Microelectrophoretic Studies of Coat-defective Spores of Bacillus megaterium

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Microelectrophoretic studies of lysozyme-resistant spores of Bacillus megaterium QM B1551 suggested that carboxyl groups were the only ionized species on the spore surface. Spores of B. megaterium NCIB 8291 have defective coats, allowing lysozyme to attack the underlying cortical peptidoglycan and initiate germination-like changes. The surface of such spores was characterized by the presence of ionized carboxyl and amino groups, suggesting that the amino groups were present on the cortical surface. Spores of B. megaterium QM B1551 rendered defective by extraction of coat protein with sodium dodecyl sulphate and dithiothreitol at pH 10 were also lysozyme sensitive and had similar electrophoretic behaviour to the naturally coat-defective spores. Since the electrophoretic behaviour of coat defective spores is qualitatively similar to that of germinated spores, holes or channels may appear in the spore coat during the early stages of germination, exposing the cortical peptidoglycan.

INTRODUCTION

The gross structure of dormant bacterial spores is well known (Tipper & Gauthier, 1972), but spore structure at the molecular level is less well understood. Germination results in physical and chemical changes within the spore (Gould, 1969; Setlow, 1981). In particular, spore components such as peptidoglycan fragments, Ca\(^2\)+ and dipicolinic acid are rapidly lost from germinating spores, suggesting changes in permeability.

Particle electrophoresis provides a method of investigating the electrical character of cell surfaces and identifying the nature of the ionizable species present at the surface (Shaw, 1969). Douglas (1955) used particle electrophoresis to show that bacterial spores were negatively charged and suggested that carboxyl groups were responsible, while Tochikubo et al. (1975) demonstrated the appearance of surface amino groups during germination of spores of Bacillus subtilis. In addition, the effect of coat modification and removal by chemical extraction on electrophoretic mobility at pH 7.2 of spores of Bacillus licheniformis has been examined by Gore et al. (1978).

METHODS

Spore preparation and maintenance of cultures. Bacillus megaterium QM B1551 was obtained from G. J. Dring (Unilever Research, Colworth House, Bedford, U.K.) and B. megaterium NCIB 8291 from the National Collection of Industrial Bacteria (Torry Research Station, Aberdeen, U.K.). Cultures were maintained on nutrient agar slopes after growth at 30 °C. Spores were produced by growth in 50 ml supplemented nutrient medium (Setlow & Kornberg, 1969) in 250 ml Erlenmeyer flasks at 30 °C on a gyratory shaker (Gallenkamp; 150 rev. min\(^{-1}\)) and harvested after 24 h by centrifugation, before washing five times with sterile glass distilled water at 4 °C, resuspension in sterile glass-distilled water and storage at 1 °C. Such cultures contained approximately 95% free spores. Electrophoretic studies were made within 14 d of preparation.

Extraction of spore coat protein. Spores of B. megaterium QM B1551 at a concentration of about 10 mg dry wt ml\(^{-1}\) were suspended in a freshly prepared solution of 0.5% (w/v) sodium dodecyl sulphate (SDS), 0.1 M-dithiothreitol (DTT) and 0.1 M-NaCl as described by Vary (1973). The pH was adjusted to 10 with NaOH.
The suspension was incubated at 37 °C for 2 h before centrifuging, washing three times and resuspending in glass-distilled water. Microelectrophoretic studies were made within 48 h of preparation.

Lysozyme resistance. Spores were incubated at 37 °C in the presence of lysozyme (100 μg ml⁻¹) and sodium phosphate buffer (0.1 M, pH 7.5). Sensitivity to lysozyme was monitored by measuring the turbidity at 600 nm and, directly, by counting dark and bright spores observed by phase contrast microscopy.

Colony formation. To determine the effect of electrophoresis buffers on viability, spores were stored at 1 °C for 4 d in the buffers before diluting into sterile distilled water and plating out on to Bacillus spore agar (Bayliss et al., 1981), using a Spiral Plate Maker (Spiral Systems, Cincinnati, Ohio, U.S.A.) as described by Bayliss & Waites (1980).

Measurement of electrophoretic mobility. Cells were diluted 100-fold into electrophoresis buffers (Miller & Golder, 1950) at a final ionic strength of 0.1 M. Electrophoretic mobilities were measured using a Rank Mark II microelectrophoresis apparatus (Rank Bros, Bottisham, Cambridge, U.K.). A rectangular cell with platinum black electrodes was used and observations were made at a stationary level (Shaw, 1969). At least 20 cells were timed over a fixed distance of 104 μm with reversal of polarity and mobilities were calculated in μm s⁻¹ V⁻¹ cm and expressed as the mean value ± standard deviation. Temperatures were maintained at 20 °C ± 1 °C.

RESULTS

Viability in electrophoresis buffers. Spores suspended in the electrophoresis buffers for 4 d did not undergo any phase change or loss in viability.

![Fig. 1](image1.png)

**Fig. 1.** pH dependence of the electrophoretic mobility of B. megaterium spores. ●, QM B1551; □, QM B1551 after extraction with SDS/DTT; ○, NCIB 8291.

