PROTEIN COMPOSITION OF CELL AND FORESPORE MEMBRANES OF *Bacillus subtilis*

Robert C. Goldman

Department of Bacteriology, University of California, Los Angeles, California 90024
(Received January 8, 1973; accepted March 1, 1973)

A sporulation mutant of *Bacillus subtilis* 168 was isolated and characterized. The mutant, designated SB-23, releases viable forespores at the end of the developmental period. Forespores were isolated on linear Renografin gradients and used as a source of forespore membranes. The protein composition of forespore membranes was found to differ from the protein composition of vegetative cell membranes by disc-gel electrophoresis. The results are discussed in relationship to morphological and physiological differentiation during bacterial sporulation.

INTRODUCTION

Morphological changes during sporulation have been characterized by electron microscopy (for reviews, see 1, 2, 3). Traditionally, seven stages have been recognized in the process. These are 1. axial filament formation, 2. septation, 3. forespore completion, 4. cortex formation, 5. coat formation, 6. maturation, and 7. liberation of the mature spore. These stages are arbitrary representations and do not adequately illustrate that sporulation is a continuous process brought about by the sequential expression of the genetic sites involved. In the first two stages an axial filament of nuclear material is present. Part of this is destined for inclusion in the forespore. Invagination of the vegetative cell membrane in the second stage results in formation of the forespore septum, which divides the cell into two compartments. The third stage terminates with the engulfment of the prespore compartment, resulting in formation of a forespore possessing an inner and outer membrane. Stage four consists of deposition of cortex muropeptide polymers between the forespore membranes. Stage five is completed upon formation of the multilayered protein coats. These form the outer most layers of the mature spore, endowing it with resistance to organic solvents. During stage six, the spore becomes refractile, dehydrated, and heat resistant; and, in stage seven, the mature spore is released as the result of cellular lysis.

There is no reason to assume that the inner and outer forespore membranes are identical to the cell membrane. They may be specialized for some aspects of sporulation (cortex and germ cell wall synthesis, coat protein binding, or metabolic regulation), and thus differ from the structural and functional characteristics of growing cells. Indeed, there is indirect evidence for assuming differences between the forespore and cell membranes. Differences in the chemical structures of cell wall and cortex peptidoglycans have suggested that at least two new membrane-associated enzymes would be required for cortex synthesis (4). Cell walls consist of N-acetylglucosamine, N-acetylmuramic acid, L- and D-alanine, D-glutamate, and meso-α,ϵ-diaminopimelic acid. N-acetylmuramic acid

Journal of Supramolecular Structure
© 1973 Alan R. Liss, Inc., 150 Fifth Avenue, New York, N.Y. 10011

residues are substituted with tetrapeptides of the sequence L-alanyl-D-isoglutamyl-(L)-meso-diaminopimelyl-(L)-D-alanine. These tetrapeptides are cross linked about 20% between alanine and meso-diaminopimelic acid residues. However, cortex peptidoglycan differs in that 10% to 20% of the muramic acid residues are substituted with C-terminal alanine residues, and another 50% exist as the muramic lactam. Furthermore, Tochikubo (5) has shown that the particulate cytochrome system of *Bacillus subtilis* is altered during sporulation and germination. In particular, succinate cytochrome c reductase was absent from spores, and concentration differences were found for other cytochrome components.

Several laboratories have recently published data suggesting that membrane alterations occur during sporulation (6). The present paper describes the analysis of cell and forespore membrane proteins by disc-gel electrophoresis, and presents evidence for alterations in membrane composition as a consequence of sporulation.

METHODS

Bacterial Strains and Growth Conditions

Bacillus subtilis 168 was employed as the parent strain (7). Mutant strain SB-23 was selected as a clone which lacked pigmentation after air dried spores of strain 168 were heated in a vacuum for 12 hr at 110°C (8) and plated on sporulation medium containing 20 gm/liter agar. Strain 168 forms heat, chloroform, and octanol resistant spores at frequencies of 80% or greater under the conditions used. Strain SB-23 is oligosporogenic. A low level of heat resistant spores is produced; however, in the majority of the cells, sporulation is blocked at some point during stage 5. *Bacillus amyloliquefaciens* H was obtained from J. Spizizen (Scripps Clinic and Research Foundation, La Jolla, California) and was used for assaying antibiotic production.

