The Structure of the Exosporium of a Pigmented Clostridium

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Exosporia surrounding spores of a pigmented *Clostridium* showed a hexagonal periodicity when negatively stained and were composed of up to 10 lamellae, each 2.6 nm thick, with a centre-to-centre spacing of 5.2 nm. Optical diffraction spectra revealed that isolated fragments of single lamellae contained hexagonal arrays of subunits with a predominant spacing of 5.2 nm and that negatively stained fragments composed of several lamellae showed interference patterns generated by slightly displaced layers of the same hexagonal lattice.

INTRODUCTION

The term 'exosporium' is used to describe the outer, membranous structure which may be either loosely or closely fitted around the spore of some species of *Bacillus* and *Clostridium* (Holt & Leadbetter, 1969; Tipper & Gauthier, 1972). During characterization of a new strain of pectolytic pigmented *Clostridium*, negative staining revealed the presence of an extensive exosporium which contained regularly arranged subunits with a hexagonal periodicity. A similar ultrastructure has been described in exosporia of several species of *Bacillus* and *Clostridium* and investigated in particular detail in *B. cereus* (Gerhardt & Ribi, 1964; Beaman, Pankratz & Gerhardt, 1971), *B. thuringiensis* (Short *et al.*, 1974; Scherrer & Somerville, 1977) and *C. pasteurianum* (Mackey & Morris, 1972).

The assembly of basic subunits to form regular arrays in biological structures is of fundamental interest. Following the development of image-processing methods applied to electron micrographs of biological materials, specimens with relatively large, flat areas of crystalline or paracrystalline form are particularly suitable for quantitative ultrastructural analysis (cf. Crowther, De Rosier & Klug, 1970; Amos, 1974; Roberts & Hills, 1976; Unwin & Henderson, 1975; Horne, 1977). In some cases bacterial exosporia may consist predominantly of such paracrystalline material and analysis of the structure and mode of assembly is of importance in relation to the formation of the exosporium during sporulation.

This paper describes the structure of the exosporium of a pigmented *Clostridium* revealed by negative staining and sectioning of whole spores and of isolated and purified exosporium material. A more detailed account of the lattice structure and its analysis by image-processing methods will be published separately.

METHODS

Bacterium. Clostridium sp. BL70/20 was isolated from potatoes and maintained in a potato infusion medium (Lund, 1972). Some properties of this organism are described elsewhere (Lund & Brocklehurst, 1978).

Preparation of spore suspensions. The organism was first grown in freshly boiled and cooled reinforced clostridial medium (Hirsch & Grinsted, 1954) and subcultured into the same medium minus agar. After

incubation for 3 d at 25 °C these cultures were used to inoculate plates of potato infusion medium by flooding with approximately 1 ml of culture. Incubation was at 25 °C under H₂:CO₂ (9:1, v/v) for 10 d, and gave a confluent lawn of pink-pigmented colonies with a high proportion of spores. The bacterial growth was washed from the surface of 50 plates with distilled water and cooled to 3 °C. After centrifugation (30 000 *g* for 20 min at 3 °C) the opalescent, pink supernatant liquid (approx. 150 ml) was discarded and the spores were washed three times with 75 ml 50 mM-potassium phosphate buffer pH 7.0 and five times with 75 ml distilled water.

Removal of the exosporium. The washed spores were resuspended in distilled water and exposed to ultrasonic treatment using an M.S.E. 60 W oscillator at 18 to 20 kHz for periods of 0.5 min until the exosporium had been detached from most of the spores (a total time of 6 to 7 min). The suspension was cooled in ice during and between treatments and the process was monitored by examination of samples under the phase-contrast microscope. After ultrasonic treatment the suspension was diluted with an equal volume of distilled water and centrifuged at 2000 *g* for 15 min to sediment the spores.