![Fig. 2](image2.png)

**Fig. 2.** Frequency of occurrence of a given mean transit time (t) within B. megaterium spore populations at pH 7.5. (a) QM B1551; (b) QM B1551 after extraction with SDS/DTT; (c) NCIB 8291. The values of t are the mean transit times, after reversal of polarity, for traversing 50 μm at an applied voltage of 20 V in a rectangular cell of length 9.8 cm. Mobility, \( \mu = 24.5/t \) μm s⁻¹ V⁻¹ cm.
Electrophoretic mobility of spores of *B. megaterium QM B1551*. Spores of strain QM B1551 did not show any evidence of germination-like changes on incubation with lysozyme. The pH dependence of the electrophoretic mobility is shown in Fig. 1 (line a). Mobility increased with increasing pH up to pH 6 and showed a plateau region at high pH values. Standard deviations were small (+12%) at neutral pH and above, but increased markedly on lowering the pH (+30% at pH 3-5). This may indicate a heterogeneous surface charge distribution amongst the population of spores, but probably reflects an increasing experimental error at lower pH, due to increased transit times and resultant errors due to electrode polarization or drifting of the particles about the stationary level.

Electrophoretic mobility of spores of *B. megaterium NCIB 8291*. Spores of strain NCIB 8291 are sensitive to lysozyme (Suzuki & Rode, 1969), which suggests that the coat is defective. The pH dependence of the electrophoretic mobility is shown in Fig. 1 (line c). Mobility increased with pH up to approximately 5.5, remained constant until a pH of about 8.4 was reached, and then showed a further increase with increasing pH. Standard deviations showed essentially the same changes as those for the mobility of strain QM B1551.

Electrophoretic mobility of coat-defective spores of *B. megaterium QM B1551*. SDS/DTT extraction of QM B1551 resulted in a spore population in which 95% of the population was observed by phase-contrast microscopy to revert from phase bright to phase dark on incubation with lysozyme for 10 min. The pH dependence of mobility is shown in Fig. 1 (line b). The curves show an increase in mobility with increasing pH up to pH 6, an ill-defined plateau region between pH 6 and 9 and some evidence for an increased mobility at higher pH. The standard deviations were markedly larger (+61% at pH 7.5) than those obtained for *B. megaterium QM B1551* (+15%) and NCIB 8291 (+25%). This results from a much broader variation in the electrical character of the spores within a population at pH 7.5 (Fig. 2).

**DISCUSSION**

Electrophoretic mobility is determined by the effective charge density at the shear plane surrounding the particle surface. The mobility thus depends on the nature of the ionizable surface groups, the nature and extent of absorption of ionic species and the screening of the surface charge by the electrical double layer. Equations relating mobilities to surface charge densities involve assumptions which generally render them inapplicable for quantitative estimates of surface charge densities of complex, irregularly shaped permeable cell surfaces. Electrophoresis is normally used to monitor the nature of surface groups through pH dependence studies (Shaw, 1969).

The present work suggests that electrophoresis provides a rapid, non-destructive method for detecting changes in spore surfaces and for determining their ionizable species. In particular, at pH > 3, spores of *B. megaterium QM B1551* are negatively charged. These results are qualitatively and quantitatively similar to those reported previously (Douglas, 1955; Douglas & Parker, 1958). Slight discrepancies would be expected in view of the differing ionic strengths of the buffers employed, variations in the species examined, the culture conditions used and the methods for washing and resuspension of the spores.

In agreement with previous work (Douglas, 1955; Douglas & Parker, 1958) the results suggest a single ionizable species consistent with surface carboxyl groups (pK 2.7). Early studies attributed this charge to a hexosamine peptide (Douglas, 1955; Douglas & Parker, 1958). However, biochemical studies of the surface layers of spore coats suggest the presence of protein containing a high percentage of disulphide linkages together with small quantities (1 to 2%) of surface lipid, phosphorus and inorganic material (Bernlohr & Sievert, 1962; Murrell, 1969; Strange & Dark, 1956; Vinter, 1960; Warth et al., 1963). Such studies suggest that the negative charge is probably due to the carboxyl groups of aspartic and glutamic acids, since the resistant coat residue of *B. megaterium*, which probably represents the outermost coat layers, is particularly rich in lysine, aspartic acid and glutamic acid (Kondo & Foster, 1967). Such an explanation has recently been proposed for *B. subtilis* spores (Tochikubo et al., 1975). These authors have
confirmed the presence of carboxyl groups by investigating the effect of diazomethane, a reagent for modifying surface carboxyl groups (Gittens & James, 1963), on the mobility of the spores.

