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Sporulation in *Bacillus subtilis*

ANTIGENIC CHANGES DURING SPORE FORMATION

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1. Antisera, prepared against extracts of cells and spores of *Bacillus subtilis*, were used in immunoelectrophoretic studies of the changes occurring in cell extracts during the course of spore formation. 2. At least 15 antigens could be detected in vegetative-cell extracts by the antiserum prepared against cell extracts and at least seven could be demonstrated in spore extracts by the homologous antiserum. 3. Cross-absorption studies showed that two of these antigens were probably completely specific for vegetative-cell extracts and that one was probably completely specific for spore extracts. The remainder were probably present in very small quantities in the heterologous extract. 4. In extracts of cells sporulating in an 'exhaustion medium' those antigens characteristic of the spore began to appear about 1hr. after the end of exponential growth. 5. In cells sporulating in a resuspension medium, spore antigens were detected at 4hr., and by 7hr. a decrease in vegetative-cell antigens was observed. 6. In an asporogenous mutant blocked early in sporulation there was neither an increase in spore antigens nor a decrease in vegetative-cell antigens. 7. In an asporogenous mutant blocked later in sporulation, there was an increase in spore antigens similar to that which occurred in the sporogenous strain.

Most previous studies of the appearance of spore antigens in bacterial cultures have been concerned with either surface antigens or crude extracts (for a review see Norris, 1962). However, with recent advances in the study of spore formation it is now more desirable and more feasible to attempt to correlate antigenic changes with those that can be detected biochemically. Among the first studies of this type was that undertaken by Baillie & Norris (1964), who were able to detect in extracts of young vegetative-cells only three of the 12 antigens found in spore extracts of *Bacillus cereus*. Norris & Baillie (1964) were also able to detect differences in catalase activities between spores and vegetative cells. Other studies have failed to show any common antigens in resting spores and vegetative cells of *Bacillus subtilis* (Cavallo, Falcone & Imperato, 1963).

The purpose of the present investigation was to determine to what extent soluble extracts of spores contained antigens differing from those of the vegetative cell, to determine at what stage in the sporulation process these antigens could be detected, and to use these new antigens to determine the points at which a number of asporogenous mutants were blocked during spore formation. Initially experiments were carried out in an exhaustion medium, but later a resuspension method was developed (see the Materials and Methods section and Mandelstam & Waites, 1968) and this system was used to study the antigen changes occurring in the asporogenous mutants. At the same time studies on protein turnover (Mandelstam & Waites, 1968), enzyme changes (Warren, 1968) and morphological development (Kay & Warren, 1968) were undertaken. Some of these results have been reported briefly elsewhere (Mandelstam, Waites & Warren, 1967; Mandelstam, Waites, Warren & Sterlini, 1968).

MATERIALS AND METHODS

Organism. B. subtilis (Marburg strain 168), which requires indole or tryptophan, was used throughout. However, this strain is competent in transformation and is sporogenic, and it is referred to here as 'wild-type'. During sporulation of the wild-type, proteolytic activity and antibiotic activity were detected extracellularly, and intracellularly there were increases in proteolytic activity, phosphatase, aconitase and glucose dehydrogenase. Protein turnover also occurs during sporulation.

Of the asporogenous mutants used in this study, mutant E22 showed none of the changes associated with spore formation whereas mutant E21 was damaged in the later stages of spore formation, i.e. it was not found to produce

Table 1. *Summary of sporulation characteristics of wild-type and asporogenous mutants*

Measurements of protease activity and protein turnover were as described by Mandelstam & Waites (1968). Assays of antibiotic, phosphatase and 2,6-dipicolinate were made as described by Warren (1968) and the results with mutants E21, E22 and 3F1 are a personal communication from Dr S. C. Warren. Other determinations were made as described in the Materials and Methods section.

	Characters							
Strain	Increase in protease activity	Presence of antibiotic activity	Increase in phosphatase activity	Protein turnover	Appearance of spore antigens	Decrease of vegetative-cell antigens	Appearance of 2,6-dipicolinate	Appearance of heat-resistant refractile spores
Wild-type	+	+	+	+	+	+	+	+
Mutant E21	+	+	+	+	+	±	–	–
Mutant E22	–	–	–	–	–	–	–	–
Mutant 3F1	–	–	?	–	?	?	–	–

2,6-dipicolinic acid or refractile heat-resistant spores (Warren, 1968; Mandelstam & Waites, 1968). These differences are summarized in Table 1.

