Endogenous Encystment of \textit{Azotobacter vinelandii}

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When young cells of \textit{Azotobacter vinelandii} are impinged on membrane filters, washed free of carbon substrate, and placed on a mineral salts basal medium, the culture will proceed to encyst although at a slower rate than if \textit{n}-butanol were supplied as a substrate. The endogenous cysts are depleted in poly\(\beta\)-hydroxybutyrate and have a narrower intine but show an increased resistance to desiccation and are susceptible to lysis by chelating agents. Membrane-supported cells reveal details of the encystment process such as the formation of a zone within the capsule prior to exine formation and the early deposition of exine structures.

The cyst of the genus \textit{Azotobacter} is a spherical resting cell produced by the shortening and rounding-up of a single rod-shaped vegetative cell and the elaboration of a thick double-layered surrounding structure. Socolofsky and Wyss (8) consider the cyst as a modified vegetative cell encompassed in a specialized capsule. This capsule affords protection from some adverse aspects of the physical environment, but does not provide the extremes of resistance to physical stresses usually associated with the bacterial endospore. \textit{Azotobacter} cysts have been shown by Socolofsky and Wyss (9) to be somewhat more resistant than are the vegetative cells to heat, sonic treatment, and ultraviolet irradiation; the most distinctive difference, however, was that, unlike vegetative cells, cysts survive drying at the temperatures associated with their mesophilic growth. Also, unlike vegetative cells, cysts are disrupted by chelating agents without the addition of lysozyme (8). Olson and Wyss (4) have presented evidence that suggests a modification of the configuration of some nucleic acids in the cysts.

Reports in the literature deal with the formation of cysts by various strains of \textit{Azotobacter} under different cultural conditions (1, 2, 8, 11, 13, 14). During a study of the effect of \textit{n}-butanol concentration on the morphology of \textit{Azotobacter} cells grown on membrane filters, it was observed that encystment occurred even when a carbon source was omitted. The characterization of these endogenous cysts is the subject of this paper.

\textbf{MATERIALS AND METHODS}

\textbf{Organisms.} Vegetative cells of \textit{Azotobacter vinelandii} ATCC 12837 were grown for 16 hr in Burk's nitrogen-free liquid medium listed by Wilson and Knight (12) with 0.5\% sucrose as a carbon source. These cells were suspended in the same medium but without the carbon source (Burk's salts) to yield a concentration of 2 \times 10^7 cells per ml. Portions (1 ml) of this cell suspension were impinged on membrane filters (Millipore Corp., Bedford, Mass.) with a diameter of 47 mm and a pore size of 0.45 \(\mu\)m. The filters were washed with two 10-ml portions of Burk's salts and then placed on a sterile 2-inch square of Whatman filter paper in sterile petri dishes containing 20 ml of sterile Burk's salts. "Normal" cysts were produced for comparison by adding 0.02\% \textit{n}-butanol to the medium. After 4 days at 33 C the filter membranes were removed from the petri dishes and dried at 33 C. At daily intervals, replicate filter membranes were removed, and the cells were washed off and plated to determine the viable count. Prior to the drying, this showed the extent of growth, and after drying it showed resistance to desiccation and therefore the extent of encystment.

\textbf{Lytic measurements.} The extent of encystment was determined by measuring susceptibility to lysis by chelating agents. The cultures were washed off the membranes, suspended in 0.01 \textit{m} tris(hydroxymethyl)aminomethane (Tris) buffer at \(pH\) 8.0, and the decrease in turbidity that followed the addition of ethylenediaminetetraacetic acid (EDTA) was measured following the technique described by Socolofsky and Wyss (8).

\textbf{Electron microscopy.} The cells were washed off of Millipore membranes with sterile Burk's salts solution after 4 days and were sedimented by centrifuging. The resulting pellet was fixed for 30 min in 2\% \textit{KMnO}_4 (3). The fixed pellet was washed in deionized water and dehydrated through a graded alcohol series, followed by two changes of acetone. Specimens were then embedded in a plastic 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 mixture used. Sections were cut on a Sorvall Porter-Blum MT-2 microtome with a diamond knife. All sections were stained with Reynolds' lead citrate (6). Specimens were viewed with a Hitachi HS-7S electron microscope with an accelerating voltage of 75 kV. The electron micrographs presented were of stained sections.
voltage of 50 kv. Photomicrographs were taken on Kodak Contrast Process Ortho film.

RESULTS

Daily plate counts on replications of the membranes, impinged with young Azotobacter cells and incubated with butanol broth, show that these cells divided about six times during the growth period, whereas the count of the endogenous cells remained constant (Fig. 1). After transfer to the desiccation environment, 8% of the endogenous culture and 34% of the butanol cells survived. Under similar conditions, nonencysting cultures consistently show less than 1% survival (9). This evidence of encystment is confirmed by the data in Fig. 2. The endogenous organisms have progressed toward encystment so that they are susceptible to lysis by EDTA alone. This pattern of lysis does not occur with vegetative cells or with immature cysts, but it corresponds to that described for an encysting culture by Socolofsky and Wyss (8).

