Outer Layers of the *Azotobacter vinelandii* Cyst

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Ruthenium red stained a capsule external to the exine of the *Azotobacter* cyst. The central body is therefore surrounded by three layers, the intine, the exine, and the capsule, all containing acid mucopolysaccharide. Vesicles that appear to originate from the contracting cell membrane of the central body may account for the lipid content of the intine. The exine is composed of laminated sheets that tend to fragment into hexagonal pieces.

*Azotobacter vinelandii* and *A. chroococcum* have the capacity to form specially structured resting cells called cysts. The cytological characterization of the formation and germination of the cysts of both species has been described previously (11, 13). Socolofsky and Wyss (9) characterized the cyst of *A. vinelandii* as a physiological entity, describing its rupture by ethylenediaminetetraacetic acid (EDTA), and its respiratory activity as compared to vegetative cells of the same species. The resistance of cysts relative to that of vegetative cells has also been described (10). Parker and Socolofsky (5) correlated cyst resistance with the presence of an intact cyst coat by showing that, after removal of the coat, the viable central bodies displayed the same resistance patterns as vegetative cells.

The cyst coat is composed of two layers, the exine and the intine. In cross sections of cysts the exine appears as a heavy barklike coat composed of laminations, whereas the intine has been described as a homogeneous layer (9). Lin and Sadoff (3) disintegrated cysts and demonstrated that the exine can be split to yield sheetlike structures.

In this study we examined the organization and fine structure of the outer layers of the cyst by electron microscopy.

**MATERIALS AND METHODS**

*A. vinelandii* ATCC 12837 was grown on the surface of Burk nitrogen-free agar with 0.2% n-butyl alcohol as a carbon source. After 4 days at 33°C, the cells were removed and washed by centrifugation, except for treatment of cells with ruthenium red where cells were removed directly from plates. In some cases cysts were lysed by the method described by Socolofsky and Wyss (9). Carbon replicas were prepared by conventional methods (8).

Negative stains were prepared with 2% uranyl acetate (pH 4.3).

For thin sectioning, cells were fixed in 0.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and postfixed overnight at 4°C in similarly buffered 1% osmium tetroxide. In some cases cells were prefixed in 0.5% glutaraldehyde in the presence of ruthenium red and postfixed with osmium tetroxide in the presence of ruthenium red as described by Pate and Ordal (6). Controls to which no ruthenium red was added were fixed at the same time. After fixation specimens were dehydrated in a graded alcohol series, followed by two changes of acetone. They were finally embedded in a mixture consisting of 70% dodecyl succinic anhydride, 20% Araldite 6005, and 10% Epon 812 with one drop of accelerator DMP-30 (Rohm and Haas Co., Philadelphia, Pa.) added per ml of plastic used. Sections were cut on a Sorvall Porter-Blum MT-2 microtome with a diamond knife. With the exception of samples fixed with ruthenium red, sections were poststained with Reynolds lead citrate. Specimens were examined with a Hitachi HS-7S electron microscope.

The electron micrographs were made with Kodak contrast process Ortho film.

**RESULTS**

A carbon replica (Fig. 1) shows the contrast between the surface of two vegetative cells and a cyst. The surface of the cyst is deeply convoluted and distinct layers can be seen, whereas the vegetative-cell surface is smooth and slightly convoluted. A carbon replica of a cyst lysed with EDTA (Fig. 2) shows that the surface of the released central body is similar to that of the vegetative cell. The flattened exine is composed of sheets or plates, some of which have angular edges.

A negative stain (Fig. 3) shows two fragments of exine which have formed hexagonal plates. The average angle in both of these plates is 120°. Ordered fine structure could not be demonstrated with this stain; however, the expected pattern of subunits is visible in the adjacent flagella fragments.
FIG. 1. Cyst showing layering (arrows) on surface. Two vegetative cells lie next to the cyst. Replica. Bar indicates 1 μm.