Media, growth of organisms and preparation of extracts. The media used were the liquid sporulation and germination media of Donnellan, Nags & Levinson (1964) as described by Mandelstam & Waites (1968). Unless otherwise stated, all preparative steps and cell and spore breakages were carried out at 4°.

Vegetative-cell extracts. Cells were grown to mid-exponential phase in aspirators (10l.) containing sporulation medium (8l.), which was aerated (8l./min.) and stirred vigorously. The temperature in these vessels varied from 30° to 32°. Vegetative-cell extracts were also prepared from cells grown in a casein-hydrolysate–yeast-extract medium containing (mg./l.): glucose, 10000; yeast extract (Difco), 5000; $MgSO_4$, 120; KH_2PO_4, 8166; $CuSO_4$, 0·1; casein hydrolysate ('casamino acids'; Difco), 5000; $ZnSO_4$, 1·3; $FeSO_4$, 0·1; $MnCl_2$, 0·04. The pH was adjusted to 7·2 with 2N-HCl.

The cells were harvested and resuspended in potassium phosphate buffer, pH 7·2 (0·15M), at 20mg. dry wt./ml. before disintegration, in either the Mickle homogenizer (H. Mickle, Gomshall, Surrey) or the Braun MSK cell homogenizer (Shandon Scientific Co. Ltd., London, N.W.10) with Ballotini no. 12 beads (Jencons Ltd., Hemel Hempstead, Herts.). Treatment in the Braun homogenizer (at 4000 oscillations/min. for 4min.) was sufficient for cell breakage (determined microscopically), but 10min. was required when the Mickle homogenizer was used. Extracts were centrifuged at 3000*g* for 20min. to remove the beads and then at 15000*g* for 20min. to remove cell-wall and cell-membrane fragments. Measurement of protein released into the final supernatants after this treatment, after breakage with the Braun homogenizer, showed about 70–90% release, and, after breakage with the Mickle homogenizer, about 50% release.

Spore extracts. For spore production, cultures were grown in flasks (5l.) containing sporulation medium (1l.) at 35° with vigorous shaking for 48hr.

Spores were harvested before complete lysis of the sporangia, washed in potassium phosphate buffer, pH 7·2 (0·15M), and then resuspended in buffer and incubated at 35° with lysozyme (50μg./ml.) and trypsin (1mg./ml.) for about 2hr. to remove as much as possible of the sporangial debris and the remaining vegetative cells. This treatment often resulted in the clumping of the spores, which were then washed twice in the phosphate buffer before a further washing in potassium phosphate buffer of different concentration (0·05M), pH 5·0. Germinated spores and any remaining vegetative cells were removed by the polyethylene glycol diphasic system of Sacks & Alderton (1961) as modified by Donnellan *et al.* (1964). The remaining spores were washed at least 20 times in water [until no catalase activity was observable after treatment of the supernatant with H_2O_2 (1%, v/v)]. The spores were then resuspended at 20mg. dry wt./ml. in potassium phosphate buffer, pH 7·2 (0·15M), and disintegrated in either the Mickle homogenizer or the Braun homogenizer with Ballotini no. 14 beads. The Braun homogenizer produced a greater proportion of damaged spores in a shorter time than the Mickle homogenizer (4min. as opposed to 15min.). Microscopic examination showed perhaps 10% of whole spores after treatment with the Braun homogenizer. However, though the Mickle homogenizer broke open the cells efficiently, only 50% spore breakage was observed under optimum conditions. Extracts were then treated in the same way as the cell extracts. Measurement of protein released into the final supernatant, after breakage in the Braun homogenizer, showed about 50% release, and, after breakage with the Mickle homogenizer, about 30% release. The Braun homogenizer was then used as a routine for spore breakage.

Time of appearance of spore antigens. First experiments were carried out in the liquid sporulation medium of Donnellan *et al.* (1964). In this medium sporulation is initiated by the exhaustion of glucose (Mandelstam & Waites, 1968) and these experiments are referred to below as exhaustion experiments. In some the sporulation medium (350l.) was aerated in a 400l. tank with vigorous stirring (300rev./min.) and aeration (9ft.^{3}/min.). The tank was inoculated with a culture in sporulation medium (16l., bacterial density 0·025mg. dry wt./ml. and the temperature controlled at 35°). Samples (1·5l.) for determinations of dry weight, antigenic study and microscopic observation were taken at intervals and the pH was recorded conti-

nuously. In other experiments cultures were grown in sporulation medium (1l.) in flasks (5l. capacity) as for spore production.

