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ANTIGEN CHANGES DURING SPORE FORMATION IN *BACILLUS CEREUS*

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ABSTRACT

BAILLIE, ANN (University of Glasgow, Glasgow, Scotland), AND JOHN R. NORRIS. Antigen changes during spore formation in *Bacillus cereus*. J. Bacteriol. **87**:1221-1226. 1964.—Ultrasonic extracts of *Bacillus cereus* cells harvested at different stages in spore formation were analyzed by immunoelectrophoresis with antisera prepared by injecting similar extracts into rabbits. Seven antigens resistant to heating at 80 C for 10 min were detected in extracts of young vegetative cells, and there were at least eight additional thermolabile antigens present at this stage. During spore formation, many of the thermolabile antigens disappeared from the cells and new antigens could be detected. Mature spores contained four of the heat-resistant antigens present in young vegetative cells and an additional five heat-resistant antigens. Only three thermolabile antigens were detected in spore extracts.

Elucidation of antigenic differences between vegetative cells and spores in the genus *Bacillus* has been the object of much of the previous work on spore antigens (Norris, 1962). DeFalle (1902) showed that *B. mycoides* spore antiserum agglutinated both viable and autoclaved spores, whereas antiserum prepared against vegetative cells gave no reaction with spores. The antigenic distinction between the two growth phases was further demonstrated by Howie and Cruickshank (1940) with a bacterium resembling *B. cereus* and with *B. mesentericus;* more recent experience has confirmed these differences in both aerobic and anaerobic sporeforming bacteria (Norris, 1962).

The majority of published observations on the antigenic differences between spore and vegetative growth phases have concerned agglutination reactions, involving only the superficial antigens of the cells, or crude chemical extracts.

The present work is a continuation of that briefly described by Norris and Baillie (1962), in which immunoelectrophoretic analysis of ultrasonic extracts of cells at various stages in spore formation has been used in an attempt to throw some light on the macromolecular changes accompanying that process.

MATERIALS AND METHODS

Organism. The strain of *B. cereus* (strain M.8; Mahmoud, 1955) used throughout this and earlier work (*see* Norris and Wolf, 1961; Norris and Baillie, 1962; Baillie and Norris, 1963; Lund and Norris, 1963) was originally isolated from Egyptian soil and has been maintained by periodic transfer on nutrient agar.

Sporulation medium. This medium was essentially the fluid sporulation medium described by Young (1958), in which well-aerated cultures of *B. cereus* show a useful degree of synchrony in spore formation. It was prepared in two parts, a salts solution and a basal medium, which were stored separately and mixed just before use.

The salts solution was prepared from the following separate solutions (%, w/v): KNO_3, 10; Na_2SO_4, 0.142; KH_2PO_4, 6.8; $MgSO_4 \cdot 7H_2O$, 0.123; $CaCl_2 \cdot 6H_2O$, 0.219; KOH, 0.33; $MnSO_4 \cdot 4H_2O$, 0.00223; $Fe_2(SO_4)_3$, 0.02; $ZnSO_4 \cdot 7H_2O$, 0.0144. These were distributed in 25-ml amounts in screw-capped bottles and sterilized by autoclaving at 15 psi for 20 min. To make the salt solution, 25-ml amounts of these sterile solutions were mixed, and 25 ml of sterile distilled water were added, by use of aseptic techniques.

Basal medium consisted of 1% (w/v) Proteose Peptone (Difco) added to distilled water containing 40% (v/v) nutrient broth (Oxoid). The medium was sterilized by autoclaving at 15 psi for 20 min.

Sporulation medium was prepared by mixing 250 ml of salts solution and 80 ml of basal medium in a sterile 1-liter flask with aseptic techniques.

Cultures. Flasks (1 liter) containing 330 ml of sporulation medium were inoculated with 1-ml amounts of overnight broth cultures of *B. cereus* M.8 and incubated at 30 C with vigorous shaking.

