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Giant Cysts and Cysts with Multiple Central Bodies in *Azotobacter vinelandii*

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Cyst germination in *Azotobacter vinelandii* ATCC 12837 was studied by using phase contrast and electron microscopy. Germination in this organism was accompanied by the formation of large cyst forms of two different types: giant cysts and cysts containing multiple central bodies. Previously, these two types have been reported only when yeast extract was added to the encystment medium. In this study, we observed giant cysts and cysts with multiple central bodies in nitrogen-free liquid medium. The germination of "normal" cysts is often preceded by enlargement to the giant form and division of the central body to produce cysts with multiple central bodies. Structures similar in appearance to ribosomal aggregates were observed only in cysts undergoing pregermination transformations.

Encystment in *Azotobacter* may be induced by placing vegetative cells on medium containing certain short-chain organic compounds, such as *n*-butanol (12). During encystment, the vegetative cell loses its motility, becomes rounded, decreases in cell volume, and acquires the intine-exine complex which characterizes the cyst (8, 10, 13). During germination in medium containing glucose, the cyst increases in volume, disrupts the intine-exine complex, and initiates vegetative outgrowth (8, 13). Two previous papers describe the formation, under certain culture conditions, of giant cysts and cysts containing multiple central bodies (1, 6). Beaman et al. (1) presented electron micrographs of giant cysts of *A. vinelandii* grown in salts medium supplemented with 0.1 to 1% yeast extract and 0.3% *n*-butanol. When cysts produced in this manner were transferred to medium containing glucose, 65% of the encysted giant organisms divided while within the cyst exine, giving rise to cysts with multiple central bodies. These workers stated that "normal" cysts never undergo central body division.

In this study, we report the formation of giant cysts and cysts containing multiple central bodies in germinating populations of normal cysts of *A. vinelandii* ATCC 12837 grown in nitrogen-free medium.

MATERIALS AND METHODS

Cultures. Vegetative cells of *A. vinelandii* ATCC 12837 were grown for 18 to 24 hr at 30 C on Burk's medium (11) supplemented with 1% glucose and solidified with 2% agar. Encystment was induced by transferring vegetative cells to the same basal agar containing 0.3% *n*-butanol instead of glucose as the carbon source. Plates were incubated for a total period of 1 week at 30 C. Encystment was studied by microscopic examination at intervals of 24 hr. Samples of the cultures were removed from the butanol medium and fixed for electron microscopy.

At the end of 7 days of incubation, when encystment was complete, composite samples from several plates were harvested, washed, and transferred from the encystment agar to Burk's nitrogen-free liquid medium containing 1% glucose. These liquid cultures were placed on a rotary shaker at 30 C, and the cells were allowed to germinate. At 4-hr intervals, samples from each germinating culture were removed, centrifuged, washed, and fixed for electron microscopy.

Electron microscopy. All cells were fixed by suspending them in a solution of 3% glutaraldehyde and 0.15% (w/v) reuthenium red (A. D. Mackay, New York) buffered with 0.1 M sodium cacodylate (4, 7) for a period of 1 hr. Fixation was continued in a solution of 1% OsO_4 and 0.15% reuthenium red in 0.1 M cacodylate buffer for another hour. The cells were washed in buffer, dehydrated by passage through a series of graded alcohol solutions, and embedded in Epon 812 (3) by using propylene oxide as the solvent. Polymerization was accomplished in 24 to 48 hr at 60 C. Ultrathin sections were obtained on a Porter-Blum MT-2 ultramicrotome fitted with diamond knives. To improve contrast, sections were poststained with lead tartrate for 45 min and uranyl acetate for 30 min. All specimens were examined and photographic negatives were obtained with an RCA-EMU-3G electron microscope at initial magnifications of 11,500 to 16,000.

Identification of giant cysts. The mean diameter of cysts cultured on butanol medium for a period of 7 days was determined by using the electron microscope. Cysts at least twice as large as the average were considered to be of the giant variety. Cysts containing two or more central bodies were identified and counted by using the electron microscope also.