Cultures were inoculated with cells grown overnight on Brain Heart Infusion agar (Difco). The sporulation medium consisted of (in gm per liter): Nutrient Broth (Difco) 8.0; KCl 1.0; $MgSO_4-7H_2O$ 0.25; $CaCl_2$ 11.0×10^{-2}, $MnCl_2-4H_2O$ 2.0×10^{-3} and glucose 1.0 (added after autoclaving separately). Cultures were incubated at 37°C, and growth was monitored using a Klett-Summerson Colorimeter with a #56 filter. Logarithmic growth ceased at 160 Klett units; one Klett unit corresponded to approximately 2.6×10^6 colony forming units per milliter.

Characterization of SB-23

Mutant strain SB-23 was compared to the parent strain with respect to sporulation specific events. This comparison involved plate tests for the production of proteases (digesting gelatin, casein, denatured albumin, protamine sulfate, hemoglobin, and native albumin), antibiotic against strain H and oxidation of triphenyl tetrazolium Cl and p-tolyl tetrazolium red (9). Dipicolinic acid was assayed by the method of Janssen et al. (10). The number of spores resistant to heat, octanol, and chloroform was determined on cultures incubated at 37°C for 18 hr after reaching the stationary phase of growth.

Isolation of Cell Membranes

Cells from a 500 ml culture were harvested during exponential phase at a culture density of 100 Klett units, washed in 0.05 M tris (hydroxymethyl) aminomethane (Tris)-1.0 mM ethylenediaminetetraacetic acid (EDTA), pH 7.5 (Buffer A) containing 0.5 M

sucrose. Lysozyme was added at 500 μg/ml, and the mixture incubated at 33°C for 60 min. Protoplasts were collected by centrifugation at 2.7×10^5 g · min in the Sorvall SS-34 rotor, and lysed by the addition of an equal volume of Buffer A. DNase (1.0 mg), RNase (1.0 mg), and M $MgCl_2$ (1.0 ml) were added and the mixture was incubated at 33°C for 60 min. The membranes were washed 3 times by suspension in Buffer A and centrifugation at 2.7×10^5 g · min, suspended in 0.5 ml of Buffer A containing 1.0% sodium dodecyl sulfate (SDS) and 2.0% 2-mercaptoethanol, and boiled for 3 min.

Isolation of Forespores and Forespore Membranes

Forespores from cultures (800 ml) of SB-23 were harvested 24 hr after the end of logarithmic growth, washed as described above for cells, suspended in 10 ml of Buffer A, and passed through a 20 gauge needle several times to disrupt clumps. An equal volume of 30% Renografin was added and 2.0 ml portions were layered onto preformed, linear 50% to 30% Renografin gradients (30 ml volume). Samples were subjected to a 1.08×10^6 g · min centrifugation at 4°C in the Spinco SW 25.1 rotor. Forespores were found to band midway in the gradients and were collected with a clean pipette, after pipetting away the upper cell layer. Forespores from nine gradients were pooled, diluted 1:1 with Buffer A and collected by a 1.08×10^6 g · min centrifugation. Forespore membranes were prepared as described for cell membranes except for the addition of another 500 μg/ml of lysozyme after the initial 30 min period of incubation.