Vegetative-cell disintegration. Cultures were harvested by centrifugation after appropriate

periods of incubation, and the cells were washed three times in distilled water. Pastes of washed vegetative cells and cells at various stages in spore formation were disintegrated by ultrasound with a 60-w ultrasonic disintegrator (Measuring and Scientific Equipment Ltd.). Control experiments showed that cell disintegration was virtually complete after exposure to ultrasonic vibration for 5 min. This period of exposure gave maximal release of antigens from vegetative cells; prolonged exposures up to 30 min had no observable effect on the antigen composition of extracts, provided adequate steps were taken to restrict temperature rise in the suspensions during disintegration. Treatment of this kind did not disrupt mature spores.

Spore disintegration. Cultures (48 hr) were harvested, and vegetative debris was broken down by exposure to ultrasonic vibration. The intact spores were washed repeatedly until neither the spore paste nor the washings showed catalase activity when tested with hydrogen

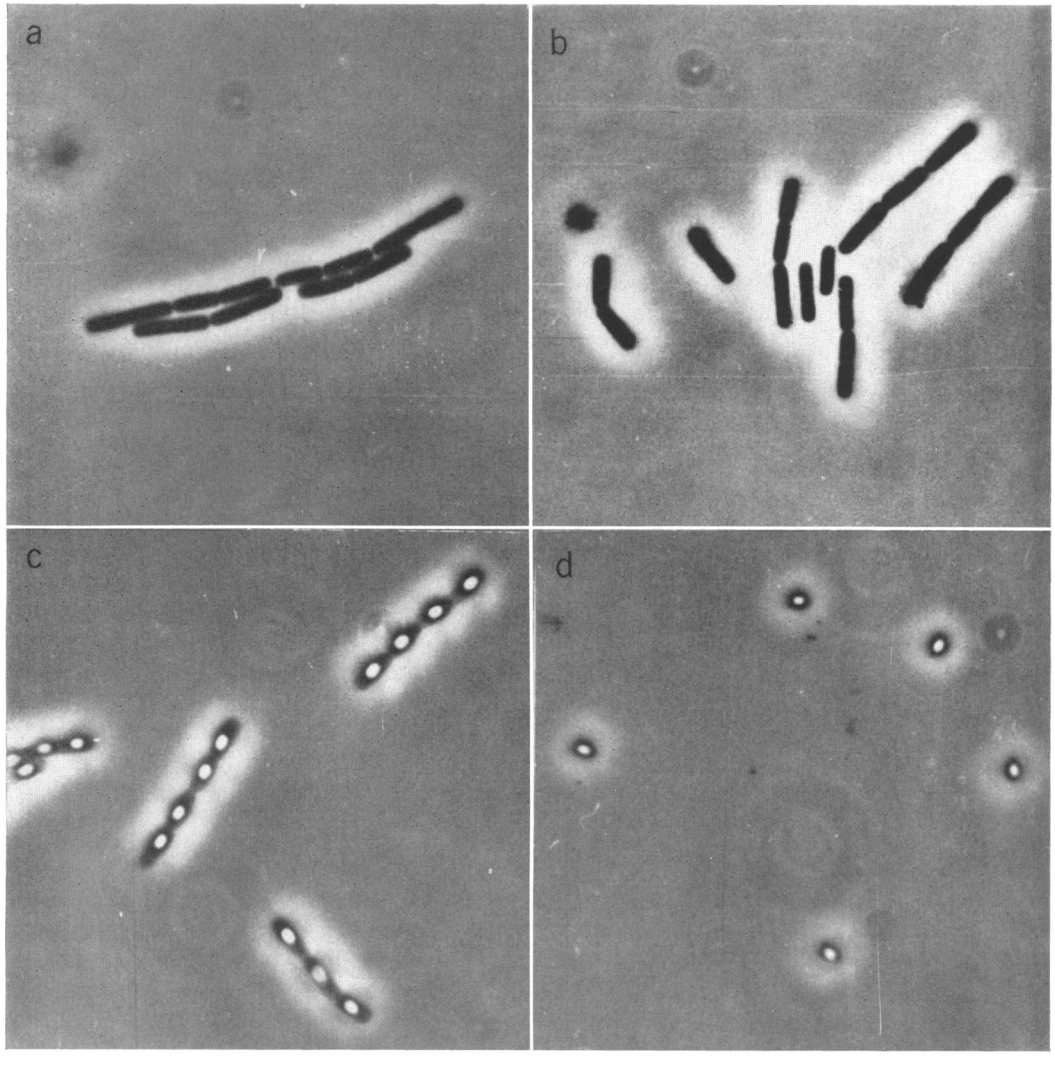


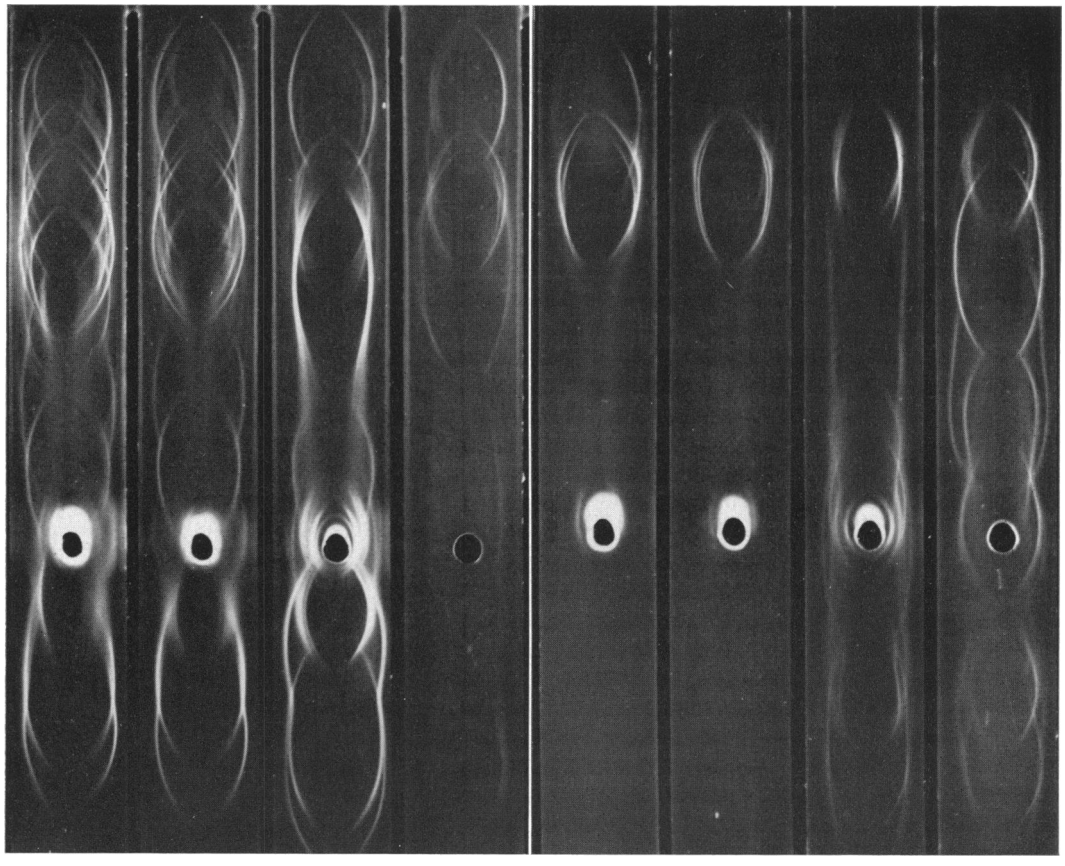
FIG. 1. *Phase-contrast photomicrographs of wet preparations of Bacillus cereus cells at various stages in spore formation. Age of culture: a, 8 hr (stage i); b, 12 hr (stage ii); c, 20 hr (stage iii); d, mature spores (stage iv).* × *1,600 approximately.*

peroxide. Ballotini beads (no. 14) were then added to an equal volume of a thick paste of washed spores, and the mixture was subjected to ultrasonic vibration when 60 to 70% disintegration occurred after about 15 min of treatment.