RESULTS AND DISCUSSION

Encysting cells from each of the seven 24-hr samples obtained from plates of the butanol medium were examined to determine the development of giant or multiple central-bodied cysts during the encystment process. The data obtained indicate that giant cysts and cysts containing multiple central bodies were not formed when vegetative cells were allowed to encyst on

the butanol medium. All samples examined showed only vegetative cells in the process of encystment, precystic forms, and normal mature cysts approximately 2 μm in diameter. Figure 1 shows a typical vegetative cell undergoing cell division prior to encystment on butanol medium. The OsO_4-reuthenium red stain shows the nature and structural distribution of capsular material and the relation of extracellular spherical vesicles (9) to the cell capsule. The juxtaposition of these elements strongly supports the mechanism of cyst coat synthesis reported previously by Hitchins and Sadoff (2). Figure 2, precystic form, shows the beginning of exine formation and the remnants of the capsule, whereas Fig. 3 depicts the completed cyst. The cysts in Fig. 3 are shown for the purpose of comparison; these are two separate mature cysts which are attached to each other, possibly by adhering capsular material, and are not to be confused with cysts containing multiple central bodies (Fig. 5). Cultures maintained on encystment (butanol) medium for periods of 1 to 4 weeks show only the morphological forms illustrated in Fig. 1 to 3. These develop in a chronological succession so that by the end of 7 days of incubation, approximately 94% of the cells appear as normal mature cysts. The remaining 6% appear as vegetative cells or as precystic cells. From the observation of several thousand cells and cysts by electron microscopy and many more by phase contrast microscopy, we conclude that giant cysts and consequently cysts with multiple central bodies are not formed during encystment on Burk's medium with butanol as sole carbon source.

Normal mature cysts transferred to glucose medium and allowed to germinate were examined at 4-hr intervals. At the end of 4 hr of incubation at 30 C in this medium, the great majority of cysts showed an increase in central body volume, but there were no giant cysts or cysts with multiple central bodies. These cultures consisted entirely of cysts of normal size (2 to 4 μm), but with central bodies which appeared to fill most of the space previously occupied by the intine matter. They were not unlike those shown in Fig. 2.

The next significant change in the ultrastructure of germinating cysts appeared 8 hr after

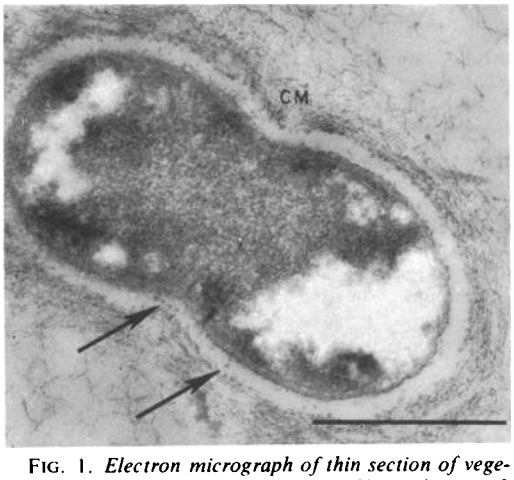

FIG. 1. *Electron micrograph of thin section of vegetative cell from 3-day culture on Burk's medium with 0.3% n-butanol. Polymerization of capsular material (CM) and distribution of extracellular vesicles indicated by arrows are plainly shown. Marker represents 1 μm.*

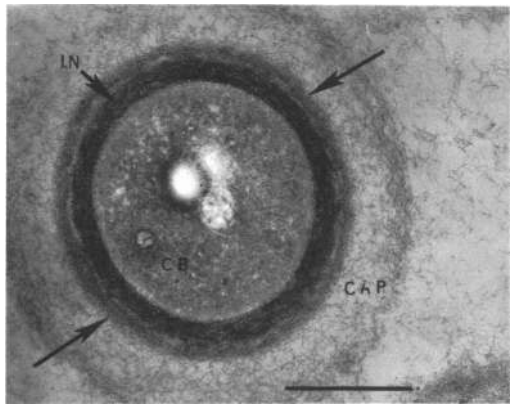

FIG. 2. *Precystic form from 5-day culture on butanol medium. Shown are the central body (CB), intine (IN), and the incomplete exine, indicated by arrows. The remnants of the capsule (CAP) are still evident. Marker represents 1 μm.*