Disc-Gel Electrophoresis

Gels, containing 1.0% sodium dodecyl sulfate, were prepared by the methods described by Davis (11) and Maizel (12). The acrylamide concentrations employed were 7.0% for the running gel, and 3.0% for the stacking gel. Samples containing 250 ug protein and 10% sucrose were applied to gels, overlayed with running buffer, and electrophoresed at 3 ma per tube for 3 hr at room temperature. Bromphenol blue was used as a tracker dye and was added to protein samples. The gels were fixed for 1 hr in a solution consisting of 5% each of trichloroacetic and sulfosalicylic acids, and stained by one of two procedures. The first consisted of staining overnight in 0.25% Coomassie Blue in methanol, acetic acid, water (5:0.7:10) followed by destaining in methanol, acetic acid, water (5:1.2:10). The second procedure, which gave more distinct banding patterns, consisted of an overnight staining in 0.25% Coomassie Blue in methanol, trichloroacetic acid, water (5:1.2:10) followed by destaining in 15% trichloroacetic acid. Densitometric scans of the stained gels were made at 600 nm using the Gilford linear transport apparatus.

Electron Microscopy

Cells were prefixed in 2% glutaraldehyde for 3 hr at 4°C, and then fixed by the method of Kellenberger et al. (13). After ethanol dehydration, cells were embedded in Spurr low-vicosity embedding media (Polysciences Inc., Warrington, Pa.). Silver to gray sections were cut using an LKB ultramicrotome, stained with 1% uranyl acetate followed by 1/10 diluted lead citrate, and examined in the Hitachi HU11A electron microscope. Membrane preparations were negatively stained with 1% phosphotungstic acid and examined immediately.

RESULTS

Sporulation of SB-23 cells results in the release of viable forespore bodies. The appearance of these bodies by phase contrast microscopy is identical to forespores which have been released from wild type cells by sonic irradiation at time T5 (unpublished data). However, the high proportion of forespores released by SB-23 cells allowed purification in sufficient quantities for protein characterization.

Characterization of SB-23

Results of tests for sporulation specific events are given in Table I. Three protease activities are overproduced by SB-23 as compared with the parent strain.* Larger halos appeared surrounding SB-23 colonies grown on plates overlayed with agar containing

TABLE I. Characteristics of *Bacillus subtilis* 168 and Mutant SB-23

Strain	Generation time 30°C	Gel	Cas	Digestion of Alb	Digestion of Hgl	AlbN	Protam	Oxidation of TZ–2	Oxidation of TZ–7	Production of Ab (BH)
168	42 min	+	+	+	+	+	+	+	+	+
SB-23	42 min	+	++	++	+	+	++	+	+	+

Gel, gelatin; Cas, casein; Alb, denatured albumin; Hgl, hemoglobin; AlbN, native albumin; Protam, Protamine sulfate; TZ–2, triphenyl tetrazolium chloride; TZ–7, p-tolyl tetrazolium red; Ab (BH), antibiotic against *Bacillus amyloliquefaciens* H.

TABLE II. Dipicolinic Acid and Spore Production of *Bacillus subtilis* 168 and Mutant SB-23

Strain	Dipicolinic acid (μg/ml at T9)	Heat resistance* (%)	Chloroform resistance* (%)	Octanol resistance* (%)
168	14.0	84	89	77
SB-23	not detectable	0.16	0.15	7.7

*Resistance expressed as resistant cells/viable count × 100.

casein, native albumin, or protamine sulfate. Table II shows the results of analysis of dipicolinic acid and spore production. Dipicolinic acid is not detectable in SB-23 cultures, and sporulation results in production of 0.16% heat resistant, 0.15% chloroform resistant, 7.7% octanol resistant, and 58.5% heat, octanol, and chloroform sensitive cells.

Isolation of Forespore Bodies and Membranes

Mature spores pellet in 50% to 30% Renografin gradients, while cells remain at the top. Since the released forespores appeared midway in the gradients, their intermediate density allowed purification. The forespore fraction from Renografin gradients contained viable, heat sensitive forespores, 13% of which were octanol resistant. Forespores first become resistant to octanol and chloroform during stage 5 of sportulation (coat formation). One hour later, they become heat resistant. This indicates that only a small fraction of the forespores reach stage 5.

*Balassa (9) concludes, on the basis of kinetic and genetic observations, that the various plate tests distinguish the activities of at least six distinct proteolytic enzymes, three of which are overproduced by strain SB-23.

