Cytochemical Studies on Alkaline Phosphatase Production During Sporulation in *Bacillus subtilis*

By A. R. GLENN* and J. G. COOTE†

Microbiology Unit, Department of Biochemistry, University of Oxford, Oxford OX1 3QU, U.K.

(Received 24 March 1975)

During sporulation of *Bacillus subtilis* 168 there is an increase in activity of alkaline phosphatase in the presence of P_i . This enzyme was shown by cytochemical techniques to be associated with the cytoplasmic membrane of the mother cell and also with the membranes of the developing prespore. There is a strong correlation between an increasing number of electron-dense deposits due to phosphatase activity and the formation of the spore septum, i.e. stage II of sporulation. Cytochemical and biochemical evidence shows that cells well advanced in spore formation can be derepressed to produce the very much higher amounts of alkaline phosphatase characteristic of phosphate-starved vegetative cells.

The biochemical sequence of events associated with sporulation of *Bacillus subtilis* has been extensively reviewed (see Schaeffer, 1969; Mandelstam, 1969; Hanson *et al.*, 1970). Among the biochemical 'marker' events described by Warren (1968) and Waites *et al.* (1970) was a 30–40-fold increase in specific activity of alkaline phosphatase at P_i concentrations that completely repress the enzyme in vegetative cells. Derepression of the enzyme in vegetative cells by phosphate starvation leads to a 1000-fold increase in specific activity (Glenn & Mandelstam, 1971). The properties of the two enzyme activities were indistinguishable when compared by a large number of criteria (Glenn & Mandelstam, 1971). It was suggested, therefore, that 'sporulation' and 'vegetative' alkaline phosphatase activities resided in a single protein species and that there was a change in the control of the enzyme specifically associated with spore formation.

There has been some dispute about the functioning of vegetative genes in sporulating cells. Hardwick & Foster (1952) reported that when cells of *Bacillus cereus* were committed to spore formation they were incapable of producing the inducible enzyme maltase. Similarly, Halvorson (1965) assumed that the vegetative genome was repressed in sporulating cells. Aubert & Millet (1963), however, reported that β -galactosidase could be induced in sporulating cells of *Bacillus megaterium*. Similar results were obtained by Sterlini & Mandelstam (1969) with histidase

induction in *Bacillus subtilis*. Most compelling, however, is the series of investigations cited by Kornberg *et al.* (1968). In these it was shown that a number of enzymes of the spore are identical with those of the vegetative cell and are newly synthesized in the course of sporulation. This must be taken to mean that a considerable number of genes for 'vegetative' functions continue to be transcribed during sporulation.

The aims of this work were twofold. First, by the use of established cytochemical methods, to determine the localization of the 'sporulation' alkaline phosphatase at the electron microscope level. Attempts could then be made to determine the stage of sporulation with which the increase in enzyme activity may most closely be associated by direct examination of individual cells at different stages in spore formation. Secondly, it was decided to determine whether sporulating cells of *B. subtilis* could be derepressed, by phosphate starvation, to produce the very much larger amounts of alkaline phosphatase characteristic of phosphate-starved vegetative cells.

The results show that the enzyme is localized both in the cytoplasmic membrane of the mother cell and in the membrane of the prespore. Evidence is also presented to show that the increase in phosphatase activity is associated with stage II (spore-septum formation) of sporulation and that cells well advanced in sporulation can be derepressed to produce very high amounts of the enzyme.

Experimental

Organism

B. subtilis 168 (*trpC2*), an indole or tryptophan auxotroph that sporulates normally, was used.

* Present address: School of Environmental and Life Sciences, Murdoch University, Murdoch, Western Australia 6153.

† Present address: Department of Microbiology, University of Glasgow, Garscube Estate, Bearsden, Glasgow, U.K.

Methods

Culture and procedure for sporulation. Cells were grown at 37°C in a rich medium (hydrolysed casein) and sporulation was induced by resuspending them in a medium containing only glutamate, tryptophan and mineral salts (Sterlini & Mandelstam, 1969). The phosphate concentration of the medium was 0.5 mM unless stated otherwise. The spore incidence was usually 70–80% after 8 h.

Nomenclature. The point of initiation to sporulation (t_0) is the time of resuspension from the growth medium: t_1 , t_2 , etc., represent 1 h intervals thereafter. Stages O–VI are the recognized cytological stages of spore formation (Ryter, 1965).

Enzyme assay. Alkaline phosphatase activity was assayed as described by Glenn & Mandelstam (1971). One enzyme unit liberates 1 nmol of product per min.