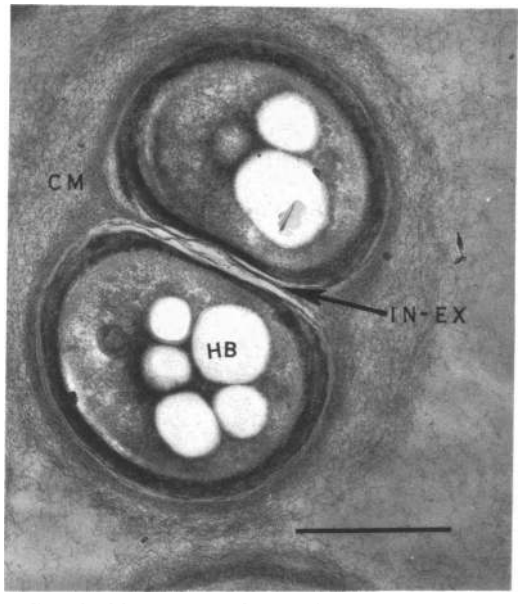

FIG. 3. *Mature cysts from 5-day culture on Burk's butanol medium. Division is complete as evidenced by intine (IN) and exine (EX) complex between the two cysts. Capsular material (CM) adhering to cysts may be reason for failure to separate. Also shown are sites of poly-β-hydroxybutyric acid deposits (HB). Marker represents 1 μm.*

transfer of the cysts into the germination medium. Approximately 69% of the total population remained in cyst form, and these showed evidence of change in that they had enlarged central bodies. Giant cysts (Fig. 4) and cysts with multiple central bodies (Fig. 5) were observed for the first time. Approximately 7% of the population was composed of giant cysts and cysts with multiple central bodies (Table 1).

Only 40% of the cells in the germination medium remained encysted after 12 hr of incubation. The number of giant cysts and multiple central-bodied cysts had increased only slightly (Table 1).

Cyst germination and the appearance of giant cysts and cysts with multiple central bodies increased as a function of time (Table 1). Also, as the frequency of giant cysts and cysts with multiple central bodies increased, the number of central bodies per cyst also increased. Figure 6 shows a cyst containing two distinct central bodies each apparently in the process of dividing, whereas Fig. 7 shows a cyst with six central bodies. The reproductive role of cysts with multiple central bodies has been suggested (1). The ordered structure in the area labeled PR in Fig. 6 is unidentified but is observed frequently in germinating cysts. To us, it is reminiscent of polyribosomal clusters (5). To our knowledge, this is the first time that this structure has been reported in cysts of *Azotobacter*.

The electron micrographs in Fig. 6, 7, and 8 confirm the report of Beaman et al. (1) that central bodies in a common exine are pleomorphic or ellipsoidal rather than spherical. On the other hand, the central body of normal cysts is spherical or nearly spherical (8, 9, 13). The fact that some cysts contain three central bodies (Fig. 8) and others six (Fig. 7) suggests independence of metabolic and, consequently, reproductive activity of the central bodies. The cell in Fig. 8 is probably the result of a cysts with a single central body which divided to give a cyst with two central bodies of which only one divided again. The result would be one large and two small central bodies as seen in Fig. 8.

At the end of the 24-hr sampling period, only 5% of the population remained in cyst form. One per cent of the population was made up of giant cysts and cysts with multiple central bodies.

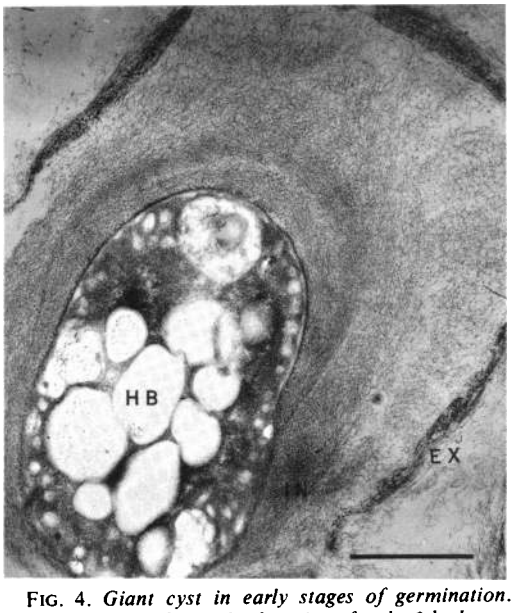

FIG. 4. *Giant cyst in early stages of germination. Central body with ample deposits of poly-β-hydroxybutyrate (HB) is seen emerging from the exine (EX) with large quantity of intine (IN) matter adhering to it. Marker represents 1 μm.*

