus* T. *FEMS Microbiology Letters* **3**, 247-251.
- CASSIER, M. & RYTER, A. (1971). Sur un mutant de *Clostridium perfringens* donnant des spores sans tuniques a germination lysozyme-dependante. *Annales de l'Institut Pasteur* **117**, 312-324.
- CLEVELAND, E. F. & GILVARG, C. (1975). Selective degradation of peptidoglycan from *Bacillus megaterium* spores. In *Spores VI*, pp. 458-464. Edited by P. Gerhardt, R. N. Costilow & H. L. Sadoff. Washington, D.C.: American Society for Microbiology.
- DUNCAN, C. L., LABBE, R. G. & REICH, R. R. (1972). Germination of heat- and alkali-altered spores of *Clostridium perfringens* type A by lysozyme and an initiation protein. *Journal of Bacteriology* **109**, 550-559.
- FRANCESCHINI, T. J. & LABBE, R. G. (1979). Sensitivity of chemically treated spores of *Clostridium perfringens* type A to an initiation protein. *Microbios* **25**, 85-91.
- 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.
- GOULD, G. W., HITCHINS, A. D. & KING, W. L. (1966). Function and location of a 'germination enzyme' in spores of *Bacillus cereus*. *Journal of General Microbiology* **44**, 293-302.
- HASHIMOTO, T., FRIEBEN, W. & CONTI, S. (1972). Kinetics of germination of heat-injured *Bacillus cereus* spores. In *Spores V*, pp. 409-415. Edited by H. O. Halvorson, R. Hanson & L. L. Campbell. Washington, D.C.: American Society for Microbiology.
- LABBE, R. G., REICH, R. R. & DUNCAN, C. L. (1978). Alteration in ultrastructure and germination of *Clostridium perfringens* type A spores following extraction of spore coats. *Canadian Journal of Microbiology* **24**, 1526-1536.
- POWELL, J. F. & STRANGE, R. E. (1953). Biochemical changes occurring during germination of bacterial spores. *Biochemical Journal* **54**, 205-209.
- STRANGE, R. & DARK, F. (1957). A cell-wall lytic enzyme associated with spores of *Bacillus* species. *Journal of General Microbiology* **16**, 236-249.
- VARY, J. C. (1973). Germination of *Bacillus megaterium* spores after various extraction procedures. *Journal of Bacteriology* **116**, 797-802.
- WARTH, A. (1972). Action of spore lytic enzymes on the cortex. In *Spores V*, pp. 28-34. Edited by H. O. Halvorson, R. Hanson & L. L. Campbell. Washington, D.C.: American Society for Microbiology.
- WYATT, L. & 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.

BBA 29778

## PARTIAL PURIFICATION AND CHARACTERIZATION OF AN INITIATION PROTEIN FOR GERMINATION FROM *CLOSTRIDIUM PERFRINGENS* SPORES

RONALD G. LABBE, SHIRLEY S. TANG and THOMAS J. FRANCESCHINI

Food Microbiology Laboratory, Department of Food Science and Nutrition and Agricultural Experiment Station, University of Massachusetts, Amherst, MA 01003 (U.S.A.)

(Received May 18th, 1981)

**Key words:** Spore germination; Initiation protein; (*Cl. Perfringens*)

An initiation protein which promoted germination of dithiothreitol-sodium dodecyl sulfate-treated spores of *Clostridium perfringens* was purified from 7-h culture supernatant fluid of cells of *Cl. perfringens*. A 3100-fold purification was obtained after cellulose-phosphate and Sephadex G-100 chromatography. The isolated product had an apparent molecular weight of 100 000, an isoelectric point of 7.9, was reversibly inhibited by HgCl<sub>2</sub> and lysed isolated cortical fragments from *Cl. perfringens* spores.

In 1969 Cassier and Sebald [1] reported that heat-injured spores of 'heat-sensitive' strains of *Clostridium perfringens* which were otherwise non-viable could germinate and form colonies if the plating medium contained lysozyme (EC 3.2.1.17). The same phenomenon occurred when sterile culture filtrates of heat-sensitive spore strains grown in a sporulation medium replaced lysozyme in the recovery medium. Duncan et al. [2] extended these observations to include spores of 'heat-resistant' strains and indicated that it was at the germination stage that lysozyme and the lytic-like factor from the culture filtrate, termed initiation protein, acted. Greater than a 10 000-fold recovery of spores from thermal injury was obtained using lysozyme or initiation protein. This phenomenon has not been reported for spore-formers of other bacterial species. This prompted us to investigate further the biochemical properties of initiation protein. We report here partial purification and some characteristics of this protein.