Purification of exosporium fragments. The supernatant liquid from the previous treatment was centrifuged at 60 000 *g* for 2 h at 3 °C. The material which sedimented was a crude preparation of exosporium fragments. This material was treated with Triton X-100 (0.5 %, v/v) in 50 mM-Tris/HCl buffer pH 7.4 for 10 min at 25 °C to remove contaminating material (Sleytr & Glauert, 1976), then sedimented by centrifugation at 110 000 *g* for 20 min at 3 °C and washed twice with the buffer and once with distilled water. The treatment with Triton X-100 and subsequent washing was repeated a further three times to give purified exosporium fragments. At each stage appropriate samples of spores or of exosporia were removed for examination by phase-contrast or electron microscopy.

Phase-contrast microscopy. Samples of spores were mounted in water and examined under a Zeiss phase-contrast microscope at a magnification of $\times 900$.

Electron microscopy. Samples of spores and of crude exosporium material for negative staining were resuspended in a drop of distilled water, transferred to a carbon-coated grid (400 mesh) and stained with a saturated aqueous solution of uranyl acetate pH 4.5. Samples of purified exosporium fragments were examined using the negative staining-carbon film technique of Horne & Pasquali-Ronchetti (1974).

Samples to be examined by sectioning were prepared by a modification of the method of Sleytr & Glauert (1976). After fixation in glutaraldehyde (2.5 %, w/v) in 0.09 M-cacodylate buffer pH 7.2 containing 3 mM-calcium chloride for 1 h at 4 °C, the samples were centrifuged and washed three times in cacodylate buffer before being left overnight in buffer at 4 °C. The pellets were then postfixed in Zetterquist veronal acetate-buffered osmium tetroxide pH 7.2 (Glauert, 1974) for 3 h at 5 °C in the dark, dehydrated in a series of increasing concentrations of acetone, washed twice (for 10 min) in 1,2-epoxypropane, once (for 2 h) in Epon:epoxypropane (1:1, v/v) and finally embedded in Epon (Luft, 1961). Sections (70 to 80 nm) were cut with a diamond knife on an LKB Ultramicrotome (model III), picked up on Formvar-coated grids (200 mesh) and poststained with a saturated aqueous solution of uranyl acetate (Watson, 1958) and 0.33 % (w/v) lead acetate (Millonig, 1961).

Electron micrographs were taken using an AEI 801 microscope operating at 60 kV, an AEI EM6B at 60 kV or a JEOL JEM 100B at 80 kV at a range of magnifications from 3000 to 40 000. The magnifications were calibrated with negatively stained catalase crystals (Wrigley, 1968). For high magnification calibration and measurements from optical diffraction spectra, the 2.3 nm periodicity of negatively stained tobacco mosaic virus was used. Optical diffraction analysis was made on an experimental diffractometer described by Horne & Markham (1973). Microdensitometer recordings from electron micrographs of sections showing multiple layers within the exosporia were made on a Joyce Loebel Mk I microdensitometer calibrated against catalase crystals photographed at the same magnification.

RESULTS

Electron microscope images of negatively stained preparations containing whole spores (Fig. 1*b*) showed well-defined exosporia. These were loosely fitted round the spore and in many cases appeared to be open, or broken, at one end. The exosporia were partially collapsed or folded, with negative stain frequently trapped in the folded areas. Examination of negatively stained exosporia at higher magnification revealed a regular ultrastructure with at least two types of pattern showing a hexagonal arrangement (Fig. 1*d*). Sections through whole spores showed that the exosporium was composed of several lamellae and there were indications of the presence of fibrous strands, 'suspensor' material, connecting the spore coat to the exosporium (Fig. 1*a, c*).

Exposure of spores to ultrasonic treatment caused many of the exosporia to fracture, initially lengthwise into two pieces (Fig. 2*a*). The treatment was terminated when a high proportion of the exosporia had been detached from the spores; judging by their appearance under the phase-contrast microscope, very few spores lost refractivity during this treatment. After separating the spores from the exosporium fraction by centrifugation, examination of sections and negatively stained preparations confirmed that a high proportion of spores lacked an exosporium (Fig. 2*a, b*); a few fragments of exosporia were visible in the spore fraction. Some sections of spores stripped of exosporia still showed strands of 'suspensor' material projecting from the spore coat (Fig. 2*a, c*).