Later experiments were carried out in resuspension medium (Mandelstam & Waites, 1968). Cultures were grown in the germination medium of Donnellan *et al.* (1964) with glucose omitted. Cultures at a density of 0·2mg. dry wt./ml. were centrifuged at room temperature and resuspended at the same density in a modified sporulation medium with glucose omitted and $MgSO_4$ (0·04M) added. From cultures (1l.) in flasks (5l.), samples (300ml.) for antigenic study, microscopic observation and determinations of dry weight were taken and extracts prepared as described above. These experiments are referred to below as resuspension experiments.

Concentrations of extracts for immunoelectrophoresis. Extracts were concentrated to about 20mg. of protein/ml. by pressure dialysis (14in. Hg/in.^{2}) in Visking tubing immersed in equimolar potassium phosphate buffer at 4°.

Determination of protein. The method of Lowry, Rosebrough, Farr & Randall (1951) was used for all protein determinations. Standards were prepared from bovine plasma albumin fraction V (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex).

Immunoelectrophoresis. The method used was similar to that of Scheidegger (1955). Cell and spore extracts (containing about 20mg. of protein/ml.) were subjected to electrophoresis in 1·2% (w/v) Ionagar no. 2 (Oxoid) buffered with barbitone (8·5g./l., adjusted to pH 8·4 with N-HCl) and supported on lantern slides. Extracts (about 0·2mg.) were subjected to electrophoresis for about 45min. at 240v, giving a current of about 20mA. After addition of antisera the gels were allowed to develop for 24hr. at room temperature and then washed, dried at 35° and stained with Amido Black (1g./l.) and Azocarmine (1g./l.) (both from G. T. Gurr Ltd., London, S.W.6). Excess of stain was removed with acetic acid (2%, v/v) in glycerol (10%, v/v) and the gels were dried again at 35°.

Production of antisera. All rabbits used (New Zealand white) were bled before inoculation and sera thus prepared did not produce any precipitin lines with either spore or cell extracts. The rabbits were injected in the gluteal muscle with complete Freund's adjuvant and cell or spore extract [about 5mg. of protein in potassium phosphate buffer, pH 7·2 (0·15M); see above]. Then 7 days later they were again injected intramuscularly with the extract–adjuvant mixture, and after a further 5 weeks six intravenous injections, containing about 0·1, 0·2, 0·4, 0·8, 1·6 and 3·2mg. of protein, were given at 2-day intervals. A series of intravenous booster injections was given when necessary and bleeding carried out on the fifth and seventh days after the last injection.

Blood was held at 35° for about 30min. and then placed in a dark cupboard for 3–4hr. before centrifuging at 2000g. The antisera were stored at −20° without added preservatives.

Absorption experiments. Some absorption experiments were carried out to decide whether spore extracts contained