All products of disintegration were clarified by centrifugation at 4,000 rev/min for 30 min and stored at −20 C.

Protein content of disintegrated cells. The amount of protein present in disintegrated cells was estimated by the method of Lowry et al. (1951), by use of lactalbumin hydrolysate (Nutritional Biochemicals Corp., Cleveland, Ohio) for calibration. Extracts were adjusted to contain approximately 0.5 mg of amino nitrogen per ml before immunoanalysis. Where necessary, preparations were concentrated by ultrafiltration by use of equipment manufactured by Membranfiltergeselschaft Göttingen.

Electrophoresis. Disintegrated cells were subjected to electrophoresis in 1% Ionagar no. 2 (Oxoid) containing barbitone-acetate buffer (Oxoid) at pH 8.6 and an ionic strength of 0.05. Thiomersalate (1:10,000) was added as a preservative. Lantern-slide cover glasses, (3.25 by 3.25 in.) were coated with a thin film of molten 0.1% agar in distilled water and allowed to dry. They were then flooded with 12.5 ml of molten electrophoresis agar which was allowed to set at room temperature and to cool for at least 2 hr before use. Serum trenches and antigen wells were cut to standard patterns with an agar cutter (Buchler Instruments Inc., Fort Lee, N.J.), and



A *B*

FIG. 2. *Photographs of immunoelectrophoretic analyses of antigen extracts. Antigens (from left to right): extracts of cells at stages i, ii, iii, and iv. Antisera: A, antiserum to stage i extract; B, antiserum to stage iv extract.*

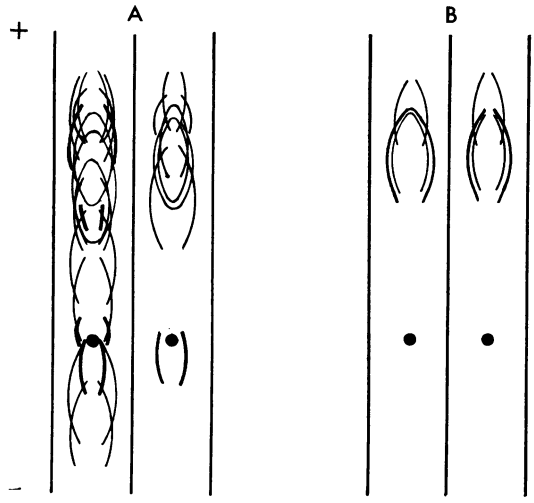


FIG. 3. *Diagrammatic summary of the effect of heating on antigens present in stage i extracts. Antisera: A, antiserum to stage i extract; B, antiserum to stage iv extract. Antigens: in each case, on the left, unheated extract of stage i cells; on the right, the same extract heated at 80 C for 10 min.*

gels were run for approximately 1.5 hr under a potential gradient of 8 to 10 v/cm. The tank buffer was identical with the gel buffer.

Antisera. Rabbits were inoculated intravenously with uncentrifuged disintegrates of cells harvested at various stages in spore formation. A primary series of six injections at 3-day intervals was followed by booster series at approximately 1-month intervals. The animals were bled 7 days after the last injection of a series. Antisera were stored at −20 C without added preservative. Two rabbits were used for each antigen preparation.

In the interests of simplicity, the results presented here are those obtained with antisera to only two of the stages. Confirmatory results were obtained with antisera against other stages.

When comparing, side by side, extracts of cells harvested at different growth stages, it was often noted that particular antigens showed slightly different electrophoretic mobilities in the different preparations. This meant that it was sometimes difficult to identify individual precipitin lines when comparing the four extracts. The technique of Osserman (1960) using terminal antigen-containing trenches was used to facilitate identification of the various antigens.