### Materials and Methods

**Organism.** *Cl. perfringens*, NCTC 8798 (Hobbs serotype 9) was originally obtained from C. Duncan, University of Wisconsin. Mutants were obtained from

M. Sebald, Pasteur Institute, Paris.

**Preparation and assay of initiation protein.** Culture supernatant fluid containing the initiation protein was prepared from the sporulation medium of Duncan and Strong [4] which was modified by the addition of 0.25% glucose. In addition, the phosphate concentration was raised to 2%. The assay involved the measurement of the germination rate of *Cl. perfringens* NCTC 8798 spores which had been pre-treated to remove spore coat protein [3].

**Chromatography.** To 8 l 7-h culture supernatant fluid of modified medium was added 8 l distilled, deionized water. To this was added sufficient amount of a slurry of charged cellulose phosphate (Sigma Chemical Co.) to bind all initiation protein activity. The cellulose phosphate had been prepared by the addition of 500 ml Buffer A (10 mM sodium phosphate (pH 6.5)/0.075 M NaCl/0.02% sodium azide) to 20 g charged cation exchanger. The material was stirred at room temperature for 30 min. After decanting the unbound culture supernatant fluid, the cellulose phosphate was poured into a 2.6 × 45 cm column and washed free of 280 nm absorbing material (about 2 l) with Buffer A. The column was eluted with buffer B (10 mM sodium phosphate (pH 6.5)/0.2 M NaCl/ 0.02% sodium azide) at a flow rate of 1.6 ml/

min with collection of 12-ml fractions. Positive fractions were pooled and concentrated to about 3 ml by ultrafiltration through PM-10 membranes (Amicon Corp.) under nitrogen pressure. This material was applied to a column (2.6 × 58 cm) of Sephadex G-100 equilibrated with Buffer C (50 mM sodium citrate (pH 6.2)/0.02% sodium azide). The column was then eluted with Buffer C (2-ml fractions, 1.0 ml/min). All chromatography was done at 20°C.

**Isoelectric focussing.** Analytical isoelectric focusing was performed using 10-cm tubes of 7.5% acrylamide (2.5% bisacrylamide). The pH range of 3–10 was achieved with 0.24% (w/v) solutions of Bio-Lyte ampholytes (Bio-Rad Laboratories) using 0.06 N H<sub>2</sub>SO<sub>4</sub> and 0.02 N Ca(OH)<sub>2</sub> plus 0.04 N NaOH as the anode and cathode electrolytes, respectively. 750 μg of the cellulose phosphate-purified, ultrafiltration-concentrated material was mixed with the acrylamide before polymerization. Focusing was carried out for 2 h at 200 V at 4°C. The gels were sliced in 0.5-cm sections and eluted in 1 ml deionized, distilled water or 0.5 ml 50 mM citrate buffer, pH 6, for determination of pH and initiation protein activity, respectively.

**Gei Electrophoresis.** Disc gel electrophoresis was performed at pH 8.9 and 4.3 as described by Maurer [5] using Pyronin Y as the tracking dye. 25 μg protein were applied to the gel. One gel was sliced in 0.5-cm sections and eluted overnight in 0.5 ml 50 mM citrate buffer, pH 6, at 4°C for determination of initiation protein activity. Another gel was stained with Coomassie Blue G-250.

**Molecular weight.** The molecular weight of the protein was obtained by gel filtration on Sephadex G-100 (2.6 × 58 cm) according to the method of Andrews [6]. The void volume was determined with Blue Dextran. Protein standards used were: lysozyme, 14 400; ovalbumin, 43 000; bovine serum albumin, 67 000 and alcohol dehydrogenase, 141 000.

**Protein assay.** Protein concentration was determined by the method of Bradford [7].

**Cortical fragments.** These were prepared by the method of Hashimoto et al. [8] from spores of *Cl. perfringens* NCTC 8798.

## Results

**Purification.** Initial purification involved elution of the crude culture supernatant fluid from a cellulose-

phosphate column with 0.2 M NaCl (Fig. 1). Approx. 1.6 g protein was applied to the column. Protein activity was eluted on the rear side of the major absorbancy peak with a 653-fold purification and a 49% recovery of activity. Fractions containing more than 600 activity units/ml were concentrated by ultrafiltration. This step reduced the yield to 23% and increased purification to 1220-fold.