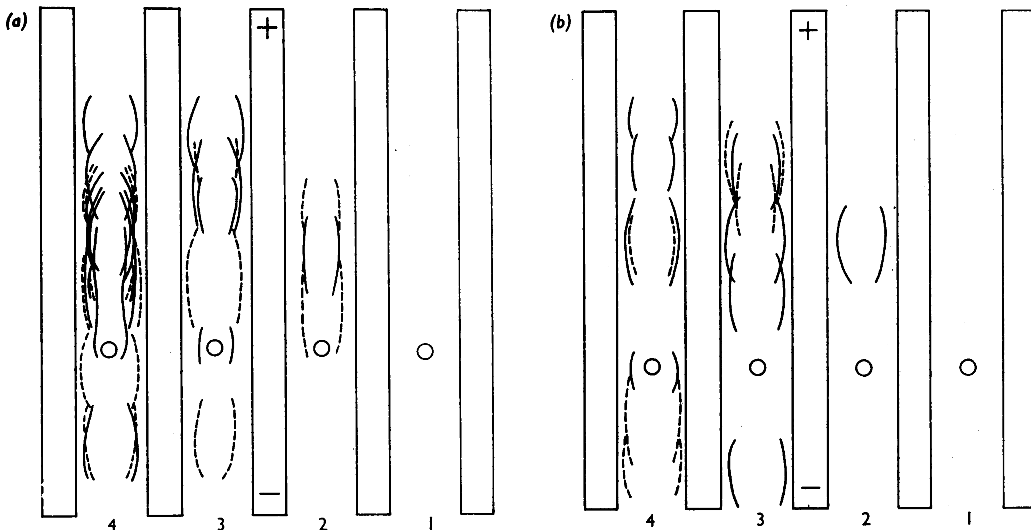

Fig. 1. Antigen analysis of extracts from vegetative cells and from spores showing cross-reaction. Extracts were prepared as described in the Materials and Methods section and were run against the homologous or heterologous antiserum, absorbed or unabsorbed, as described. (*a*) Analysis with antiserum prepared against vegetative-cell extracts: 1, spore extract run against antiserum previously absorbed with excess of spore extract; 2, vegetative-cell extract run against antiserum previously absorbed with excess of spore extract; 3, spore extract run against unabsorbed antiserum; 4, vegetative-cell extract run against unabsorbed antiserum. (*b*) Analysis with antiserum prepared against extracts of spores: 1, vegetative-cell extract run against antiserum previously absorbed with excess of vegetative-cell extract; 2, spore extract run against antiserum previously absorbed with excess of vegetative-cell extract; 3, vegetative-cell extract run against unabsorbed antiserum; 4, spore extract run against unabsorbed antiserum. Continuous and broken lines represent heavy and light precipitin arcs respectively.

very small amounts of vegetative-cell antigens or vice versa. Thus antiserum made against spore extract was absorbed with extracts of cells until no further loss of antigens could be detected on immunoelectrophoresis. It was then run against spore extract. This method would remove both identical and cross-reacting antigens, and some non-specific precipitation might also occur, so that the number of precipitin arcs found in the immunoelectrophoresis can be expected to represent the minimal number of antigens unique to the extract against which the antiserum was prepared. The detailed method was as follows.

Antisera (0·2ml.) and extracts (0·2ml. containing 4mg. of protein) were mixed, kept at 35° for 30min. and then at 0° for 24hr. The mixtures were centrifuged at 2000*g* and the supernatants stored at −20°. A second or third cycle of absorption was used in some experiments.

RESULTS

Presence of spore antigens in vegetative-cell extracts. At least 15 precipitin lines were formed when the anti-(vegetative-cell) serum was tested against vegetative-cell extracts (4 in Fig. 1*a*) and at least seven precipitin lines with the antiserum prepared against spore extracts run against spore extracts (4 in Fig. 1*b*). However, the spore antiserum was able to detect a number of antigens in vegetative-cell extracts (e.g. see 3 in Fig. 1*b*). To decide how many antigens were common to both extracts, spore and cell antisera were absorbed with an excess of the heterologous extract and then immunoelectrophoresis was carried out against the extract to which the antiserum was prepared. Treated in this way spore antiserum could detect only one, or in some experiments two, antigens in spore extracts (2 in Fig. 1*b*), whereas the vegetative-cell antiserum was able to detect two or three antigens in vegetative-cell extracts (2 in Fig. 1*a*).

It has been suggested that many antigens are common to both extracts because of the presence of low numbers of spores during exponential growth and hence of spore antigens in the vegetative-cell extracts used in the production of the antiserum. This was considered unlikely because it was never possible to detect spores microscopically in vegetative cells from actively growing cultures. Nevertheless, vegetative-cell extracts were also made from cells growing exponentially in the casein-hydrolysate–yeast-extract medium (see the Materials and Methods section), a medium in which spore formation is even lower than in sporulation medium. In this medium, spore incidence (detected as colonies produced after heating at 80° for 10min.) was only about 4 in 10^6 cells during exponential growth. With antisera pre-absorbed with this extract it was still possible to detect one, or in some experiments two, precipitin lines with the homologous extract. However, as shown (2 in Fig. 1*b*), the extract from the culture grown in casein-hydrolysate–yeast-extract medium, used to pre-absorb the spore antiserum, removed all but one precipitin line when the absorbed antiserum was run

