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Structure of *Methylosinus trichosporium* Exospores

WILLIAM M. REED, JEFFREY A. TITUS, PATRICK R. DUGAN,* AND ROBERT M. PFISTER
Department of Microbiology, The Ohio State University, Columbus, Ohio 43210

Methylosinus trichosporium exospores did not display a well-defined cortex or an exosporium. A thick, electron-dense exospore wall was characteristic of the exospores. Located on the exterior of the exospore wall was a cell wall to which a well-defined capsule was attached. An extensive lamellar intracytoplasmic membrane system characteristic of the kind in vegetative cells of this bacterium was present along the interior periphery of the exospore wall. Upon germination of *M. trichosporium* exospores, the thick exospore wall gradually disappeared and a germ tube formed. The intracytoplasmic membranes of the exospores extended into the germ tube which did not possess the extensive fibrillar capsule observed on the dormant exospore. Cup-shaped exospores which have an ultrastructure similar to that of mature exospores except that they are invaginated also germinated upon exposure to methane.

Methylosinus trichosporium sporulates by budding off an exospore from one end of the vegetative cell. These exospores are similar to bacterial endospores in that they demonstrate heat and desiccation resistance (9). It has also been shown that the external surface of the exospores is antigenically different from the external surface of the vegetative cells (4). The objective of this study was to examine the structure of *M. trichosporium* exospores in comparison to the structure of vegetative cells and to bacterial endospores.

MATERIALS AND METHODS

Culture methods. Liquid cultures of *M. trichosporium* OB3b originally obtained from R. Whittenbury were maintained in rubber-stoppered 250-ml flasks containing 50 ml of CM medium (8) and an atmosphere of air-methane (1:1). The cultures were incubated at 22 ± 2°C.

Exospore production. Aseptic technique was used throughout the following procedure. Fifty milliliters of a 3-day culture of *M. trichosporium* was inoculated into a rubber-stoppered 3-liter flask containing 750 ml of CM medium in an atmosphere of methane-air (1:1) and incubated on a rotary shaker at 150 rpm at 30°C for 48 h. This flask was used to inoculate a 14-liter fermentor (Model MF-107, New Brunswick Scientific Co., New Brunswick, N.J.) containing 7 liters of CM medium. The fermentor was maintained at 22 ± 2°C and aerated with a glass wool-filtered methane-air (2:1) gas mixture at a flow rate of 20 ml/min per liter and mixed at 150 rpm. After 72 h, the ratio of methane-air was changed to 1:2 and maintained for 12 h to enhance spore formation. Finally, the culture was aerated with air and no methane for 12 h. The broth from the fermentor was centrifuged at 7,000 × *g* for 15 min in a Sorvall RC-2B centrifuge. The cells were suspended in 40 ml of CM medium and passed through a French pressure cell at 21,000 lb/in^2 three times. The suspension was centrifuged at 7,000 × *g* for 30 min and washed once with 40 ml of CM medium. A portion of the virtually vegetative cell-free pellet was saved for electron microscopy, and the remainder was used to obtain germinating exospores by suspension in 10 ml of CM medium and inoculation into a rubber-stoppered 3-liter flask containing 100 ml of CM medium under methane-air (1:1). The flask was incubated on a rotary shaker at 150 rpm at 30°C for 18 to 24 h. The germinated exospores were harvested by centrifugation at 7,000 × *g* for 15 min and washed twice with distilled water.

Fluorescent-antibody staining procedure. The fluorescent-antibody staining procedure was the same as that used in a previous study (4).

Electron microscopy techniques. Dormant and germinated exospores were fixed using Luft ruthenium red procedure (3) and were embedded in Spurr (6) low-viscosity embedding medium. Thin sections were stained with lead citrate (5) and uranyl acetate (7) and were viewed with a Philips EM-300 electron microscope.