The concentrated, partially purified initiation protein was passed through a Sephadex G-100 column (Fig. 2). Protein activity was located on the tail side of the major protein peak. Fractions 59–62 containing the majority of the activity were pooled. In an attempt to stabilize the activity, glycerin (1%) and sodium thioglycollate (0.1%) were included in the elution and equilibration buffers. However, these reduced activity levels and were not used further. A summary of the purification procedure is given in Table I. A final 9% yield with 3400-fold purification was obtained. Disc gel electrophoresis (pH 4.3) revealed the presence of two bands (Fig. 3, insert). A third diffuse band was also faintly visible.

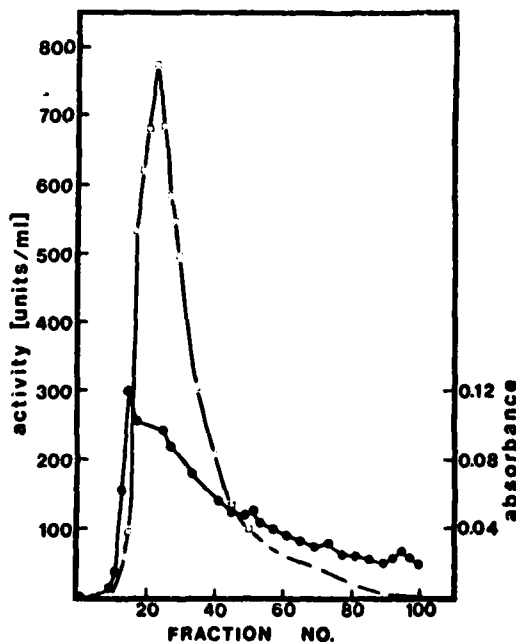


Fig. 1. Chromatography of 7 h culture supernatant fluid of *Cl. perfringens* NCTC 8798 on cellulose phosphate. ○—○, activity; ●—●, A<sub>280</sub>.

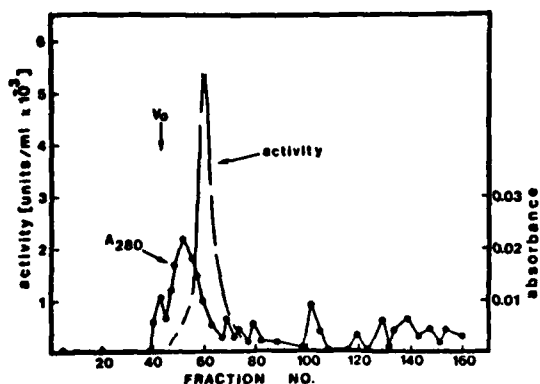


Fig. 2. Chromatography of partially purified initiation protein from cellulose phosphate on a column of Sephadex G-100.  $V_0$ , void volume.  $\square$ — $\square$ , activity;  $\bullet$ — $\bullet$ ,  $A_{280}$ .

#### Properties of initiation protein

**Thermal stability.** Sephadex G-100 material was concentrated by ultrafiltration and placed at low temperatures. Initiation protein held at 4°C slowly lost activity during a 9-day period (Table II). Activity was also lost when the material was held at -20°C, about half during frozen storage for 5 months. Heating at 65°C resulted in a 17% loss of activity. No activity remained after heating at 75°C for 20 min.

**Effect of sulfhydryl agents.** (Table III) Three sulfhydryl active reagents were tested for their effect on the protein.  $\text{HgCl}_2$  completely removed activity but its inhibition was partially reversible by thioglycollate. *p*-Chloromercuriphenyl sulfonate inhibited 51% of the activity. Its inhibition was also partially reversible. On the other hand, *N*-ethylmaleimide was ineffective.

**Lysis of cortical fragments.** Fig. 3 shows the

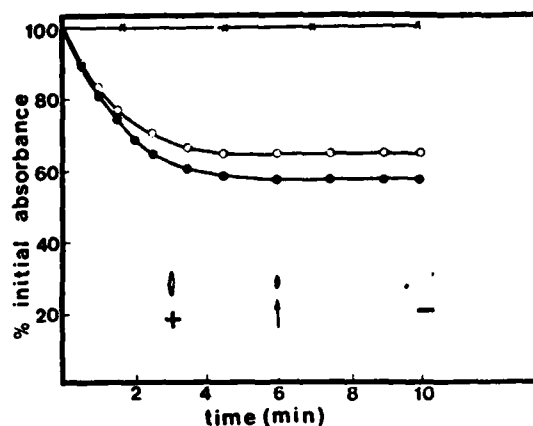


Fig. 3. Lysis of cortical fragments of *Cl. perfringens* spores by lysozyme and initiation protein. Insert: polyacrylamide gel electrophoresis of Sephadex G-100-purified initiation protein. The protein was obtained following elution of the gel slice corresponding to arrow ( $R_f = 0.48$ ). Assay conditions: 42 mM citrate buffer, pH 6.0, at 45°C. Concentrations of lysozyme and initiation protein were 100  $\mu\text{g}/\text{ml}$  and 750 units/ml, respectively. Initial absorbance was 0.5. X—X, control, o—o, gel; •—•, lysozyme.

effect of initiation protein and lysozyme on lysis of isolated cortical fragments. Treatment of this material with the protein resulted in a 35% decrease in absorbance within 5–6 min. As expected, lysozyme was very effective in hydrolyzing the cortical material. In this case 40% of the initial absorbance was lost within 4 min.