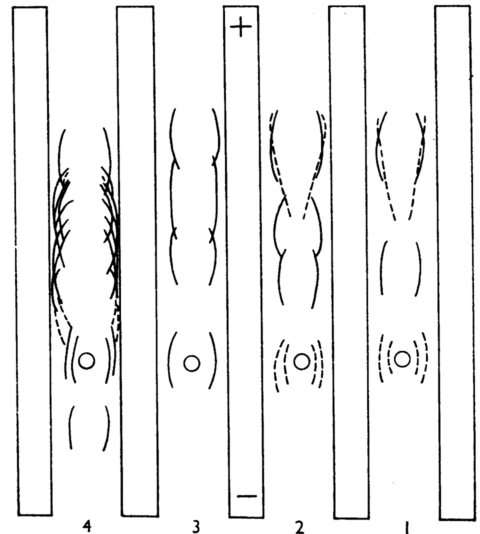

Fig. 2. Comparison of heat-stability of antigens of extracts from vegetative cells and from spores. Extracts were prepared as described in the Materials and Methods section, heated at 80° for 10min. and then subjected to immunoelectrophoresis: 1, heated spore extract run against spore antiserum; 2, unheated spore extract run against spore antiserum; 3, heated vegetative-cell extract run against vegetative-cell antiserum; 4, unheated vegetative-cell extract run against vegetative-cell antiserum.

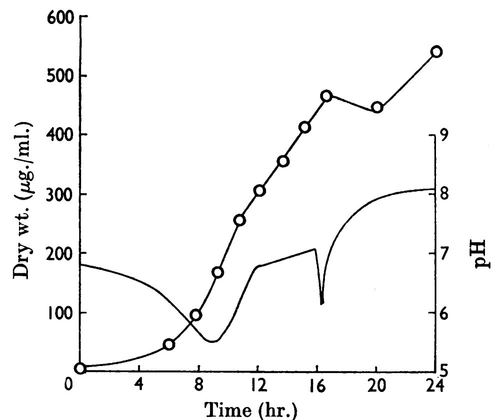

Fig. 3. Changes in pH and cell mass during sporulation in an exhaustion medium. A culture in sporulation medium was aerated vigorously at 35° (see the Materials and Methods section). Samples for determinations of dry weight (○) were taken at intervals, and the pH (——) was recorded continuously.

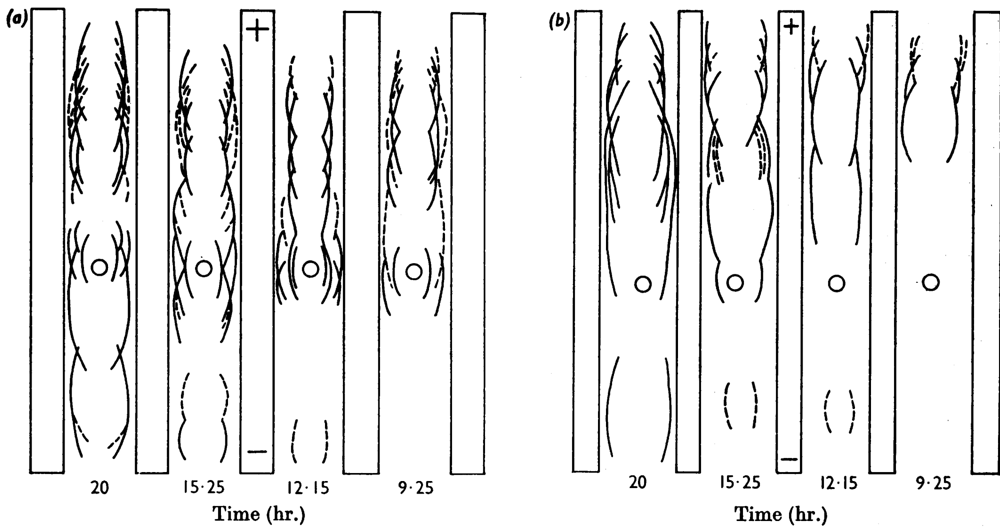

Fig. 4. Changes in antigen pattern during sporulation of wild-type in an exhaustion medium. The experiment was that described in Fig. 3. Samples for antigen analysis were removed at 9·25, 10·75, 12·15, 13·75, 15·25, 16·75 and 20 hr. and treated as described in the Materials and Methods section. Four of the immunoelectrophoretic patterns obtained with samples removed at 9·25, 12·15, 15·25 and 20 hr. are illustrated. (*a*) Extracts run against vegetative-cell antiserum; (*b*) extracts run against spore antiserum. Spore antigens appeared during the experiment, but there was no loss in the number of vegetative-cell antigens (see the text). This result can be compared with the loss of vegetative-cell antigens found during sporulation in the resuspension medium (see Fig. 5*a*).