RESULTS

Exospore preparations were obtained which had less than 1% vegetative cells, as determined by microscopic examination. Upon exposure to methane-air the first visible signs of outgrowth appeared after approximately 12 h with complete outgrowth of a germ tube in 18 to 24 h. Figure 1A shows a stationary culture of *M. trichosporium* with a mixture of spores and vegetative cells. Figure 1B shows the preparation of exospores obtained after disruption of vegetative cells followed by centrifugation of the remaining spores. Figure 1C shows the germinating exospores after 18 h of exposure to methane. The germination of the exospores was synchronous, with greater than 99% of the spores germinating

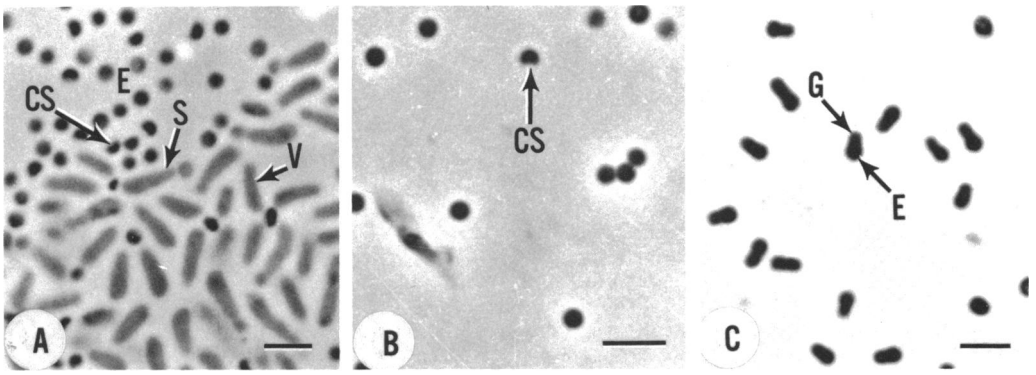


FIG. 1. *Phase-contrast photomicrographs of various developmental stages of M. trichosporium. The bars indicate 3 μm. (A) M. trichosporium stationary culture. CS, Cup-shaped exospores; E, exospores; S, sporulating cell; V, vegetative cell. (B) Isolated exospores. (C) Germinating exospores. G, Germ tube.*

within 12 h. This was not the case if spore production took place over a protracted period of time. For example, if the inoculum for the fermentor was significantly less than 750 ml, the incubation period had to be extended from 96 h to between 5 and 9 days to obtain 50% spore production. Exospores produced under such conditions demonstrated asynchronous germination with less than 10% of the spores germinating in a period of 1 to 4 weeks.

Embedding exospores in Epon 812 proved inadequate, since proper infiltration was not obtained. Spurr embedding medium (6) with the fixation of Kellenberger et al. (2) was used, and good penetration was obtained. However, by using this technique the capsule was not visible. Therefore, Spurr embedding medium was used with the ruthenium red procedure (3) which rendered the exospore capsule clearly visible.

Bacillus endospores were used as a standard for comparison with the *M. trichosporium* exospores. Ultrastructural characteristics of *Bacillus* endospores include a plasma membrane, a cortex, a spore coat, and sometimes an exosporium (1).

The ultrastructure of an *M. trichosporium* exospore is shown in Fig. 2. A well-defined cortex is absent, and an exosporium is not evident. A thick, electron-dense layer internal to a distinct outer cell wall is defined here as the exospore wall and is characteristic of the exospores. On the exterior of the exospore wall is a cell wall to which a well-defined fibrous capsule is attached. Intracytoplasmic membranes are present.

Upon exposure to methane the exospores germinated. Figure 3 shows a germinating *M. trichosporium* exospore. The exospore wall is still present in the nonvegetative part but greatly diminished as compared to the exospore wall of the dormant exospore. The exospore capsule is present and extends to the origin of the germ tube. The germ tube is covered by a compact, dense capsule. Figure 4 shows a germinating exospore at a further stage of development than the exospore in Fig. 3. The same ultrastructure is evident with the exception that the exospore wall has been further diminished. It can also be seen that the intracytoplasmic membranes extend out from the exospore into the germ tube in a parallel array.

Crescent-shaped bodies which demonstrated an ultrastructure similar to that of the exospores, except for the crescent shape, were observed in many of the thin sections (Fig. 5). Upon closer examination of some late stationary cultures, cup-shaped spores were observed (Fig. 1A). These spores were also observed in some of the exospore preparations and in many cases at a higher ratio to spherical spores than that observed in the stationary cultures. When stationary cultures of *M. trichosporium* were harvested and subjected to the exospore isolation procedure immediately after incubation for 72 h under an atmosphere of methane-air (2:1) followed by 12 h under an atmosphere of methane-air (1:2), the resulting exospore preparation contained a majority of cup-shaped exospores as compared to spherical exospores. When the incubation period was extended to include an additional 12 h under an atmosphere of air and no methane, the exospore preparation contained a majority of spherical exospores as compared to cup-shaped exospores. The cup-shaped spores demonstrated a positive reaction to anti-*M. trichosporium* exospore fluorescent-antibody stain. Figures 5 and 6 represent cup-shaped exospores which were thin sectioned in different planes relative to the area of invagination. The cup-shaped exospores were viable and germinated upon exposure to methane (Fig. 7).