**Molecular weight.** The  $K_{av}$  value obtained for the Sephadex G-100-purified protein corresponded to an apparent molecular weight of 100 000 (data not shown).

**Other characteristics.** Both divalent and monovalent cations inhibited protein activity to various

TABLE I

SUMMARY OF THE PURIFICATION OF INITIATION PROTEIN FROM *C. PERFRINGENS*

Purification	Total activity (U $\times 10^{-3}$ )	Total protein (mg)	Spec. act. (U/mg $\times 10^{-3}$ )	Purification	Yield (%)
Culture supernatant fluid	288	1 600	0.18	—	100
Cellulose-phosphate chromatography	141	1.2	117.5	653	49
Concentration by ultrafiltration	66	0.3	220	1 220	23
Sephadex G-100 chromatography	26.8	0.048	558	3 100	9

TABLE III  
INACTIVATION OF INITIATION PROTEIN BY TREATMENT WITH SULFHYDRYL REAGENTS

Activity (initially 95–110 units/ml) was measured in the presence or absence of 10 mM sodium thioglycollate. Inhibitors were added to assay mixture before addition of initiation protein. Sodium thioglycollate was mixed with the protein for 1 min before their addition to the assay mixture. Abbreviation, *p*-CMPS, *p*-chloromercuriphenylsulfonic acid.

Treatment <sup>a</sup>	Activity (% of original)	
	Thioglycollate absent	Thioglycollate present
None	100	100
HgCl <sub>2</sub> (0.1)	0	67
<i>p</i> -CMPS (1.0)	49	70
<i>N</i> -Ethylmaleimide (5)	95	99

<sup>a</sup> Concentration in mM given in parenthesis.

extents. Those tested included MgCl<sub>2</sub>, MnCl<sub>2</sub>, CoCl<sub>2</sub>, CaCl<sub>2</sub>, LiCl, NaCl, KCl, CsCl and NH<sub>4</sub>Cl, all at 1 mM. It is likely that this inhibition is an ionic strength effect. A broad pH optimum of between 6 and 7 was found. Activity fell off sharply on either side of this range. Isoelectric focusing on polyacrylamide gels revealed an isoelectric point of 7.9. Finally, activity

TABLE II  
THERMAL STABILITY OF INITIATION PROTEIN

Initiation protein activity was measured at pH 6.0. The reaction mixture contained 110–160 U/ml initially. Comparisons were with untreated material.

Refrigeration (4°C)	% Original activity
Refrigeration (4°C)	
1 day	100
4 days	63
9 days	27
Freezing (-20°C)	
4 days	57
5 months	47
Heat (°C) <sup>a</sup>	
45	100
55	97
65	83
75	0

<sup>a</sup> Samples were heated for 20 min at indicated temperatures.

was completely lost following a 30-min treatment with pronase (50 µg/ml) at 37°C (data not shown).

## Discussion

Agents such as calcium and starch have long been known to promote recovery of bacterial spores from thermal injury but none approach the effectiveness of lysozyme or initiation protein with regard to *Cl. perfringens* spores [2]. The protein is similar to lysozyme in that it causes germination and promotes dramatic recovery of heat-injured *Cl. perfringens* spores. As reported here both products hydrolyze isolated cortical fragments of this organism. They differ in terms of isoelectric point, pH optimum, molecular weight and, as previously reported, in the inability of initiation protein to lyse *Micrococcus lysodeikticus* cells [2], the substrate used for lysozyme assays.

The source of initiation protein in this study was 7-h cultures of *Cl. perfringens* cells. An intriguing question is whether this protein is a sporulation-specific product. We have noticed that the greater the percentage of sporulating cells in the culture the greater the concentration of the protein in the crude supernatant fluid. On the other hand we have also observed that a mutant blocked at Stage 0 of sporulation still produced initiation protein in modified sporulation medium (Tang, S. and Labbe, R.G., unpublished data). Further, little sporulation (less than 10%) was observed in the cultures used as a source of the protein in this study. However, initiation protein is not produced by vegetative cells growing in a glucose-rich medium [2]. Perhaps its production is a function of the slow growth rate associated with cells growing in modified sporulation medium. The use of a chemostat would be useful in resolving this question.