The spores of *B. megaterium* NCIB 8291 were found to be negatively charged at pH > 3. The pH dependence indicates the presence of at least two ionizable species consistent with surface carboxyl (pK 2.7) and surface amino (pK 10.1) groups. The appearance of amino groups at the surface of the spore may be the major cause of the reduction of the mobility at near neutral pH within the plateau region. One plausible explanation for the results is that the defect in the coat structure results in conformational changes of the outermost coat proteins exposing amino groups (possibly attributable to the high lysine content). An alternative explanation, favoured by Gore *et al.* (1978) for spores of *B. licheniformis* extracted with SDS–DTT, is that the defect may arise due to the appearance of 'holes' within the protein coat, which expose the underlying cortical peptidoglycan. Such an explanation would not only account for the appearance of amino groups but would readily explain the lysozyme sensitivity of *B. megaterium* NCIB 8291.

Fitz-James (1971) showed that extraction of spores of *B. megaterium* KM with SDS/DTT removed the coats exposing the cortex, and Vary (1973) found that a similar extraction of *B. megaterium* QM B1551 removed 46% of protein and permitted lysozyme to initiate germination-like changes. We therefore extracted spores of *B. megaterium* QM B1551 with SDS/DTT. This treatment produced a heterogeneous population with electrical characteristics spanning the range from those obtained for *B. megaterium* QM B1551 to *B. megaterium* NCIB 8291 (Fig. 2), suggesting that the extraction acts non-uniformly and that the distribution of coat protein remaining on the treated spores varies irregularly from spore to spore. However, the fact that 95% of the population showed lysozyme sensitivity suggests that in the majority of the population the cortex is exposed in at least one site.

In view of the variation in the population we can only draw qualitative conclusions from the pH dependence of the mean mobility on the effect of cortex exposure. We note that at neutral pH the mean mobility is reduced, as compared to *B. megaterium* QM B1551, and there is some evidence for a rise in mobility at higher pH. Although the present results are not definitive they do suggest that chemical removal of the coat protein and exposure of the cortex can simulate the effects observed in coat-defective spores (strain NCIB 8291). Variations in the mobility due to adsorption of SDS on to the surface of the spores are unlikely to explain the observed results. Biochemical evidence suggests a small lipid content in the spore coat, and early workers (Douglas, 1955; Douglas & Parker, 1958) noted a slight rise in mobility of *B. megaterium* spores due to adsorption of SDS (Shaw, 1969). In the present studies a decrease in mobility at pH 7.5 was observed. Extraction with SDS/DTT at 1 °C for periods of 1 h or 2 h produced progressively increasing lysozyme sensitivity and progressively decreasing electrophoretic mobilities.

Electrophoretic data may suggest conformational changes in proteins resulting in the exposure of amino groups. This is a subtle change, unlikely to be detected by electron microscopy. However, gross morphological changes arising from the formation of holes or channels in the spore coat exposing cortical peptidoglycan might be detected. Ultrastructural studies of spore coats of *Bacillus cereus* T and *B. megaterium* KM suggests the presence of three distinct layers (Aronson & Fitz-James, 1976). The innermost layer, the undercoat (UC), appears to be amorphous whereas the middle pitted layer (P) and the outermost crosspatched layer (CP) are structured. The pitted layer contains pits or holes estimated to comprise 35% to 40% of the coat layer. Electron micrographs of sectioned spores of *B. megaterium* QM B1551 obtained in our laboratories suggest that the coat is qualitatively similar. The ultrastructure of coat of wild-type and modified spores of *B. cereus* have been studied in detail by Aronson & Fitz-James. Spores of wild-type *B. cereus* T show intact CP, P and UC (Aronson & Fitz-James, 1976). Treatment with DTT to break disulphide bonds removes P and depletes UC but leaves CP intact (Aronson & Fitz-James, 1971; 1973; Aronson & Horn, 1972). Extraction with DTT/SDS modifies CP and removes both P and UC, exposing the cortex (Aronson & Fitz-James, 1971; 1973; Aronson & Horn, 1972). Spores of mutant 10**a** formed at a non-permissive temperature and those of mutant 13**L** are both lysozyme sensitive, having a modified CP, a reduced P and some decrease in the density of UC (Aronson & Fitz-James, 1975). Thus there is direct evidence that drastic modifications of the spore coat expose the underlying cortex.
In our study the sensitivity to lysozyme suggests that the cortex of spores of \emph{B. megaterium} NCIB 8291 and those of \emph{B. megaterium} QM B1551, extracted with SDS/DTT, were also exposed. The differences in the electrophoretic behaviour of such spores with defective coats and unextracted spores of \emph{B. megaterium} QM B1551 are qualitatively similar to those observed between dormant and germinated spores of \emph{B. subtilis} (Tochikubo et al., 1975). In addition, the sensitivity to lysozyme parallels the observed electrophoretic changes. It is therefore tempting to propose that the changes during germination result from similar gross structural changes in the spore coat. There is clear evidence that such changes occur during germination of spores of \emph{B. cereus} T, since Aronson & Fitz-James (1976) found depletion of UC and loss of P. We cannot rule out the possibility that the electrophoretic changes we observed may have resulted from configurational alterations in the coat proteins which exposed amino groups, but in view of the morphological evidence, it seems that exposure of cortical peptidoglycan occurred.

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