Bacterial density. This was measured spectrophotometrically from a calibration curve relating extinction at 600 nm to dry weight.

Refractile spores. These were counted in the phase-contrast microscope.

Premature release of prespores. Cells from a resuspension culture at t_5 (150 ml) were centrifuged and resuspended in warm 0.1 M-KH₂PO₄, pH 7.8, containing 0.01 M-MgCl₂ and lysozyme (250 µg/ml). After incubation for 20 min at 37°C the free immature spores were collected by centrifugation and washed twice with resuspension medium.

Preparation of membranes from sporulating cells. Cells from a resuspension culture (500 ml) were centrifuged and the pellet was taken up in 0.05 M-Tris-HCl, pH 7.8, containing 5 mM-MgCl₂ and lysozyme (250 µg/ml). After incubation at 37°C with shaking for 35–40 min, final concentrations of 0.05 M-EDTA, deoxyribonuclease (4 µg/ml) and ribonuclease (4 µg/ml) were added to the suspension which was then shaken for a further 15 min. At the end of this period MgCl₂ (0.05 M final concentration) was added to the membrane preparation. Intact prespores or whole cells were removed by centrifugation in a bench centrifuge. Membranes were then collected by centrifugation at 25000g for 60 min, washed twice with 0.05 M-Tris-HCl, pH 7.8, containing 5 mM-MgCl₂ and stored at –80°C.

Cytochemical methods. Sporulation alkaline phosphatase activity was detected either by a modification of the method of Sabatini *et al.* (1963) or by the method of Ghosh *et al.* (1971). Samples (2.0 ml) of culture were taken at various times after resuspension, centrifuged and the cells fixed at 4°C in glutaraldehyde (2.5%, w/v, in 0.1 M-cacodylate buffer, pH 7.4) for 60 min. Preliminary experiments showed that denaturation of the enzyme followed biphasic kinetics with an initial rapid loss of activity. After this time 25–30% of the initial activity remained and this was stable.

If the cells were stained by the modified method of Sabatini *et al.* (1963), the fixed cells were then centrifuged, washed in 0.1 M-cacodylate buffer, pH 7.4, and resuspended in a mixture containing 0.015 M-sodium β -glycerophosphate (or 0.015 M-sodium *p*-nitrophenyl phosphate), 0.02 M-veronal, 0.04 M-CaCl₂, 2 mM-MgCl₂·6H₂O and 0.05 M-cacodylate buffer, pH 7.4. The final pH of the mixture was 9.2 and did not fall significantly during incubation. After incubation at 37°C for various lengths of time the samples were washed with distilled water and treated with 0.08 M-CoCl₂·6H₂O for 30 min at 37°C.

When cells were stained by the alternative method of Ghosh *et al.* (1971) the cells (prefixed in glutaraldehyde) were resuspended at 37°C in a mixture containing 0.05 M-Tris, 2 mM-MgCl₂, 0.03 M-sodium potassium tartrate, 3 mM-Pb(NO₃)₂ and 0.05 M-sodium *p*-nitrophenyl phosphate; the final pH was adjusted to 8.5. After the cells had gone through either of the cytochemical procedures they were fixed at room temperature in 1% (w/v) OsO₄ in Kellenberger buffer, pH 6.1 (Kellenberger *et al.*, 1958). The fixed material was then stained at room temperature with 0.5% (w/v) uranyl acetate in Kellenberger buffer, dehydrated in graded ethanol and embedded in Araldite. Only those sections used to correlate the appearance of the enzyme with a particular stage of sporulation were stained with lead citrate (Reynolds, 1963).

Control samples were prepared by incubating cells in mixtures lacking substrate, CaCl₂ or Pb(NO₃)₂. Cells at t_0 were also taken as a control since they have only a basal amount of enzyme. As another control, cells at t_5 were treated with 1% (w/v) OsO₄ in Kellenberger buffer for 20 min to destroy the enzyme before incubating them in the substrate mixture.

Counting procedure for the stage of sporulation. Counts of cells exhibiting the various stages of sporulation were performed by using the electron microscope and traversing across a single section which had been stained with lead citrate. Cells were assessed for their stage of sporulation only if they exhibited a complete longitudinal section sufficiently near the equatorial level to give a representative view of the whole cell.