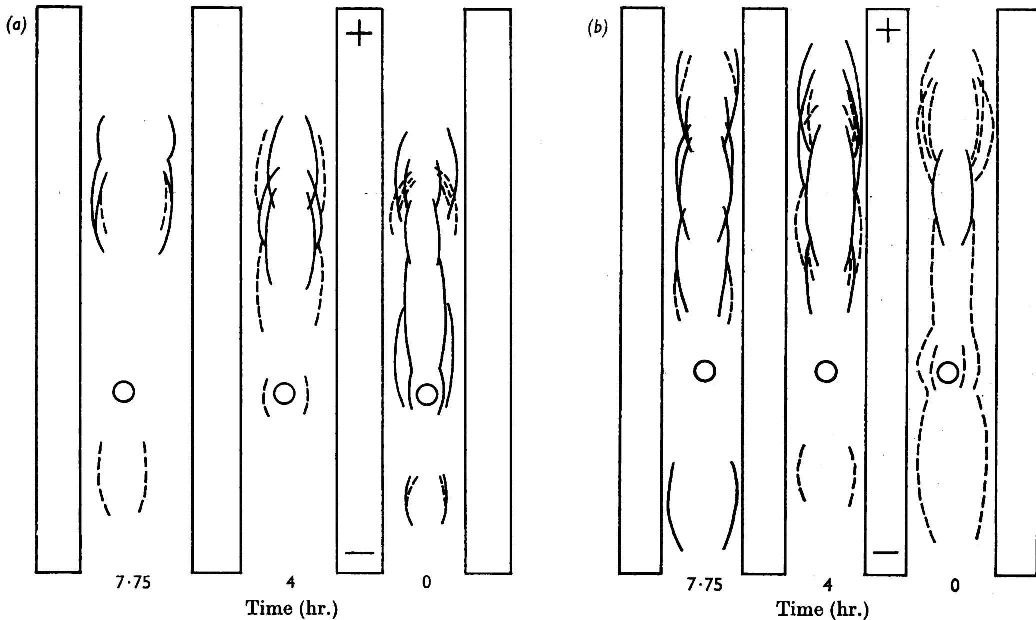

Fig. 5. Appearance of spore antigens and disappearance of vegetative-cell antigens in a resuspension experiment. Cells growing exponentially in a medium containing hydrolysed casein were resuspended in a medium containing glutamate and inorganic ions to initiate sporulation (see the Materials and Methods section). Samples for antigenic analysis were removed at 0, 1, 2, 4, 6 and 7·75 hr. after resuspension and treated as described in the text. Three of the immunoelectrophoretic patterns obtained with samples removed at 0, 4 and 7·75 hr. are illustrated. (*a*) Extracts run against vegetative-cell antiserum; (*b*) extracts run against spore antiserum.

against spore extracts. Hence, with regards to the presence of spore antigens, vegetative cells grown in the casein-hydrolysate–yeast-extract medium and essentially spore-free present a similar result to those grown in the defined medium of Donnellan *et al.* (1964). It seems, however, that cultures grown in the more complex medium may lack one of the antigens present in the vegetative cells grown in the defined medium.

Heat-stability of spore and cell antigens. Cell and spore extracts (10mg. of protein in 0·5ml. of phosphate buffer, prepared as described in the Materials and Methods section) were placed into pre-warmed tubes held at 80° and incubated for 10min. Samples were then subjected to electrophoresis and compared with unheated samples (Fig. 2). At least 14 antigens were detected in the unheated cell extracts by the homologous antiserum, whereas with heated extracts only four precipitin lines could be demonstrated. The spore antiserum was able to detect at least six antigens in the unheated spore extract and five in the heated sample.

Time of appearance of spore antigens: exhaustion experiments. Initially *B. subtilis* 168 was grown in 350l. of the sporulation medium of Donnellan *et al.* (1964) in a 400l. tank (as described in the Materials and Methods section). The pH was recorded automatically and at intervals samples were taken for determinations of dry weight, microscopic study and antigen determinations. The pH and dry-weight curves are shown in Fig. 3. The precipitin lines formed by the extracts (produced as described in the Materials and Methods section) with antisera prepared against vegetative-cell extracts and spore extracts are shown in Figs. 4(*a*) and 4(*b*) respectively. Exponential growth stopped at about 11hr. after the time of inoculation, though the dry weight continued to increase at a low rate after this time. A fall in pH occurred in the early exponential phase, but after about 9hr. there was a rise, similar to that shown in *B. subtilis* by Szulmajster & Hanson (1965) and in *B. cereus* by Kominek & Halvorson (1965). Cells having darkened poles, a sign of incipient sporulation, first appeared microscopically after 14hr., and the first refractile spores were detected after 20hr. The end of exponential growth and the rise in pH were rapidly followed by the appearance of antigens not detected previously by the spore antiserum, though at least three could be detected in all stages of growth. Extracts harvested throughout the experiment showed similar numbers of vegetative-cell antigens, a result to be expected in view of the high numbers of vegetative cells remaining at the end of the experiment. More clear-cut results were obtained in the resuspension experiments described below.

