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Study of Developmental Stages of *Methylosinus trichosporium* with the Aid of Fluorescent-Antibody Staining Techniques

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When stained by using an indirect fluorescent-antibody technique, *Methylosinus trichosporium* displayed an uneven fluorescence. Exospores and the polar tips of some vegetative cells displayed a more intense fluorescence than the other cells. Cross-absorption of the specific anti-*M. trichosporium* immunoglobulin G with exospores resulted in no fluorescence of exospores or exospore regions of sporulating vegetative cells. This demonstrated that antigens were present on exospores and exospore regions of vegetative cells that are different from vegetative cell antigens. Taking advantage of this phenomenon, three fluorescent-antibody staining techniques were developed which were used to study the life cycle of *M. trichosporium*.

Members of the genus *Methylosinus* are among the few genera of true bacteria besides *Clostridium* and *Bacillus* that produce heat- and dessication-resistant spores. *Methylosinus* spores are budded off at one end of the vegetative cell and are therefore referred to as exospores which are also distinguished from the endospores by the absence of dipicolinic acid (3).

The objective of this study was to determine (i) through the use of indirect fluorescent-antibody (FA) staining techniques, if vegetative cell surface antigens differ from the surface antigens on exospores and exospore-related areas of sporulating vegetative cells and (ii) whether indirect FA staining techniques would permit differentiation among spores, vegetative cells, regions on vegetative cells which contain spore-specific antigens, and regions containing nonspore antigens on the same cell.

MATERIALS AND METHODS

Culture methods. *M. trichosporium* OB3b was cultured as described by Weaver and Dugan (2).

FA staining procedure. The specific anti-*Methylosinus trichosporium* immunoglobulin G (IgG) was developed during a previous study (1). Stationary-phase *M. trichosporium* cells were used as inoculum for antibody production in rabbits. IgG specific for vegetative cell antigens was produced by cross-absorption of anti-*M. trichosporium* IgG with washed *M. trichosporium* exospores (approximately 10^6 spores per milliliter of concentrated IgG) overnight at 4°C.

A smear of the sample under examination was heat fixed to a glass microscope slide. Bovine serum albumin solution (0.05 ml, 20 mg of 0.1 M phosphate buffer per ml) was layered over the smear which was held for 30 min at 37°C in a glass petri dish containing a piece of moist sponge. The slide was then washed for 5 min in a Coplin jar containing 0.1 M phosphate-buffered saline. This was repeated two times, and the slide was blotted dry. The smear was stained similarly with 0.05 ml of each of a series of reagents as shown in Table 1. Each of the reagents was titrated weekly with the titer representing the greatest dilution of the reagent which resulted in a +4 fluorescence (fluorescence intense enough to produce good photographs).

The FA-stained smears were examined microscopically and photographed as previously described (1).

Exospore isolation procedure. A dense suspension of stationary *M. trichosporium* cells was passed through a French pressure cell at 21,000 lb/in^2, three times. This resulted in the breakage of vegetative cells. The suspension was centrifuged at 7,000 × *g* for 15 min in a Sorvall RC-2B centrifuge. The pellet was resuspended in 3 ml of distilled water and placed in a centrifuge tube over 6 ml of a solution of 65% Renografin 76 (E. R. Squibb & Sons, Princeton, N.J.). The suspension was centrifuged in a swinging bucket centrifuge head on a Sorvall RC-2B centrifuge at 13,000 × *g* for 15 min. The pellet was discarded. The supernatant was combined with 4 ml of distilled water, mixed thoroughly, and centrifuged at 7,000 × *g* for 15 min. This procedure was repeated. Finally the centrifugation procedure was repeated with 38% Renografin-76. The supernatant was discarded. The pellet, which contained isolated exospores, was washed three times in distilled water.