Results

Biochemical localization in sporulating cells

When sporulation was induced, alkaline phosphatase activity began to increase at $t_{1.5}$ – t_2 (some 2.0–2.5 h before the appearance of refractile spores) and reached a maximum at about t_5 . A peripheral localization of the enzyme in the cell was suggested by the following observations. The enzyme could be assayed in whole cells, yet bacterial cells are relatively impermeable to phosphate esters. Treatment

EXPLANATION OF PLATE I

Cytochemistry of alkaline phosphatase in sporulating cells

Cells of *B. subtilis* at t_5 of sporulation stained cytochemically (2.5 h incubation with β -glycerophosphate) in the medium of Sabatini *et al.* (1963). (a) A cell in longitudinal section and part of another in cross-section show discrete deposits in the peripheral membrane of the mother cell. (b) In addition to the deposits in the peripheral membrane, a finer deposit of electron-dense material is visible around the membranes of the developing prespore. (c) Cell pretreated with toluene (2 drops added to 2.0 ml of cell suspension and the tube shaken vigorously for 1 min) before cytochemical staining. *p*-Nitrophenyl-phosphate replaced β -glycerophosphate in the incubation mixture. Sites of enzyme activity are visible at the peripheral membrane and the prespore membranes. Each bar represents 0.2 μ m.

EXPLANATION OF PLATE 2

Cytochemistry of alkaline phosphatase in sporulating cells

(a) Cell at t_5 of sporulation stained cytochemically (2.0h incubation with *p*-nitrophenylphosphate) in the medium of Ghosh *et al.* (1971). Large areas of enzyme activity are visible at the cell surface and associated with the prespore membranes. (b) Cell prepared as in Plate 1(a) except that the substrate (β -glycerophosphate) was omitted from the cytochemical incubation mixture. (c) Cell prepared as in Plate 1(a) except that before the cytochemical staining the cells were treated with 1% (w/v) OsO₄ to destroy all alkaline phosphatase activity. Each bar represents 0.2 μm.

EXPLANATION OF PLATE 3

Cytochemistry of alkaline phosphatase in sporulating cells

(a) Immature prespore prepared at t_5 of sporulation by liberation from the mother cell after treatment with lysozyme. Alkaline phosphatase activity was detected by incubation in the medium of Sabatini *et al.* (1963) (3.0h incubation with β -glycerophosphate). Heavy deposits are associated with the outside surface of the inner membrane of the spore protoplast. The outer electron-dense area is the developing coat layers of the immature spore. (b) Immature prespore prepared as described above except that the substrate (β -glycerophosphate) was omitted from the cytochemical incubation mixture. The electron-dense outer spore coat layer is still visible, but no deposits due to enzyme activity are associated with the spore protoplast. (c) Activity of the vegetative enzyme in a pair of cells undergoing sporulation. Cells at t_5 were incubated in a phosphate-depleted medium for 65 min. The cells were then stained cytochemically (45 min incubation with p -nitrophenylphosphate) in the medium of Ghosh *et al.* (1971). Heavy deposits are associated with the peripheral and prespore membranes. Each bar represents $0.2\mu\text{m}$.

A. R. GLENN AND J. G. COOTE

with toluene (which destroys the selective permeability) resulted in a small (20–25%), but reproducible increase in phosphatase activity over that of untreated cells. The enzyme was removed by washing cells in 0.01 M-Tris buffer, pH 7.4, containing 0.3 M-MgCl₂·6H₂O. When membranes were prepared, 90% or more of the cell-bound phosphatase activity remained associated with the membrane fraction. Sporulating cells (treated with toluene) had a specific activity for the enzyme of 52.5 units/mg of protein at t_5 . Prespores, isolated from these cells, had a specific activity of 30.2 units/mg of protein. These biochemical data indicated that enzyme activity was associated with both the mother cell and the developing spore.

Cytochemical localization in sporulating cells

Cultures were induced to sporulate and samples taken at t_5 for cytochemical treatment. Plates 1(a) and 1(b) show the results obtained by the modified method of Sabatini *et al.* (1963). There are clearly localized deposits of electron-dense material in the membrane of the mother cell. In addition a fine deposit was sometimes observed around the prespore membrane. To overcome any permeability barrier to the passage of staining reagents, cells were treated with toluene and then stained cytochemically. This treatment produced clearer deposits around the spore protoplast (Plate 1c). Experiments carried out by the method of Ghosh *et al.* (1971) produced similar results except that electron-dense deposits were visible in prespore membranes without prior treatment with toluene (Plate 2a). The difference in results may reflect variations in permeability of the heavy metals used in the two methods. Similar electron-dense deposits were obtained with either β -glycerophosphate or *p*-nitrophenyl phosphate as substrate. No such deposits were found in any of the controls (Plates 2b and 2c).

Immature prespores were prepared from a culture undergoing sporulation and treated cytochemically. Heavy deposits of electron-dense material were found on the inner membrane of the spore protoplast (Plate 3a). No such deposits were found in a control sample incubated without substrate (Plate 3b).