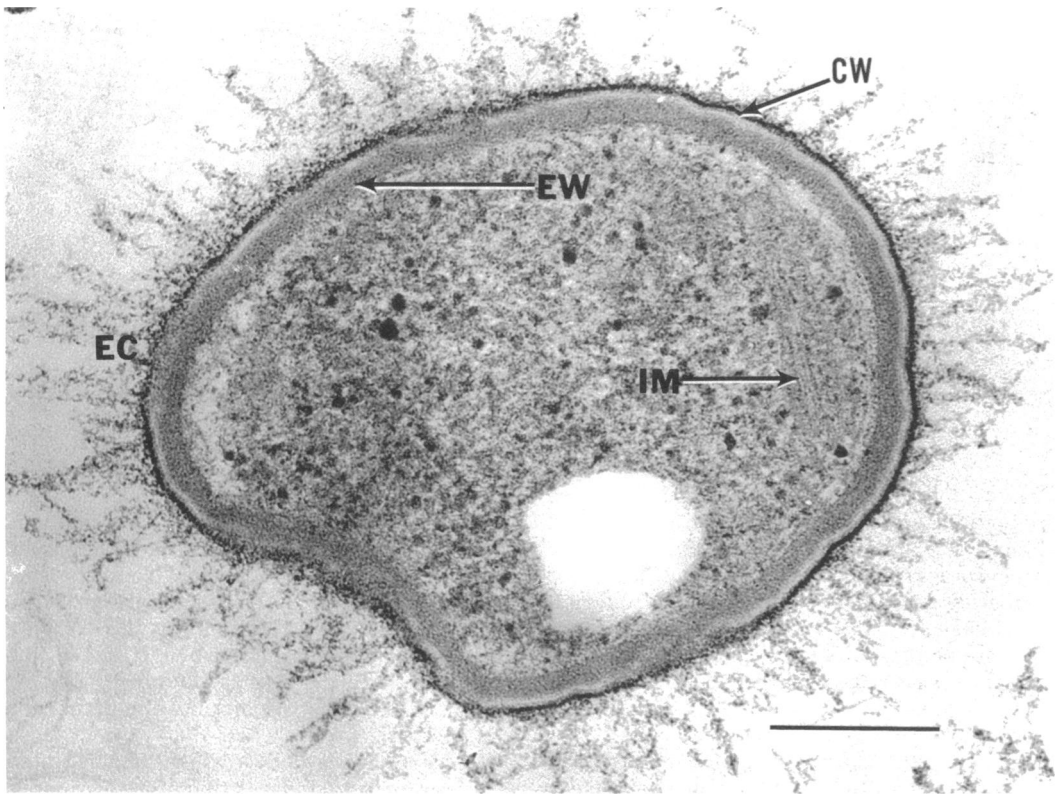


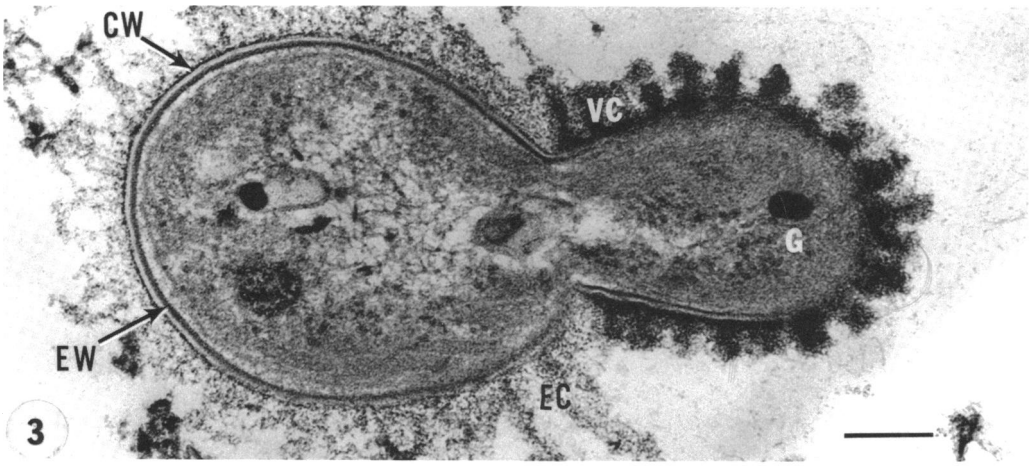
FIG. 2. *Spherical M. trichosporium exospore. Bar indicates 0.2 μm. CW, Cell wall; EC, exospore capsule; EW, exospore wall; IM, intracytoplasmic membranes.*