Experiments in resuspension medium. To increase the rate of spore formation, to provide a more definite time for the start of sporulation and to enable a defined medium to be used, experiments were also carried out in a resuspension medium as described in the Materials and Methods section. The immunoelectrophoretic patterns produced by the extracts from these experiments were similar to those obtained in the experiments in the exhaustion medium, except that about 7hr. after resuspension when refractile spores began to appear there was a marked loss of vegetative-cell antigens (Fig. 5). There was no decrease in the extinction of the cultures and the result is attributable to turnover of protein. These results are reported in the preceding paper (Mandelstam & Waites, 1968), but are presented here for comparison. In similar experiments with mutant E21, which is blocked late in sporulation, there was an increase in spore antigens (Fig. 6), but the decrease in vegetative-cell antigens did not always occur. Mutant E22, which is blocked in the earliest stages of sporulation,

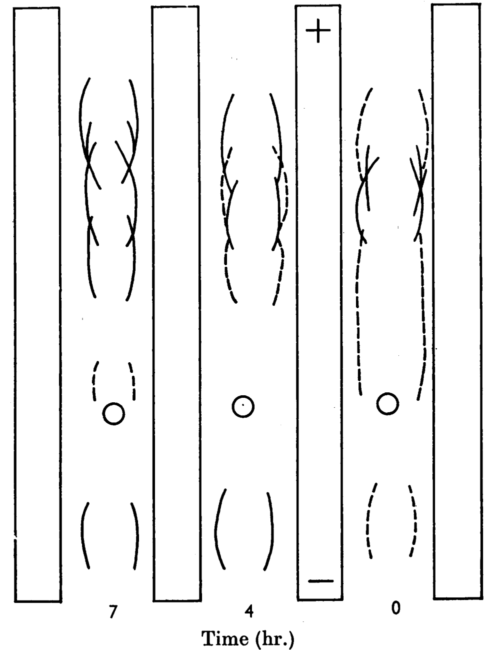

Fig. 6. Changes in antigen pattern in a resuspension experiment with the asporogenous mutant E21. The method was the same as that described in Fig. 5. Samples for antigen analysis were removed at 0, 1, 2, 4, 6 and 7hr. and three of the immunoelectrophoretic patterns obtained with samples removed at 0, 4 and 7hr. are illustrated. Extracts were run against spore antiserum. This mutant is blocked at a late stage of sporulation and shows an increase in spore antigens. Loss of vegetative-cell antigens occurred in some experiments but not others and is not shown.