Negative staining of fractions containing crude exosporia revealed angular-shaped fragments with a hexagonal surface pattern (Fig. 3*b, d*) together with other contaminating material, including fragments of spore coat. In sections, exosporium fragments appeared as sheets, consisting of several lamellae, or as coiled, tube-like fragments (Fig. 3*a, c*). In transverse sections through individual lamellae there was evidence of a periodic structure. Treatment with Triton X-100 and subsequent washing removed most of the contaminating material and the purified exosporium fraction consisted mainly of angular fragments with the characteristic ultrastructure (Fig. 4). The fragments were present either as flat sheets (Fig. 4*b*) or as tube-like structures of the type shown in Fig. 4(*d*). Sections through the exosporium of whole spores and through purified fragments showed that the structure was composed of up to 10 lamellae.

The purified exosporium fraction contained some fragments consisting of a single lamella (Fig. 5*a*). Negative staining showed a fine, granular structure and slender strands appeared to be separated from the fragment where the edges were partially disrupted. Analysis of the electron micrograph in Fig. 5(*a*) by optical diffraction (Fig. 5*b*) revealed a hexagonal periodicity in which the first-order spectrum showed a weak array of spots at a distance of 10.4 nm and the second-order spectrum consisted of a predominant array of spots spaced at 5.2 nm. A hexagonally arranged ultrastructure was clearly visible on the negatively stained fragment shown in Fig. 5(*c*). This fragment was probably composed of several lamellae, two of which had been penetrated by the negative stain. The optical diffraction pattern (Fig. 5*d*) derived from the electron micrograph in Fig. 5(*c*) gave two sets of spectra (A-A, B-B and A'-A', B'-B') representing hexagonal arrays displaced by 26° with respect to each other. Interference between these arrays generated the ultrastructure shown in Fig. 5(*c*), which resulted from a reinforcement of the basic hexagonal periodicity and also the introduction of moiré patterns. A study of optical diffraction spectra generated by the electron micrograph in Fig. 1(*d*) showed that the regular arrays resulted from at least four superimposed stained lamellae. This Figure probably represents two or more layers of the exosporium, each layer consisting of several lamellae; it is likely that the negative stain only penetrated to a limited number of these lamellae.

A diagram of the spacings and structural features of the exosporium at a resolution of about 2 nm is shown in Fig. 6. This is based on the measurements made from sections and from optical diffraction spectra of negatively stained fragments. It should be stressed that the diagram represents averaged spacings observed and no attempt has been made to describe the shape or dimensions of the structural components assembled to form the exosporium. A more detailed study of the lattice structure and its analysis by image-processing methods is in progress and the results will be published separately.

Fig. 1. Washed spores of *Clostridium* bl70/20 before removal of the exosporium: (a, c) sections of spores with exosporia; (b) negatively stained spores with exosporia; (d) detail of negatively stained intact exosporium. ex, Exosporium; c, spore coat; s, 'suspensor' material. Bar markers represent 500 nm

Fig. 2. Spores after removal of the exosporium: (a) sections of spores; (b) negatively stained spores; (c) section of a spore showing attached 'suspensor' material. ex, Exosporium fragments; c, spore coat; s, 'suspensor' material. Bar markers represent 500 nm.

Fig. 3. Crude preparation of exosporium material: (a, c) sections; (b, d) negatively stained. ts, Transverse section through flat or coiled fragment of exosporium; c, fragment of spore coat; la, lamella; sh, sheet of exosporium material; m, contaminating material. Bar markers represent 500 nm.

Fig. 4. Purified preparation of exosporium material: (a, c) sections; (b, d) negatively stained. ts, Transverse section through flat or coiled fragment of exosporium; c, fragment of spore coat; la, lamella; sh, sheet of exosporium material; tu, fragment of exosporium coiled to form a tube. Bar markers represent 500 nm.