DISCUSSION

The ultrastructure of *M. trichosporium* exospores and *Bacillus* endospores is quite dissimilar. However, there is a distinct resemblance between the ultrastructures of *M. trichosporium* exospores and *Rhodomicrobium vannielii* exospores (10). Whittenbury and Dow (10) showed that the exospores of *R. vannielii*, a purple nonsulfur photoheterotroph, display a poorly defined cytoplasmic region, a cell wall, an exospore wall, a lamellar arrangement of membranes, and often a fibrous capsule, all of which are evident in *M. trichosporium* exospores. Upon germination of *R. vannielii* exospores, the thick exospore wall disappears. This phenomenon is also characteristic of germinating *M. trichosporium* exospores.

Observing the cup-shaped exospores indicates that during sporulation an invaginated sphere is formed at one end of the vegetative mother cell. A composite drawing of an *M. trichosporium* cup-shaped exospore is shown (Fig. 8) and illustrates that sectioning the cup-shaped exospores through a plane parallel to the direction of the invagination (Fig. 8A) results in the production of crescent-shaped bodies (Fig. 5). Figure 8B shows that thin sectioning the cup-shaped exospores through a plane perpendicular to the direction of the invagination and through the invagination results in the production of doughnut-shaped bodies as shown in Fig. 6.

Once detached from the vegetative mother cell, the cup-shaped exospore is a separate, viable organism as evidenced by its ability to germinate upon exposure to methane. The composite drawing of a germinating *M. trichosporium* cup-shaped exospore (Fig. 9) illustrates that sectioning through a plane parallel to the direction of invagination results in the production of a section such as that shown in Fig. 7. The cup-shaped exospore appears to represent an earlier stage of spore development than the spherical exospores. This is supported by the fact that extending the incubation period of the stationary-phase cultures from which exospore preparations were made resulted in a change from predominantly cup-shaped exospores to pre-



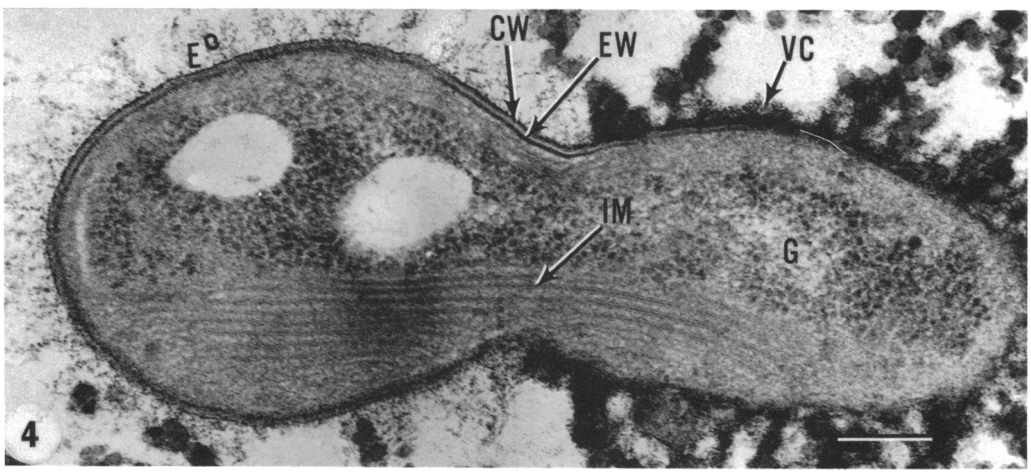


FIG. 3 and 4. *Germinating M. trichosporium exospores at different stages of development. CW, Cell wall; EC, exospore capsule; EW, exospore wall; G, germ tube; IM, intracytoplasmic membranes; VC, vegetative capsule. Bar indicates 0.2 μm.*