FIG. 2. Cyst lysed with EDTA showing central body (CB) and flattened exine. Replica. Bar indicates 1 μm.
Cross sections of young encysting cells (Fig. 4) show that in 2-day-old cultures it is possible to see fibrous material around the cell as well as vesicles which appear to originate from the surface of the central body. In a cross section of a more mature cyst (Fig. 5), these vesicles appear to be distributed throughout the intine region, indicating that, if their origin is as suggested, they must be formed during the development of the intine. The layered exine is also visible. In a section through the cyst coat which does not cut through the central body (Fig. 6), vesicles can be seen throughout the intine; however, in the region of the central body there is a greater concentration of vesicles, suggesting again that their origin is from the surface of that structure.

The occurrence of polysaccharide-like capsular material (Fig. 7) external to the exine region is revealed in a section of a mature cyst treated with ruthenium red. There is also dense staining in the vicinity of the exine; however, there are areas in that region which have not stained. The intine is heavily stained with ruthenium red. There is no detail revealed within the central body, with the exception of dense osmiophilic bodies which also show up in the control sample (Fig. 8) fixed in the same manner, except that ruthenium red was not present in the glutaraldehyde or osmium fixative. The increased contrast due to staining by ruthenium red is evident. In the control there is little difference in density between the cytoplasm, the intine, and the exine.

**DISCUSSION**

We formerly thought that the mature Azotobacter cyst consisted of a central body enclosed by two distinct layers, a fibrous intine similar in nature to the vegetative cell capsule on whose
FIG. 4. Two-day-old encysting cells with fibrous material and vesicles (arrows) surrounding the cell. Glutaraldehyde-osmium fixation. Uranyl acetate and lead citrate poststain. Section. Bar indicates 0.5 μm.

FIG. 5. Mature cyst. Identifiable structures external to the central body include the exine (EX) and the intine (I) containing vesicles (arrows). Glutaraldehyde-osmium fixation. Uranyl acetate and lead citrate poststain. Section. Bar indicates 0.5 μm.

FIG. 6. A section through the cyst coat showing vesicles (arrows) distributed throughout the intine and concentrated in the region of the central body. Glutaraldehyde-osmium fixation. Uranyl acetate and lead citrate poststain. Bar indicates 0.5 μm.
FIG. 7. Section through a cyst stained with ruthenium red showing capsular material (C) external to the exine (EX) and the heavily stained intine (I). Glutaraldehyde-osmium fixation in the presence of ruthenium red. No poststain. Bar indicates 0.5 μm.

Fig. 8. Contrasting section through a cyst not stained with ruthenium red. Glutaraldehyde-osmium fixation. No ruthenium red. No poststain. Bar indicates 0.5 μm.
surface a rigid exine was precipitated. It now appears that the fibrous capsule extends beyond the exine. This capsule external to what had been regarded as the outer cyst coat has also been noted in India ink preparations by Parker and Socolofsky (5).

A micrograph (Fig. 4) of a sample treated with ruthenium red in the presence of osmium showed marked increase in density of all three layers surrounding the central body. In samples in the presence of osmium alone, the whole cyst was approximately the same density, which was considerably less than the ruthenium red-osmium samples. Dense osmiophilic bodies were present in both samples. It was postulated by Luft (4) that ruthenium red can be bound in sufficient quantity by acid mucopolysaccharide so that, in the presence of osmium tetroxide, it produces an observable density in an electron microscope. Most of the work at this time has been done on cartilage, since cartilage matrix contains acid mucopolysaccharide. Jones, Roth, and Sanders (2) used ruthenium red in their study of slime layers in streams. Pate and Ordal (6) used ruthenium red to stain Chondrococcus columnaris.

No substructure is evident in our photomicrographs of the surface of the exine; however, the tendency of the exine to form hexagonal fragments suggests that such ordered fine structure may exist. The structure revealed in our studies with Azotobacter cysts is reminiscent of the para-crystalline exosporium fragments from spores of Bacillus cereus described by Gerhardt and Ribi (1).

The intine appears to be fibrous in nature, and staining with ruthenium red confirms that this fibrous structure contains acid polysaccharides. Distributed within the fibrous matrix of the intine are spherical structures which correspond to the vesicle-like structures around the central body. It is possible that the compaction and membrane-shortening involved in the process of encystment is accomplished by pinching off vesicles from the surface of the cell membrane. This could account for the loosely bound lipid reported by Lin and Sadoff (Bacteriol. Proc., 1969, p. 42) to be present in the intine along with polysaccharide and protein.

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LITERATURE CITED