Heat-resistant antigens. In the course of this investigation, the heat resistance of the various antigens was examined by heating extracts to various temperatures for periods of 10 min immediately prior to analysis. Results presented here were obtained with extracts heated at 80 C for 10 min. This time-temperature combination was chosen for special study, because it kills vegetative cells of *B. cereus* but has no lethal effect on mature spores.

RESULTS

Antigenic composition of the various growth stages. Four stages in growth and spore formation were selected for study: (i) vegetative cells, from an 8-hr culture, staining uniformly gram-positive and appearing uniformly dense under phase contrast; (ii) vegetative cells from a 12-hr culture, showing distinct granulation but no mature spores or recognizable fore-spores; (iii) sporulating cells from a 20-hr culture; (iv) mature spores

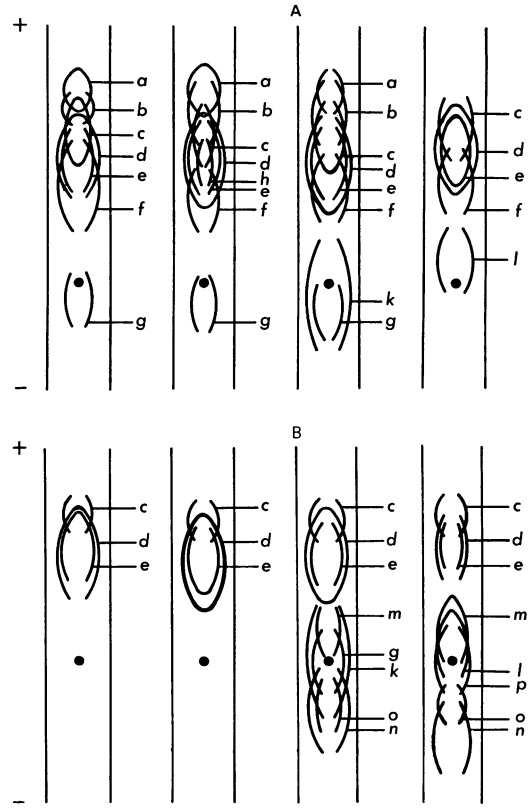


FIG. 4. *Diagrammatic summaries of the heat-resistant antigen composition of the four stages. Antisera: A, antiserum to stage i extract; B, antiserum to stage iv extract. Antigens: in each case, from left to right, extracts of stage i, ii, iii, and iv cells heated at 80 C for 10 min.*

released by dissolution of the sporangia and washed free from debris (Fig. 1). The two antisera used were prepared against disintegrated cells (stage i) and disintegrated spores (stage iv).

At least 15 antigens could be detected in extracts of young vegetative cells when these were analyzed against the homologous antiserum. These antigens differed widely in their electrophoretic mobility; the fastest moved more rapidly than human serum albumin, and several moved toward the cathode. Seven vegetative-cell antigens resisted heating at 80 C for 10 min.

Spore antiserum revealed clearly only three antigens present in young vegetative cell extracts, but there was a progressive increase in numbers of spore antigens as spore formation proceeded; mature spore extracts showed some 12 antigens when tested against homologous antiserum. During spore formation, many of the thermolabile antigens disappeared from the cells; spore extracts contained four of the thermostable antigens present in young vegetative cells, an additional five thermostable antigens, and only three thermolabile antigens. The appearance of the precipitin lines and the overall pattern of changes from stage-to-stage are shown in Fig. 2.

Heat-resistant antigens. Figures 3 and 4 show diagrammatic summaries of the changes affecting antigens resistant to heating at 80 C for 10 min. The antigens, which were detected at all stages in the sporulation cycle (antigens *c*, *d*, and *e*), showed moderately high electrophoretic mobility towards the anode; the close relationship between the behavior of antigens *d* and *e* under these conditions probably indicates that they are similar molecules. The slower-running components showed the greatest variation from stage to stage.