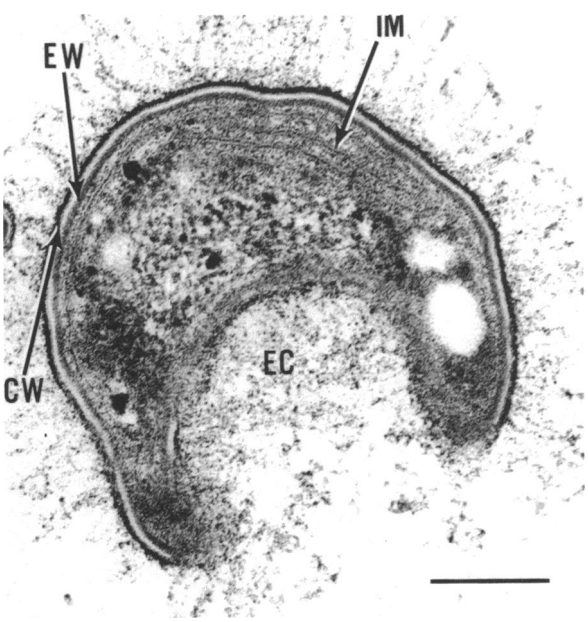


FIG. 5. *Crescent-shaped thin section of a cup-shaped M. trichosporium exospore. CW, Cell wall; EC, exospore capsule; EW, exospore wall; IM, intracytoplasmic membranes. Bar indicates 0.2 μm.*

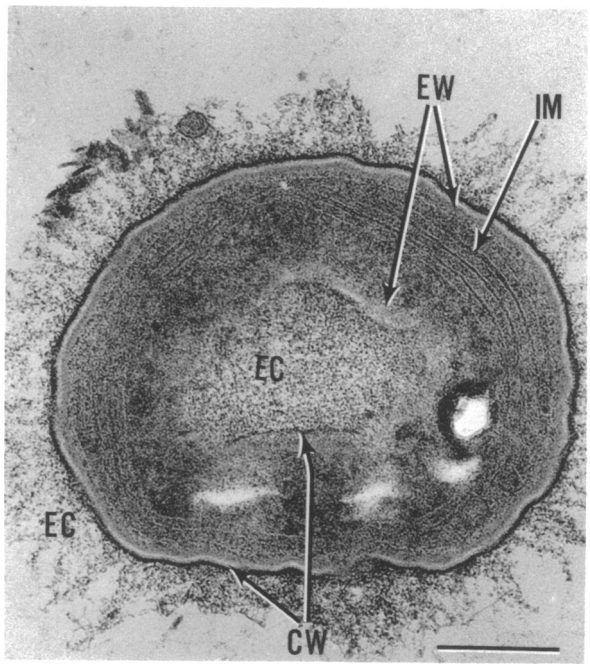


FIG. 6. *Thin section of a cup-shaped exospore cut perpendicular to the direction of invagination. CW, Cell wall; EC, exospore capsule; EW, exospore wall; IM, intracytoplasmic membranes. Bar indicates 0.2 μm.*