Since isolated forespores lack protein coats which protect mature spores from enzymic digestion, they are lysozyme sensitive. Digestion of any cortex or germ cell wall material should leave membrane preparations similar to those obtained from vegetative cells. Negatively stained preparations of forespore and cell membranes revealed collapsed ghosts, indicating successful digestion of peptidoglycan material (data not shown).

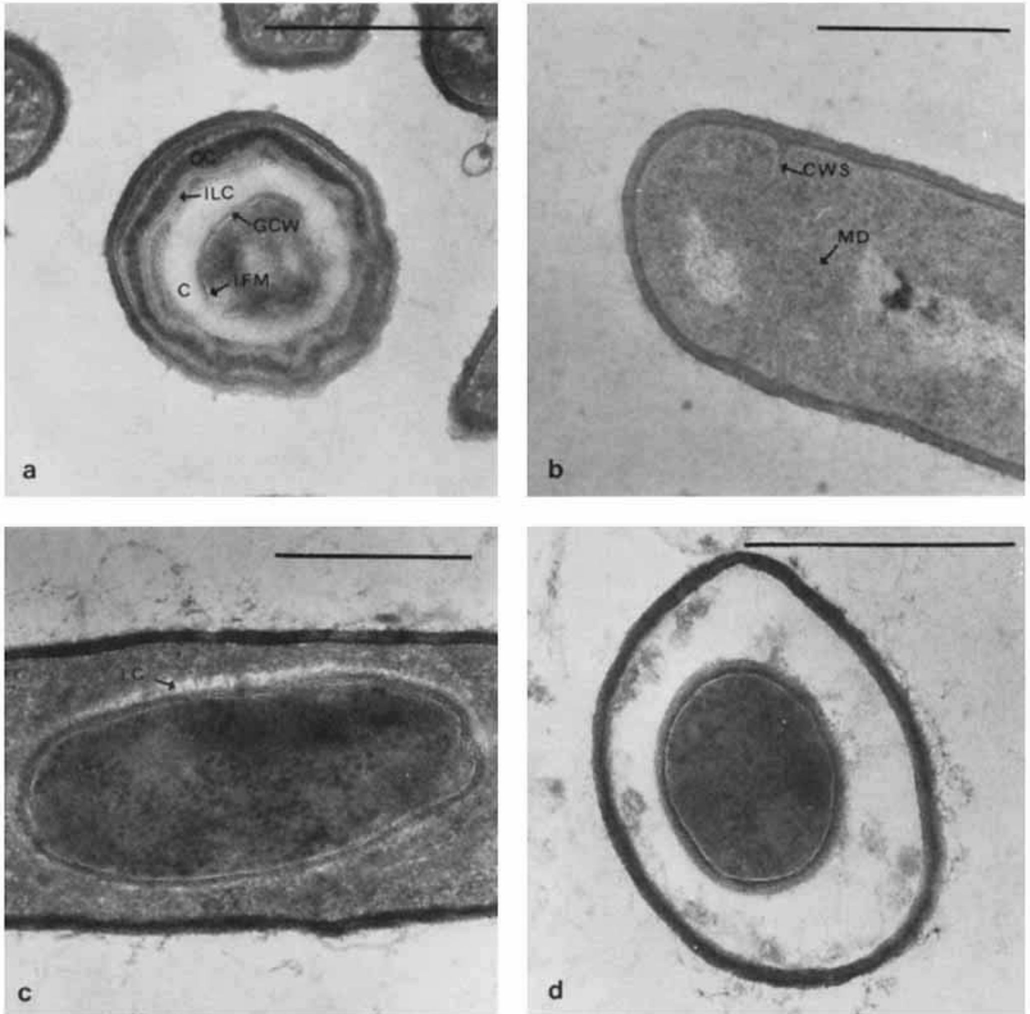

Fig. 1. Bars indicate 0.5 μm. Electron micrographs of parent strain 168 (a and b) and mutant strain SB-23 (c and d). (a) A mature spore showing an inner forespore membrane (IFM), germ cell wall (GCW), cortex (C), inner lamellar coat (ILC) and outer coat (OC). (b) A portion of a cell representative of those at stage 2 of sporulation. The membrane diaphragm (MD) is still associated with the cell wall spikes (CWS). The prespore compartment is engulfed by the mother cell at a later time (3). (c) While still within the mother cell, the forespore possesses an inner forespore membrane, and germ cell wall. Some immature cortex is present (IC); however, protein coats (ILC and OC, Fig. 1a) are not observed. (d) A lysed mother cell. Note the absence of all spore layers which are usually present outside the germ cell wall.