DISCUSSION

Cavallo, Falcone, and Imperato (1963) appear to be the only workers who have carried out a comparable study of the detailed antigen structure of sporeforming bacteria. They detected three antigens in *B. subtilis* spores and demonstrated the appearance of additional antigens during spore germination. They did not find any of the antigens present in the resting spores to be present also in vegetative cells. Our observations also reveal a complex system of antigens in *B. cereus*. Some of these antigens are only synthesized at certain stages in the growth cycle, but some appear to be present at all stages.

The complex pattern of changes in the cell which make up processes of spore formation and spore germination, and the heat resistance of the spore itself, have attracted the attention of many workers (Halvorson, 1957, 1961; Jacobs and Clegg, 1957). As a result of this interest, we have learned a great deal about the processes involved in spore formation, particularly from the cytological and biochemical points of view. Relatively little attention, however, has been paid to the changes which occur in the large molecules, the proteins, polysaccharides, and enzymes, of the cell. In an earlier paper (Baillie and Norris, 1963), we reported changes in the molecular forms of catalase and esterase in *B. cereus* during spore formation. The work reported here is of an exploratory nature, aimed at using the high sensitivity of serological techniques to study the nature of changes in other molecules.

Work now in progress concerns the locating of antigens within the intact cell at different stages and at correlating antigenic and enzymatic changes during spore formation.

ACKNOWLEDGMENTS

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LITERATURE CITED

BAILLIE, A., AND J. R. NORRIS. 1963. Studies of enzyme changes during sporulation in *Bacillus cereus* using starch gel electrophoresis. J. Appl. Bacteriol. **26**:102–106.

CAVALLO, G., G. FALCONE, AND S. IMPERATO. 1963. Antigenic changes in bacterial spores during germination. Bacteriol. Proc., p. 25.

DEFALLE, W. 1902. Recherches sur les anticorps des spores. Ann. Inst. Pasteur **16**:756–774.

HALVORSON, H. O. [ed.] 1957. Spores. Am. Inst. Biol. Sci. Publ. 5.

HALVORSON, H. O. [ed.] 1961. Spores II. Burgess Publishing Co., Minneapolis.

HOWIE, J. W., AND J. CRUICKSHANK. 1940. Bacterial spores as antigens. J. Pathol. Bacteriol. **50**:235–242.

JACOBS, S. E., AND L. F. L. CLEGG [ed.] 1957. Symposium on the formation and germination of bacterial spores. J. Appl. Bacteriol. **20**:305–472.

LOWRY, O. H., N. J. ROSEBROUGH, L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.

LUND, B. M., AND J. R. NORRIS. 1963. Enzyme and antigen changes during germination of bacterial spores. Proc. Intern. Symp. Physiol. Ecol. Biochem. Germination, Greifswald.

MAHMOUD, S. A. Z. 1955. A study of sporeformers occurring in soils. Their germination and biochemical activity. Ph.D. Thesis, University of Leeds, Leeds, England.

NORRIS, J. R. 1962. Bacterial spore antigens: a review. J. Gen. Microbiol. **28**:393–408.

NORRIS, J. R., AND A. BAILLIE. 1962. Antigenic changes accompanying spore formation in *Bacillus cereus*. Intern. Congr. Microbiol. 8th, Montreal, Abstr. p. 17.

NORRIS, J. R., AND J. WOLF. 1961. A study of antigens of the aerobic sporeforming bacteria. J. Appl. Bacteriol. **24**:42–56.

OSSERMAN, E. F. 1960. A modified technique of immunoelectrophoresis facilitating the identification of specific precipitin arcs. J. Immunol. **84**:93–97.

YOUNG, I. E. 1958. Chemical and morphological changes during sporulation in variants of *Bacillus cereus*. Ph.D. Thesis, University of Ontario, Toronto, Canada.