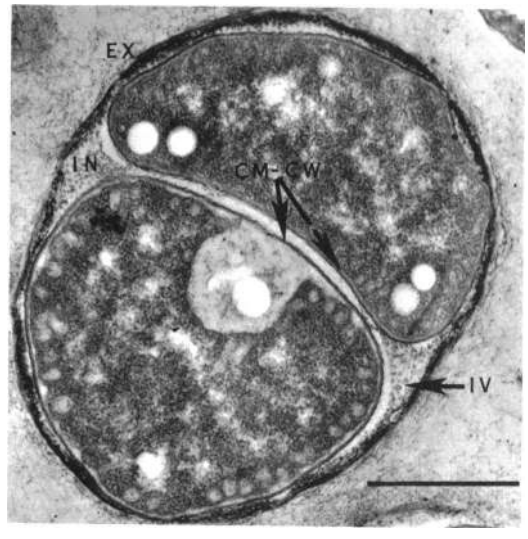

FIG. 5. *Thin section of cyst with two central bodies from 8-hr culture in germination medium. Exine (EX) and remaining intine (IN) with intine vesicles (IV) are shown. The cell membrane-cell wall complex (CM-CW) of each of the central bodies are also indicated. Marker represents 1 μm.*

TABLE 1. *Per cent of giant cysts and cysts containing multiple central bodies in a population of cysts germinating in a glucose medium at 30 C*[a]

Time (hr)	Per cent cysts	Per cent giant forms	Per cent multiple-central bodied cysts
0	94[b]	0	0
4	92	0	0
8	69	1.5	5.9
12	40	0.9	7.3
16	30	1.0	10.6
20	23	1.4	9.8
24	5	0.2	0.8

[a] Three hundred cells were counted at each time interval.

[b] Six per cent of the population was made up of vegetative and other nonencysted cells.

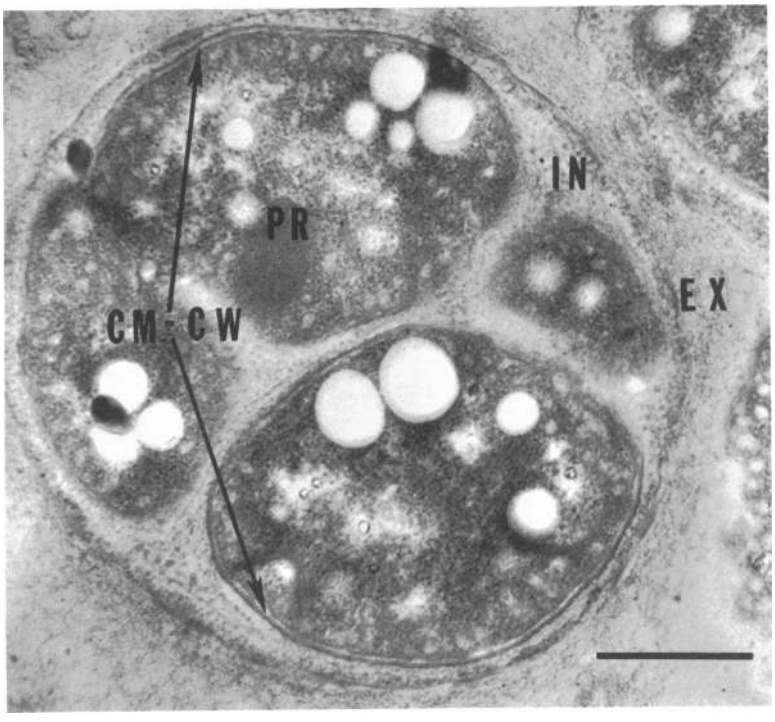

FIG. 6. *Thin section of Azotobacter cyst in the process of dividing. Cyst exine (EX) is intact, but intine (IN) has almost disappeared. The cell membrane-cell wall complex (CM-CW) of the individual central bodies is evident. The area labeled PR is probably a polyribosome cluster. Marker represents 1 μm.*

Repetition of these observations yielded some quantitative variation, but, in essence, the morphological sequence was identical in all tests.

The results of this study indicate that giant cysts are not normally produced during the encystment process of *A. vinelandii* cultured on Burk's medium with 0.3% butanol as carbon source. On the other hand, it is evident that giant cysts and cysts with multiple central bodies occur commonly during the germination process in Burk's medium with 1% glucose as carbon source. The nature of these giant cysts is of interest in this connection. Beaman et al. (1) claim that *Azotobacter* grown on medium containing butanol and yeast extract produce giant cysts and that it is only these giant forms which undergo central body division to produce multiple central-bodied cysts. We present data which clearly show that normal cysts grown on medium with butanol have the ability to form giant cysts (Fig. 4) during germination in glucose medium. These giant cysts apparently undergo central body division to produce multiple central body forms (Fig. 5-8) undoubtedly indistinguishable from those reported by Beaman et al. (1).