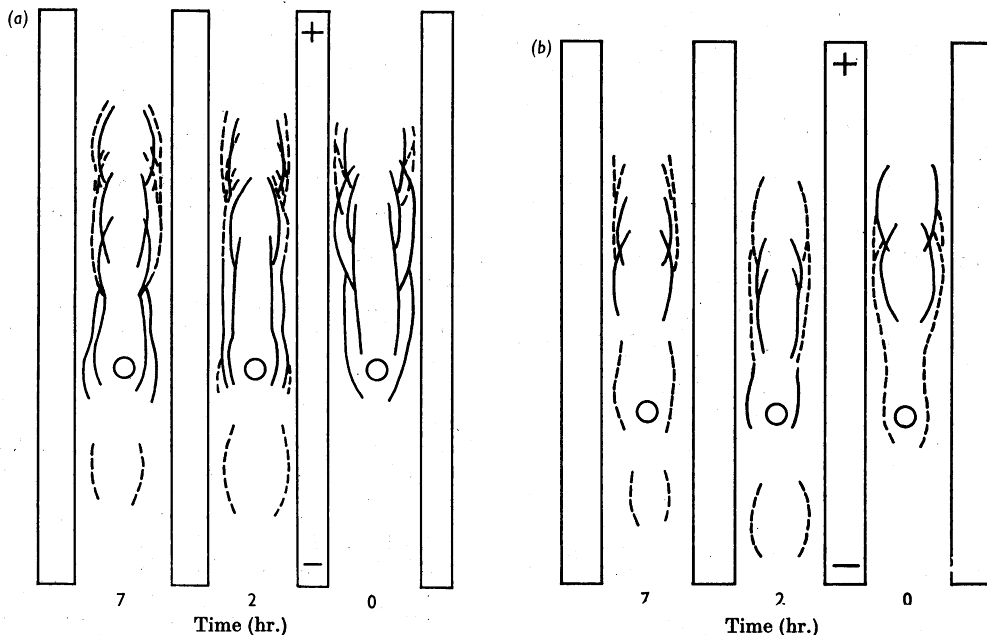

Fig. 7. Antigen pattern in a resuspension experiment with the asporogenous mutant E22. The method was the same as that described in Fig. 5. Three of the immunoelectrophoretic patterns obtained with samples removed at 0, 2 and 7hr. are illustrated. (*a*) Extracts run against vegetative-cell antiserum; (*b*) extracts run against spore antiserum. This mutant is blocked at an early stage of sporulation. Spore antigens did not appear nor did vegetative-cell antigens disappear.

showed no increase in spore antigens nor any loss of vegetative-cell antigens (Fig. 7).

DISCUSSION

The studies reported here and by Calvallo *et al.* (1963) with *B. subtilis* and also those of Baillie & Norris (1964) with *B. cereus* have all shown that some of the antigens detected in spores differ from those found in vegetative cells, and that during spore formation the appearance of the previously undetected antigens can be followed. In the present work, of the seven antigens detected by the spore antiserum at least three could be demonstrated in all stages of growth. However, it was uncertain whether the remaining four antigens were present at concentrations too low to be detected by immunoelectrophoresis. Cross-absorption methods were used in an attempt to resolve this problem and the results suggest that of these four antigens at least two and probably three are present in vegetative cells but only in very low concentrations (see 2 in Fig. 1*b*). Thus of the seven antigens demonstrated by the spore antiserum in spore extracts five or six can be found in vegetative-cell extracts, and the low concentrations in vegetative cells of two or three of them suggest a situation analogous to the basal concentrations of inducible enzymes. It is shown in the next paper (Warren, 1968) that a number of enzymes that increase during sporulation are present in low, but detectable, concentrations in exponentially growing cells. Of the enzymes that are present in spores, a number are comparatively thermostable [e.g. the catalase of *B. cereus* (Sadoff, 1961) and the glucose dehydrogenase (Sadoff, Bach & Kools, 1965)], and this may be related to the presence of greater numbers of heat-resistant antigens in spore extracts than in vegetative-cell extracts (see Baillie & Norris, 1964; and also Fig. 2).

Although spore antigens have been detected at different times when different media have been used for sporulation, the same qualitative changes occurred in both exhaustion and resuspension experiments and it is likely that the results discussed below are usual in sporulation of this organism. The disappearance of vegetative-cell antigens after resuspension of the wild-type strain (Fig. 5*a*) can be correlated with the ^{35}S-labelling experiments on protein turnover (Mandelstam & Waites, 1968), which show that 50% or more of the protein present at zero time has been broken down by 7hr. By contrast mutant E22, which is blocked early in

sporulation, showed no loss of vegetative-cell antigens (Fig. 7*a*) and also no protein turnover (Mandelstam & Waites 1968). No increase in spore antigens could be shown (Fig. 7*b*) and it may be that this mutant is defective in the initiation of spore formation.

In mutant E21, which is blocked late in sporulation, an increase in spore antigens was observed (Fig. 6) and this mutant has also been shown to undergo protein turnover (Mandelstam & Waites, 1968). Warren (1968) has shown that this mutant undergoes enzymic changes similar to those demonstrated in the wild-type during sporulation. However, loss of vegetative-cell antigens was variable, and this may be related to the different enzyme concentrations present in this mutant and in the sporogenic strain (S. C. Warren, personal communication).

These results are summarized in Table 1 and are discussed more fully in accompanying papers (Mandelstam & Waites, 1968; Warren, 1968).

In principle it seems that immunoelectrophoresis could help to distinguish mutants blocked early in sporulation from those blocked late in sporulation, but to obtain finer discrimination it would be necessary to have a larger number of antigens. This might be achieved by preliminary separation of the extracts into a number of fractions, each of which would then be used for immunization.

The earlier experiments were done at the National Institute for Medical Research, London, where I obtained advice and help from members of the Immunology Division and from Mr J. Lee. I am grateful to Dr S. C. Warren for mutants E21 and E22. The later experiments were done in Oxford with the support of the Science Research Council and the assistance of Mrs D. Torgersen.

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