Alkaline phosphatase and the stages of sporulation

The discrete areas of electron-dense material found in the membranes afforded a semi-quantitative method of correlating the appearance of enzyme activity with a particular stage of spore formation. Cultures at t_5 were sampled and treated cytochemically. At this time enzyme activity was high and the culture was likely to contain cells at all stages of

sporulation up to stage V. Thin sections were examined in the electron microscope and cells showing a complete longitudinal section were assigned to a stage of sporulation and the number of discrete areas of electron-dense material in each cell counted. Some difficulty was experienced in separating stages 0 and I of sporulation and they were therefore grouped together. In all, 135 complete longitudinal sections were examined (30 at stages 0 or I, 29 at stage II, 53 at stage III and 23 at stage IV). The average numbers of distinct deposits per cell for the various stages of sporulation were as follows. Stage 0 or I, 1.2; stage II, 4.9; stage III, 7.3 and stage IV, 8.9. The number of electron-dense deposits per cell increased sharply in cells at stage II.

Derepression of the phosphate-repressible alkaline phosphatase in sporulating cells

Cells were resuspended to induce sporulation and immediately (t_0) a 10 ml sample was filtered through a Millipore filter, washed with warm phosphate-free resuspension medium and the cells were then incubated at 37°C in the same phosphate-free medium. Samples were taken at intervals for extinction at 600 nm and alkaline phosphatase assay. The procedure was repeated at t_5 when the incidence of refractility in the culture was 30%. The final degree of refractility at t_7 was 75%, i.e. most of the cells at t_5 were well on the way to forming refractile spores.

The rates of synthesis of the derepressed enzyme at t_0 and t_5 were very similar (Fig. 1). This did not prove unambiguously that sporulating cells were being derepressed, especially as there was a pronounced lag period exhibited by the cells at t_5 (Fig. 1). There are in any population 15% or more of cells that never become initiated to sporulation and it could be reasonably argued that these cells were producing enzyme at an increased rate after the initial lag period. Although this seemed an unlikely explanation for the results, cytochemical experiments were carried out to examine enzyme production in individual cells.

A sample of cells at t_5 was resuspended in the phosphate-free medium at 37°C for 65 min. After this time the specific activity of the enzyme was 610 units/mg dry wt. A sample of cells was fixed in glutaraldehyde and stained cytochemically. Heavy deposits of electron-dense material were found associated with the cytoplasmic and prespore membranes of sporulating cells starved of phosphate in this manner (Plate 3c). The relatively short incubation time with the staining reagents eliminated interference from the low amounts of sporulation phosphatase. Sections of control cells not subjected to a period of phosphate starvation never showed electron-dense deposits after short incubation periods.

Fig. 1. Derepression of the phosphate-repressible vegetative alkaline phosphatase in cells undergoing sporulation

Cells were resuspended and at t_0 a sample was filtered, washed and resuspended in a phosphate-depleted medium and assayed at 15 min intervals for alkaline phosphatase activity. A sample was similarly treated at t_5 when 30% of the cells were refractile. Alkaline phosphatase activity: ○, phosphate depletion at t_0 ; ●, phosphate depletion at t_5 .

Discussion

The biochemical data indicated that the sporulation alkaline phosphatase was associated with both the cytoplasmic and prespore membranes. This suggestion was confirmed by the cytochemical studies. Deposits of electron-dense material, caused by enzyme activity, were clearly associated with the cytoplasmic membrane of the mother cell and with the developing prespore membranes (Plates 1, 2 and 3).

Sporulation alkaline phosphatase thus has a membrane localization similar to that described for the vegetative cell enzyme of *B. subtilis* by Wood & Tristram (1970) and Ghosh *et al.* (1971). The enzyme gave rise to discrete deposits similar to those described by other workers in e.g. *Myxococcus xanthus* (Voelz & Ortigoza, 1968); *Escherichia coli* (Done *et al.*, 1965); *B. subtilis* (Ghosh *et al.*, 1971). An obvious, but unsubstantiated, suggestion is that there are specific areas on the membrane for synthesis of the enzyme.

A strict correlation between a biochemical event and the stage of sporulation at which it occurs cannot be achieved satisfactorily by normal enzyme assay procedures because of the asynchrony of the population undergoing sporulation. Therefore an attempt was made by cytochemical means to correlate the increase in alkaline phosphatase activity in individual cells with a particular stage in the sporulation process. A strong correlation was found between the

average number of deposits per cell and the stage of spore formation and there was a sharp increase in the number of deposits per cell at stage II (spore-septum formation). This association of enzyme activity with stage II agrees with previous observations made from examination of the properties of mutants unable to sporulate (Waites *et al.*, 1970; Coote & Mandelstam, 1973). In addition, Dawes *et al.* (1969) showed that the time taken for cells to begin septum formation was 1.4 h, a value that agrees well with the time that phosphatase activity begins to increase in resuspension cultures.