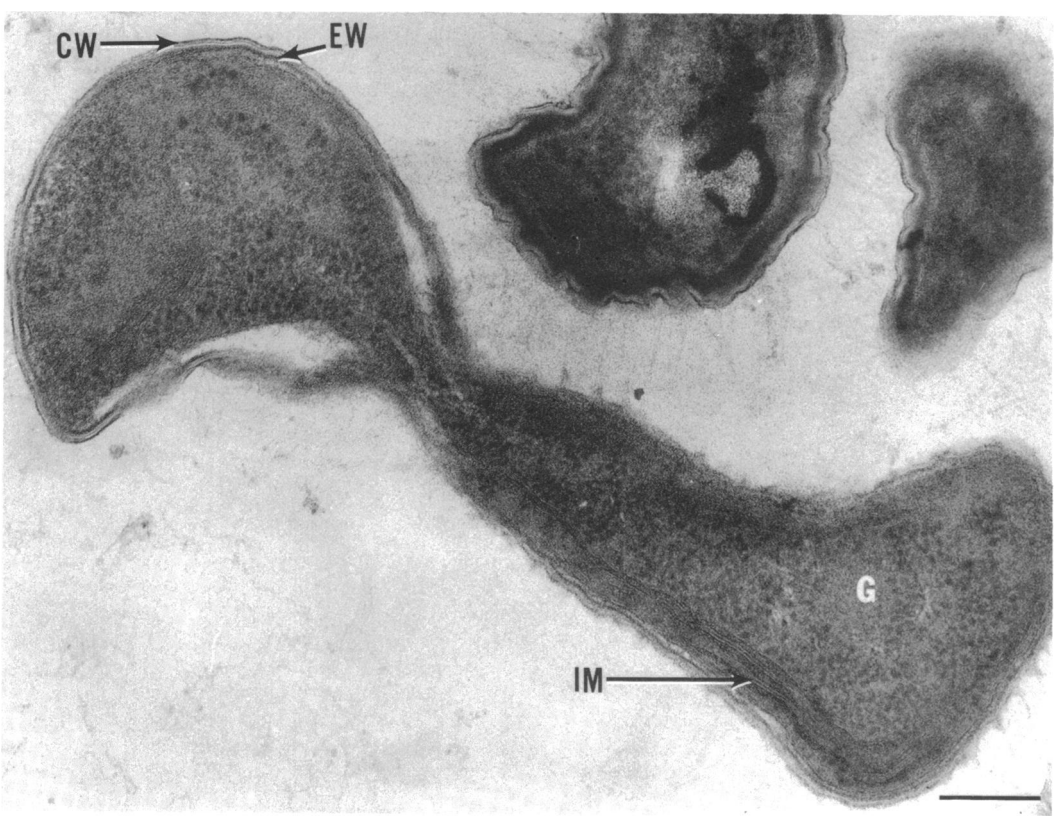


FIG. 7. *Germinating cup-shaped exospore. Kellenberger fixation and Spurr embedding resin. CW, Cell wall; EW, exospore wall; IM, intracytoplasmic membranes; G, germ tube. Bar indicates 0.2 μm.*

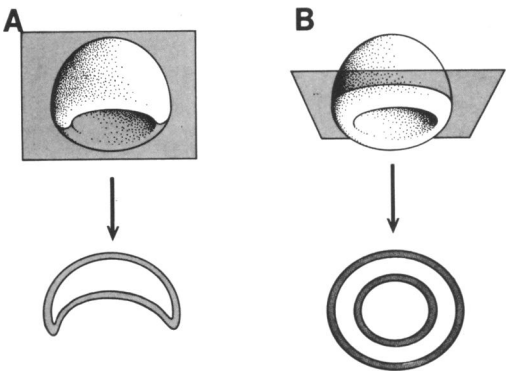


FIG. 8. *Composite drawing of an M. trichosporium cup-shaped exospore and thin sections which can be obtained when the exospore is sectioned through different planes relative to the invagination. (A) Sectioning through a plane parallel to the direction of the invagination results in crescent-shaped section. (B) Sectioning through a plane perpendicular to the direction of the invagination results in doughnut-shaped bodies.*

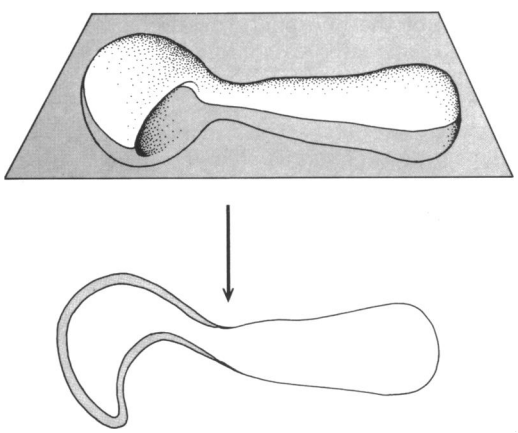


FIG. 9. *Composite drawing of a germinating M. trichosporium cup-shaped exospore and the thin section which is obtained when the exospore is sectioned through a plane parallel to the direction of the invagination and through the germ tube.*

dominantly spherical exospores in the exospore preparation. This indicates that, if maintained under a methane-deficient environment, the cup-shaped exospore develops into a spherical exospore. In addition, the mature spherical exospore displays a much thicker exospore wall than the cup-shaped exospore. Therefore, the cup-shaped exospores are different from the spherical exospores not only in shape but also in internal development. As such, the cup-shaped exospore represents an earlier stage in spore maturation than the spherical exospore and is therefore defined as an immature exospore.

The uniqueness of the developmental stages of *M. trichosporium* and the successful synchronization of growth as achieved in this study by isolation of fresh exospores free of vegetative cells demonstrates that *M. trichosporium* can be considered a useful model for the study of morphogenesis and differentiation.

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