Electron Microscopy

Figures 1a and b show electron micrographs of parent strain 168 for comparison. Electron micrographs of sporulating SB-23 cells revealed incomplete spores lacking protein coats. Forespores found in intact cells sometimes contained immature cortex material (Fig. 1c), whereas those forespores found in lysed cells possessed only a germ cell wall (Fig. 1d). This indicates that spore cortex material is digested from coatless spores during lysis of the mother cell, releasing forespores with an inner forespore membrane and germ cell wall.

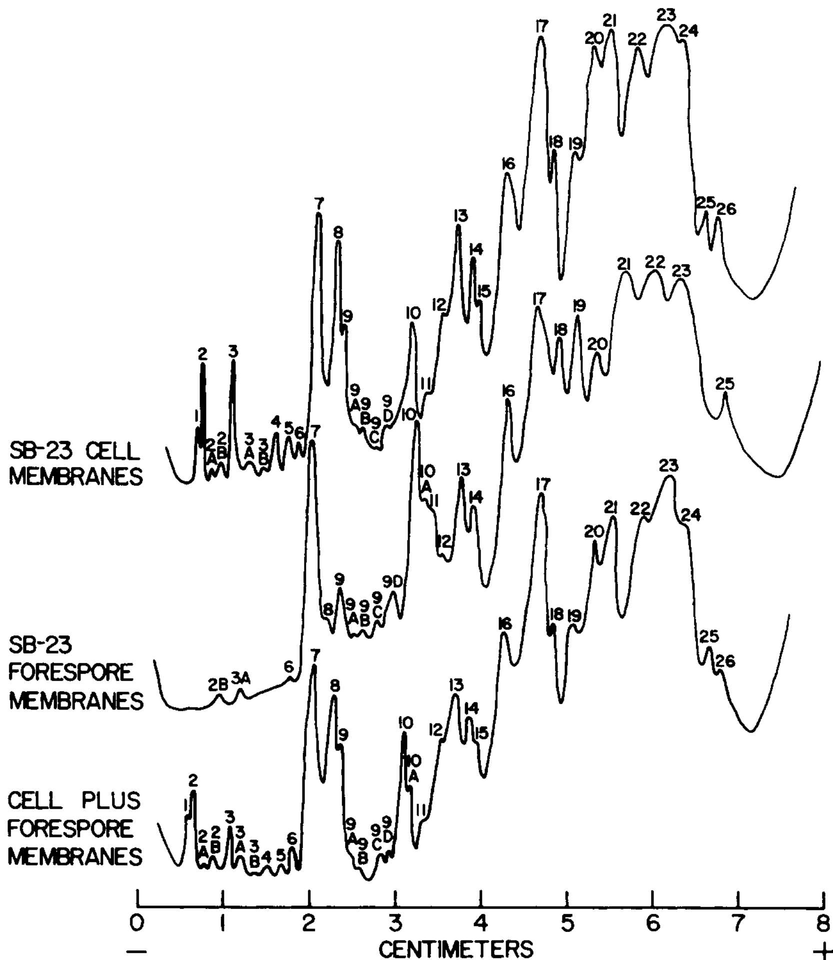

Fig. 2. Densitometric tracings of acrylamide gels stained for protein. Cell membranes from strain SB-23 (top curve, 250 μg protein per gel) reveal a protein pattern identical to cell membranes of strain 168. Forespore membranes isolated from strain SB-23 show an altered protein composition (middle curve, 250 μg protein per gel). Cells and forespores were mixed before membrane preparation (bottom curve, 135 μg cell membrane protein and 96 μg forespore membrane protein per gel) and reveal the pattern expected of this mixture. A similar pattern was obtained when membrane proteins were prepared separately from cells and forespores and then mixed immediately before electrophoresis.