Various studies (Nelson & Kornberg, 1970; Glenn & Mandelstam, 1971; Ichikawa & Freese, 1974) have tended to eliminate phosphate starvation as the mechanism of induction of sporulation phosphatase. The direct demonstration of enzyme activity in individual cells at stage II of spore formation, when phosphate transport is normal and there is no closed spore protoplast, provides an additional reason for rejecting phosphate starvation as the inducing mechanism.

The phosphate-repressible 'vegetative' alkaline phosphatase could be derepressed by phosphate starvation in sporulating cells (Fig. 1). Individual cells well advanced in spore formation showed heavy deposits of electron-dense material owing to vegetative enzyme activity (Plate 3c). This was a direct and unambiguous demonstration that sporulating cells were able to synthesize normally the phosphate-repressible enzyme characteristic of growing cells. It is noteworthy that the deposits caused by this vegetative enzyme activity had a similar location on the cytoplasmic and prespore membranes as the sporulation specific enzyme activity.

The use of cytochemical techniques at the electron-microscope level offers a direct method of demonstrating that individual cells, which have become morphologically differentiated, can be induced to produce vegetative enzymes. Further application of the technique might be useful for determining the relative contribution of the mother cell and the developing spore to the activity of various enzymes associated with spore formation.

We thank Dr. D. Kay and Dr. P. Dyer for advice on electron microscopy and Professor J. Mandelstam for many helpful discussions. The work was supported by the Science Research Council.

References

- Aubert, J.-P. & Millet, J. (1963) *Colloq. Int. C. N. R. S.* **124**, 545-551.
- Coote, J. G. & Mandelstam, J. (1973) *J. Bacteriol.* **114**, 1254-1263.
- Dawes, I. W., Kay, D. & Mandelstam, J. (1969) *J. Gen. Microbiol.* **56**, 171-179.
- Done, J., Shorey, C. D., Loke, J. P. & Pollak, J. K. (1965) *Biochem. J.* **96**, 27C-28C.

- Ghosh, B. K., Wouters, J. T. M. & Lampen, J. O. (1971) *J. Bacteriol.* **108**, 928–937
- Glenn, A. R. & Mandelstam, J. (1971) *Biochem. J.* **123**, 129–138
- Halvorson, H. O. (1965) *Symp. Soc. Gen. Microbiol.* **15**, 343–368
- Hanson, R. S., Peterson, J. A. & Yousten, A. A. (1970) *Annu. Rev. Microbiol.* **24**, 53–90
- Hardwick, W. A. & Foster, J. W. (1952) *J. Gen. Physiol.* **35**, 907–927
- Ichikawa, T. & Freese, E. (1974) *Biochim. Biophys. Acta* **338**, 473–479
- Kellenberger, E., Ryter, A. & Séchaud, J. (1958) *J. Biophys. Biochem. Cytol.* **4**, 671–676
- Kornberg, A., Spudich, J. A., Nelson, D. L. & Deutscher, M. P. (1968) *Annu. Rev. Biochem.* **37**, 51–78
- Mandelstam, J. (1969) *Symp. Soc. Gen. Microbiol.* **19**, 377–402
- Nelson, D. L. & Kornberg, A. (1970) *J. Biol. Chem.* **245**, 1137–1145
- Reynolds, E. S. (1963) *J. Cell. Biol.* **17**, 208–212
- Ryter, A. (1965) *Ann. Inst. Pasteur Paris* **108**, 40–60
- Sabatini, D., Bensch, K. & Barnett, R. (1963) *J. Cell Biol.* **17**, 19–58
- Schaeffer, P. (1969) *Bacteriol. Rev.* **33**, 48–71
- Sterlini, J. M. & Mandelstam, J. (1969) *Biochem. J.* **113**, 29–37
- Voelz, H. & Ortigoza, R. O. (1968) *J. Bacteriol.* **96**, 1357–1365
- Waites, W. M., Kay, D., Dawes, I. W., Wood, D. A., Warren, S. C. & Mandelstam, J. (1970) *Biochem. J.* **118**, 667–676
- Warren, S. C. (1968) *Biochem. J.* **109**, 811–818
- Wood, D. A. W. & Tristram, H. (1970) *J. Bacteriol.* **104**, 1045–1051