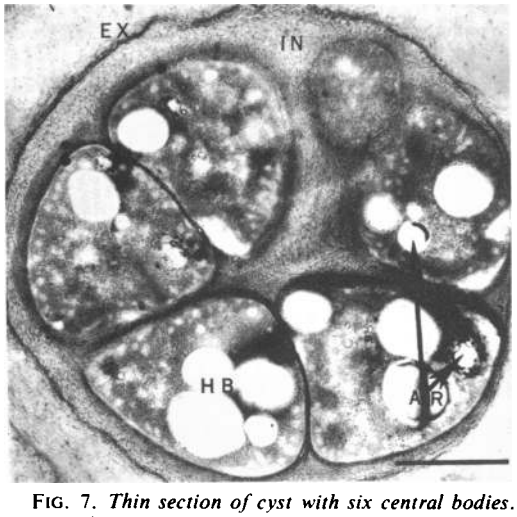

FIG. 7. *Thin section of cyst with six central bodies. Discernable structures are the cyst exine (EX), intine (IN), and sites of poly-β-hydroxybutyrate (HB) deposits.* Several artefacts are labeled AR. Marker represents 1 *μm.*

On the basis of this, we propose that yeast extract is not essential for central body division and that giant cysts represent a morphological step in the pregermination processes of some of the cysts in a germinating population.

The possibility exists that certain vegetative cells in an encysting population may be predisposed to form giant cysts and cysts with multiple central bodies. This hypothesis may be refuted by observing that only 6% of the cells fail to form normal cysts on butanol but nearly 12% giant forms and cysts with multiple central

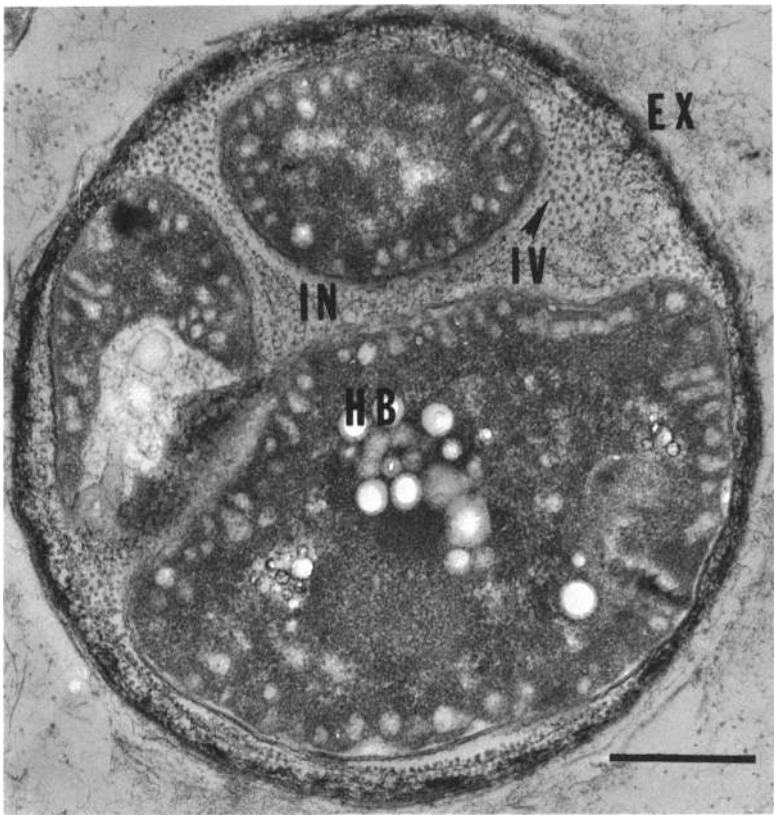

FIG. 8. *Cyst with three elliposidal central bodies showing intact exine (EX), intine (IN), intine vesicles (IV), and dispersed poly-β-hydroxybutyrate (HB). Marker represents 1 μm.*

bodies are found after 16 hr in the glucose medium. This indicates strongly that giant cysts and cysts with multiple central bodies develop not from nonencysted forms in a predominately cyst population but rather from normal cysts (Table 1).

We also feel justified in stating that the *Azotobacter* cyst possesses the capacity for growth without germination. This is tenable because normal cysts produced on butanol medium (2 to 4 μm) and then transferred to glucose medium will become giant cysts (4 to 6 μm) during germination. While the central body enlarges and divides, the exine also enlarges to assume the giant form and, probably, the intine is consumed in the process.

ACKNOWLEDGMENTS

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