Electron micrographs confirmed the occurrence of encystment in endogenous cultures but showed the process to proceed more slowly, since the 4-day cultures exhibited various intermediate stages in the encystment process. Some of the endogenous cells were rounded-up and surrounded by a large fibrous capsule (Fig. 3) that showed a concentration of the fibrous material in the capsule in that region where the exine will be formed. More common were the forms shown in Fig. 4 in which fragments of exine were deposited within the more fibrous area of the capsule. There were also many cysts in which encystment was almost complete in that a thick exine had been deposited completely around the cells (Fig. 5). In the final stage the central body is compacted still further, and the intine appears to widen to fill the space between the central body and the exine. The exine is not formed on the outer edge of the capsule since material external to the exine is quite evident in these pictures. In these endogenously-produced cysts polyβ-hydroxybutyrate (PHB) globules are almost nonexistent. Cells grown for the same length of time on membranes placed on Burk's salts medium enriched with 0.02% n-butanol yielded cysts (Fig. 6) with a completed exine, wider intine, and extensive deposits of PHB within the central body.

DISCUSSION

Winogradsky (13) reported that short chain alcohols enhanced encystment in Azotobacter and published photographs showing cultures where cysts accounted for 5 to 10% of the cell population. In this study we have used the strain selected by Socolofsky and Wyss (8) which yields nearly 100% encystment with the use of n-butanol in Burk's agar medium. Stevenson and Socolofsky (10) reported cyst formation on substrates other than n-butanol and Lin and Sadoff (2) have also shown encystment on intermediate metabolic products of n-butanol most of which are metabolized as slowly as butanol itself. The dissolution of cysts by chelating agents prescribes their accumulation in the presence of a substrate which is rapidly metabolized to yield extracellular products with chelation properties, or when employing strains or conditions of culture (e.g. low aeration on media enriched in organic matter) which result in such extracellular substances. Endogenous encystment of washed young cells of A. vinelandii strain 12837 is not unexpected even though Sobek et al. (7) found that no cysts were formed endogenously with A. vinelandii strain O.

Fig. 1. Plate counts of A. vinelandii ATCC 12837 impinged on membranes and grown 4 days with and without a carbon source. At 4 days they were transferred to a desiccation environment and incubated 4 more days wherein cysts survive and vegetative cells die.

Fig. 2. Decrease in optical density of 4-day-old cultures grown on membranes with and without a carbon source upon exposure for the indicated times to 0.25 mM EDTA.
**FIG. 3.** Thin section of endogenously cultured *Azotobacter* showing a rounded-up cell surrounded by a fibrous capsule. Bar = 0.5 μm.

**FIG. 4.** Thin section of endogenously encysting *Azotobacter* showing a rounded-up cell surrounded by a fibrous capsule in which fragments of exine are deposited. Bar = 0.5 μm.
Fig. 5. Thin section of endogenously encysting Azotobacter showing a layer of fibrous material extending beyond the exine. Bar = 0.5 μm.

Fig. 6. Thin section of an Azotobacter cyst grown on 0.02% n-butanol. A central body containing several PHB deposits is surrounded by a typical exine and intine. Bar = 0.5 μm.
The lower resistance to desiccation of an endogenous cyst culture can be explained by the presence of large numbers of cysts in which the exine is incomplete and possibly by the absence of globules of PHB. Parker and Socolofsky (5) found the cyst coat to be primarily responsible for the resistance of encysted cells to deleterious agents and uncovered a correlation between encystment and PHB reserves. Furthermore, Sobek et al. (7) have shown that vegetative cells with low amounts of PHB reserves rapidly lose viability upon starvation in an aerated buffered suspension. Although Stevenson and Socolofsky (10) have reported that the accumulation of PHB is essential to the encystment process, our electron micrographs (Fig. 3–5) of endogenous cysts reveal no observable inclusions of stored PHB material as are typical of n-butanol-grown cysts (Fig. 6).

The endogenous encystment on membrane filters expands the time scale of the cyst-forming process and reveals several stages not emphasized heretofore. The accumulation of material at a definite distance between the outer wall of the central body and the outer edge of the capsule (Fig. 3) appears significant. It is in this region that the cut edges of well-defined segments of exine first become visible as in the electron photomicrographs of the thin section shown in Fig. 4. Such a result might be expected if a product diffusing into the capsule from the medium meets and reacts with a material diffusing out from the central body. Our original suggestion was that the exine might be a complex of capsular material reacting with the divergent cations from the Burk's salts in the medium. However, the recent report by Lin and Sadoff (Bacteriol. Proc., p. 42, 1969) indicates that although the Azotobacter capsule is primarily an acid polysaccharide, the exine and intine of the cyst contain large portions of lipid and protein. Since this species is essentially devoid of proteolytic and lipolytic activity (although rich in enzymes that act on the \( \beta \)-hydroxybutyrate polymer) the central body, during its compaction and membrane-shortening stage, extrudes protein and lipid into the capsular material to make up the intine, and possibly, upon reaction with cations from the medium, the exine is precipitated. The present paper supports the view that such a reaction can begin with young cells and continue to completion, even when the encysting cells are deprived of an exogenous carbon source.

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LITERATURE CITED