Disc-Gel Electrophoresis

The protein profile obtained from solubilized cell membranes of strain SB-23 revealed 26 major and 8 minor bands (Fig. 2) and was identical to the profile for strain 168 cell membranes (data now shown). Major differences are observed in the protein profiles of SB-23 cell and forespore membranes (Fig. 2). Major bands 1 to 5, 15, 24, and 26 are absent in the gels of forespore membranes, as are minor bands 2A and 3B. Band 8 is present to a lesser extent; whereas band 10 is increased. A new band appears (minor band 10A). When mixtures of cell and forespore membrane porteins were electrophoresed, band 10A was the only new band detected. Furthermore, when forespores and cells were mixed prior to lysis, the resultant solubilized membranes revealed a protein composition qualitatively identical to that expected from a summation of the profiles obtained when the cells and forespores were processed separately (Fig. 2). This indicates that the missing bands in the gel profiles of forespore membranes are unlikely to result from proteolysis during membrane preparation.

DISCUSSION

Membranes from vegetative cells of strains SB-23 and 168 consist of the same 26 major and 8 minor protein components which can be visualized by SDS disc-gel electrophoresis. Mutant strain SB-23 and parent strain 168 also have identical generation times. However, during the postlogarithmic period, the mutant strain overproduces three sporulation specific protease activities, produces no detectable amounts of dipicolinic acid, and releases incomplete spores (Tables I and II). Although the specific site of the mutation is unknown, it is evident that some regulatory aspect of sporulation is altered. Balassa (9) characterized protease overproducers and found that many are abnormal in coat formation. Strain SB-23 is defective in some aspect of coat formation as assessed by the appearance of incomplete spores by electron microscopy. The lack of coat material leaves the cortex subject to attack by the lytic enzymes which lyse the mother cell. Forespores observed by electron microscopy, within intact cells, are characterized by an inner forespore membrane, germ cell wall, and immature cortex; whereas forespores observed in lysed cells are characterized by an inner membrane and germ cell wall (Figs. 1c and d). The fact that only 13% of the heat sensitive forespores become octanol resistant, and none become chloroform resistant, also indicates a defect in coat formation.

The characteristics of SB-23 (Tables I and II) are all consistent with a late block at about T 4.5 (middle of stage 5). All events expressed prior to this time are present (all protease activities, factors oxidizing tetrazolium salts, antibiotic against strain H, germ cell wall, and early cortex formation), while all events expressed by the parent strain after this time are absent or imparied (dipicolinic acid production, coat formation, production of octanol, chloroform, and heat resistant spores). The fact that three early-expressed protease activities are overproduced indicates that the mutation may be in a regulatory gene giving rise to a pleiotropic block in late stage sporulation events and hyperproduction of some early stage products.

The precise effect of protease overproduction by SB-23 has not been determined. One possibility which has not been experimentally ruled out is proteolysis of membrane proteins. Indirect evidence against this possibility includes the fact that the forespores remain viable. Furthermore, Balassa (9) reports that some mutants which overproduce early-expressed protease activities, as does SB-23, reach late stages of sporulation, suggesting

that overproduced proteases do not indiscriminately degrade cellular proteins. In addition, we have shown (Fig. 2) that the missing proteins of the forespore membranes are unlikely to result from proteolysis occurring during membrane preparations.