The results reported here suggest that initiation protein is an enzyme which causes lysis of the spore cortex. Production of a lytic enzyme by *Cl. perfringens* is not surprising since lytic enzymes from *Bacillus* sp. have been isolated from culture supernatant fluid [9–11] or by extraction of intact or disrupted cells [12–17]. Such lytic enzymes have been implicated in the turnover of cell wall peptidoglycan during growth [18–25], in the separation of daughter cells at the end of cell division [21,22], in the pro-

cessing of spore cortex during sporulation [14,17], in the release of the mature bacterial spore from its sporangium [23,24] and in spore germination [25, 26]. Initiation protein may be involved in these processes but in its ability to promote recovery from thermal injury (reflected here in its ability to lyse cortical fragments) it probably acts to replace the naturally occurring spore-lytic enzyme which may be inactivated by heat. In this way heat-injured spores could be germinated by (and subsequently outgrow) the exogenously added protein. We are attempting to isolate and purify spore-lytic enzyme from intact spores in an attempt to compare it with initiation protein.

#### Acknowledgement

This work was supported by U.S. Army Research Office.

#### References

- 1 Cassier, M. and Sebald, M. (1969) *Ann. Inst. Pasteur (Paris)* 117, 312-324
- 2 Duncan, C.L., Labbe, R.G. and Reich, R.G. (1972) *J. Bacteriol.* 109, 550-559
- 3 Franceschini, T.J. and Labbe, R.G. (1979) *Microbios* 25, 85-91
- 4 Duncan, C.L. and Strong, D.H. (1968) *Appl. Microbiol.* 16, 82-89
- 5 Maurer, H.R. (1971) *Disc Electrophoresis*, 2nd edn., pp. 44-46, Walter de Gruyter, New York
- 6 Andrews, P. (1964) *Biochem. J.* 91, 222-233
- 7 Bradford, M. (1976) *Anal. Biochem.* 72, 248-254
- 8 Hashimoto, T., Frieben, W.R. and Conti, S.F. (1972) in *Spores V* (Halvorson, H., Hanson, R. and Campbell, L.L., eds.), pp. 409-415, American Society for Microbiology, Washington
- 9 Richmond, M.H. (1959) *Biochim. Biophys. Acta* 33, 78-91.
- 10 Hlawiger, J. (1968) *J. Bacteriol.* 95, 376-384
- 11 Arvidson, S., Holme, T. and Wadstrom, T. (1970) *J. Bacteriol.* 104, 227-233
- 12 Brown, W.C. (1972) *Biochem. Biophys. Res. Commun.* 47, 993-996.
- 13 Forsberg, C.W. and Ward, J.B. (1972) *J. Bacteriol.* 110, 878-888
- 14 Guinand, M., Michel, G. and Balassa, G. (1976) *Biochem. Biophys. Res. Commun.* 68, 1287-1293
- 15 Vacheron, M., Guinand, M., Arminjon, F. and Michel, G. (1977) *Biochimie* 59, 15-21
- 16 Fein, J. (1979) *J. Bacteriol.* 137, 933-946
- 17 Guinand, M., Vacheron, M.J., Michel, G. and Tipper, D.J. (1979) *J. Bacteriol.* 138, 126-132
- 18 Mauck, J., Chan, L. and Glaser, L. (1971) *J. Biol. Chem.* 246, 1820-1827
- 19 Pooley, H.M. (1976) *J. Bacteriol.* 125, 1127-1138
- 20 Glaser, L. and Lindsay, B. (1977) *J. Bacteriol.* 130, 610-619.
- 21 Forsberg, C.W. and Rogers, H.J. (1974) *J. Bacteriol.* 118, 358-368
- 22 Fein, J. and Rogers, H.J. (1976) *J. Bacteriol.* 127, 1427-1442
- 23 Kingan, S.L. and Ensign, J.C. (1968) *J. Bacteriol.* 96, 629-638
- 24 Strange, R.E. and Dark, F.A. (1957) *J. Gen. Microbiol.* 17, 525-537
- 25 Gould, G.W. and King, W.L. (1969) in *Spores IV* (Campbell, L.L. and Halvorson, H.O., eds.), pp. 213-221, American Society for Microbiology, Ann Arbor
- 26 Warth, A.D. (1972) in *Spores V* (Halvorson, H., Hanson, R. and Campbell, L.L., eds.), pp. 28-34, American Society for Microbiology, Washington

**ATE  
LMED**