Fig. 5. (a) Negatively stained fragment of an exosporium consisting of a single lamella. *f*, Fine strand of material separated from the lamella. Bar marker represents 500 nm. (b) Optical diffraction pattern from the exosporium fragment shown in (a). The hexagonal array of spots shows a weak first-order spectrum at A-A, 10.4 nm, and a strong second-order spectrum at B-B, 5.2 nm. This array is interpreted as evidence that the fragment from which it was derived consisted of a single lamella. (c) Negatively stained fragment of an exosporium consisting of at least two lamellae. The basic lattice present in a single lamella has been reinforced, showing a clear hexagonally arranged ultrastructure, and interference patterns are evident. Bar marker represents 500 nm. (d) Optical diffraction pattern from the exosporium fragment shown in (c). This is interpreted as being derived from two similar arrays displaced by 26° with respect to each other. The spectrum from the first hexagonal array of spots is indicated at A-A, 10.4 nm, B-B, 5.2 nm. The spectrum from the second hexagonal array is indicated at A'-A', B'-B'.

Fig. 6. Diagrammatic representation of the structural features seen in electron micrographs of exosporia prepared from sections and by negative staining techniques. The dimensions shown are averaged values obtained from microdensitometer recordings of transverse sections and optical diffraction spectra from negatively stained material.

DISCUSSION

In negatively stained preparations the exosporium appeared as an open-ended sac surrounding the spore; in this respect it resembled exosporia of *C. pasteurianum* (Mackey & the Morris, 1972), *C. botulinum* (Stevenson & Vaughn, 1972) and *C. bifermentans* (Pope, Yolton & Rode, 1967). The strands of material, seen in sections, apparently connecting the spore coat to the exosporium and projecting from the spore coat after removal of the exosporium, may function as 'suspensors' by which the spore is suspended within the exosporium sac (Mackey & Morris, 1972). A comparison of electron micrographs of whole spores and of purified exosporia showed that the dimensions of the lamellae and the paracrystalline arrays were not affected by the purification procedure.

The interpretation of the basic structural components within the exosporium lattice which we have examined is obviously dependent on several factors including the preparation of single layers (i.e. single lamellae), focussing of the electron microscope lens and penetration of electron-dense staining material. The regular arrays seen in the electron micrographs in Figs 1(d), 3(d) and 5(c) are clearly the result of interference patterns generated by slightly displaced layers of the same hexagonal lattice. This is evident from the negatively stained preparations and corresponding diffraction patterns shown in Fig. 5.

The structure of this exosporium resembled that described by other workers in the inner layer of the exosporium of *B. cereus* var. *terminalis* (Gerhardt & Ribí, 1964; Beaman *et al.*, 1971; Gerhardt, Pankratz & Scherrer, 1976) and of *B. thuringiensis* (Short *et al.*, 1974; Scherrer & Somerville, 1977) and in the exosporium of *C. botulinum* types E and F (Hodgkiss, Ordal & Cann, 1967) and *C. pasteurianum* (Mackey & Morris, 1972). The centre-to-centre distance between lamellae of 5.2 nm is close to the value of 5 nm reported by Mackey & Morris (1972). The predominant spacing between hexagonally arranged subunits of 5.2 nm and the weaker periodicity at 10.4 nm, estimated by optical diffraction, are similar to the spacings of 5 nm and 10 nm found by these workers in negatively stained preparations and by optical diffraction. Further work is required in order to determine the shape and dimensions of the subunits which form the hexagonal lattice and in particular to investigate the possible presence of globular subunits similar to those described in freeze-etched exosporia of *B. thuringiensis* (Short *et al.*, 1974), *C. pasteurianum* (Mackey & Morris, 1972) and of several other species of *Clostridium* (Walker *et al.*, 1976). The electron-transparent region separating layers containing the hexagonal arrays is of uniform thickness but no structural features were visible in the sections examined in the present series of experiments.

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