Lysozyme degrades cortex and germ cell wall material when spore coats are absent. Membrances prepared from forespores and cells of strain SB-23 by lysozyme digestion appear as collapsed ghosts when they are negatively stained with phosphotungstic acid and examined by electron microscopy. This indicates that similar membrane preparations were obtained from both cells and forespores. However, the gel electrophoresis protein profiles of cell and forespore membranes were different. Forespore membranes lack 8 major and 2 minor bands. One major protein component is present to a lesser extent, and another is increased. A protein band not observed in cell membrane preparations is present, suggesting de novo synthesis of a new protein or alteration of a preexisting one.

The precise meaning of these results is obscured by the fact that nothing is known about the structural or functional characteristics of the proteins observed in the gels. The known chemical differences between cortex and cell wall peptidoglycan material suggests that at least two new enzymes are required for cortex synthesis (4). Band 10A, which is unique to the forespore membrane, could be a protein with this or other sporulation-related function. In addition, Tochikubo (5) has shown that the particulate cytochrome system of *B. subtilis* spores lacks at least one component found in cells.

The membrane associated glucose phosphotransferase system, of the class initially described by Kundig et al. (14) declines at the end of vegetative growth (15). The loss of this transport system may be required if cells are to become committed to sporulation, since glucose strongly suppresses sporulation.

Alterations such as those noted for the particulate cytochrome and phosphotransferase systems may account for some of the alterations in protein composition between SB-23 cell and forespore membranes. The observed modifications could take place during spore development by release (16) or degradation (17) as well as by the addition of sporulation specific membrane components. The protein composition of strain SB-23 cell and forespore membranes, as shown by gel electrophoresis, shows that the inner forespore membrane is basically a vegetative cell membrane, lacking several protein components, and containing at least one new minor one.

ACKNOWLEDGEMENTS

This investigation was supported by research grants GM-18233, GM-42359, and AI-10733 from the U.S. Public Health Service. R. Goldman is a predoctoral trainee in microbiology supported by training grant GM-01297 from the U.S. Public Health Service.

The author sincerely thanks Judy P. Delafield for technical assistance and C. Fred Fox for his invaluable help in preparing this manuscript.

REFERENCES

1. Fitz-James, P., and Young, E., In "The Bacterial Spore" (G. Gould and A. Hurst, Eds.) p. 39. Academic Press, N.Y. (1969).
2. Tokuyasu, K., and Yamada, E., J. Biophys. Biochem. Cytol. 5:129 (1959).
3. Yamamoto, I., and Balassa, G., Molec. Gen. Genetics 106:1 (1969).
4. Tipper, D., and Pratt, I., J. Bacteriol. 103:305 (1970).

5. Tochikubo, K., J. Bacteriol. 108:652 (1971).
6. Halvorson, H., Hanson, R., and Campbell (Ed.). Spores V. American Society for Microbiology (1972).
7. Spizizen, J., Proc. Nat. Acad. Sci., U.S.A. 44:1072 (1958).
8. Northrup, J., and Slepecky, R., Science 155:838 (1967).
9. Balassa, G., Molec. Gen. Genetics 104:73 (1969).
10. Janssen, F., Lund, A., and Anderson, L., Science 127:26 (1958).
11. Davis, B., Ann. N. Y. Acad. Sci. 121:404 (1964).
12. Maizel, J., In "Fundamental Techniques in Virology" (K. Habel and N. Salzman, Eds.) p. 334. Academic Press, N.Y. (1969).
13. Kellenberger, E., Ryter, A., and Séchaud, J., J. Biophys. Biochem. Cytol. 4:671 (1958).
14. Kundig, W., Ghosh, S., and Roseman, S., Proc. Nat. Acad. Sci. U.S.A. 52:1067 (1964).
15. Freese, E., Klofat, W., and Galliers, E., Biochem. Biophys. Acta 222:265 (1970).
16. Sussman, M., and Lovgren, N., Exp. Cell Res. 38:97 (1965).
17. Kornberg, A., Spudich, J., Nelson, D., and Deutscher, M., Annual Review of Biochemistry. p. 51 (1968).