RESULTS AND DISCUSSION

In a previous study we developed an indirect FA staining technique which was used to identify and quantitate *M. trichosporium* in environmental samples (1). When *M. trichosporium* was FA stained it was observed that some of the

vegetative cells stained unevenly (Fig. 1). The polar tips of some of the cells in the rosette fluoresce more intensely than the remainder of the cell. The isolated exospores also demonstrated this intense fluorescence which facilitated the observation of *M. trichosporium* exospores in various stages of development (Fig. 2). The intensely fluorescing exospores and sporulating vegetative cells often displayed a halo-like appearance (Fig. 1). The intensity and the extent to which the halo-like fluorescence encompassed the polar tip of sporulating vegetative cells correlated with the extent to which sporulation had progressed. Therefore, Fig. 1 shows sporulating cells ranging from those just beginning sporulation to those which have nearly completed sporulation. Ungerminated spores can also be observed separate from cells. The fibrillar nature of the halo-like fluorescence and its relationship to sporulation suggest that the FA may be binding to the exospore capsule. Whittenbury et al. (3) observed that *M. trichosporium*, which is encapsulated, forms a second capsule at the polar tip opposite the holdfast at the onset of sporulation, and this capsule increases as sporulation progresses. This could explain the increasing fluorescence intensity relative to the stage of the sporulation process at

TABLE 1. *Differential FA staining methods for M. trichosporium spores and vegetative cells*

FA method	First reagent	Second reagent	Third reagent	Fourth reagent	Fluorescence results	
					Spore	Vegetative cell
1	Reagent 1[a]	Reagent 2[b]	—[c]	—	—	+4 green
2	Reagent 1	Reagent 3[d]	Reagent 4[e]	Reagent 2	+4 green	+4 orange
3	Reagent 1	Reagent 5[f]	Reagent 4	Reagent 2	+4 green	—

[a] Exospore cross-absorbed anti-*M. trichosporium* IgG.
[b] Fluorescein isothiocyanate-conjugated goat anti-rabbit IgG.
[c] —, No reagent added; no reaction.
[d] Rhodamine isothiocyanate-conjugated goat anti-rabbit IgG.
[e] Unabsorbed anti-*M. trichosporium* IgG.
[f] Goat anti-rabbit IgG.

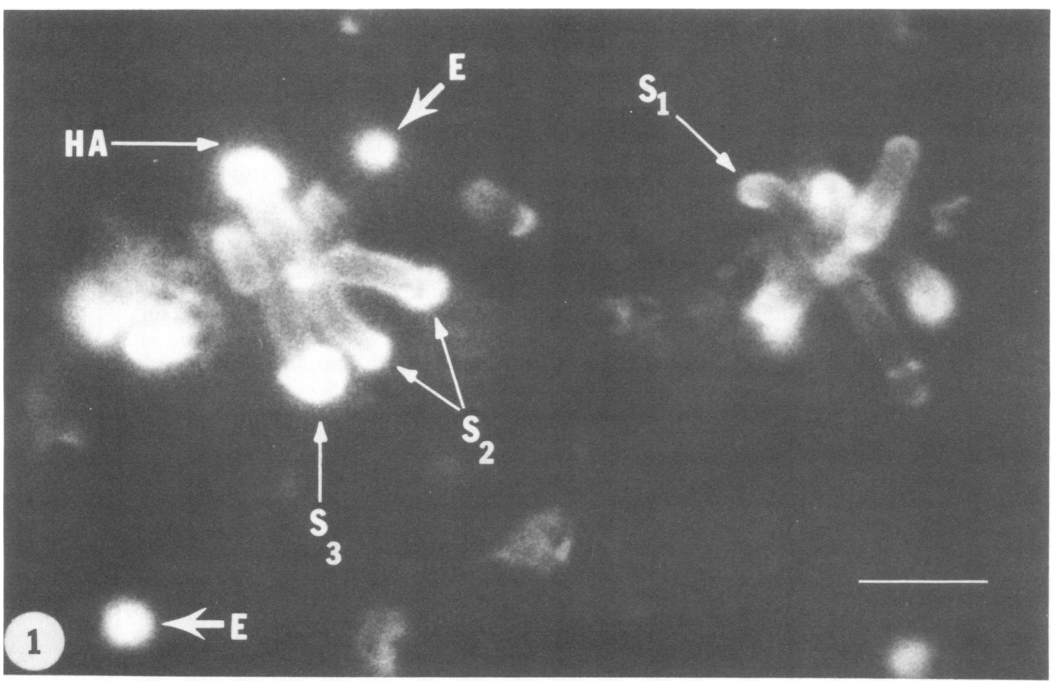

FIG. 1. *FA-stained M. trichosporium in various stages of sporulation. Bars in this and all subsequent figures indicate 3 μm. E, Exospore; HA, halo-like fluorescence; S_1, early stage of sporulation; S_2, intermediate stage of sporulation; S_3, late stage of sporulation.*

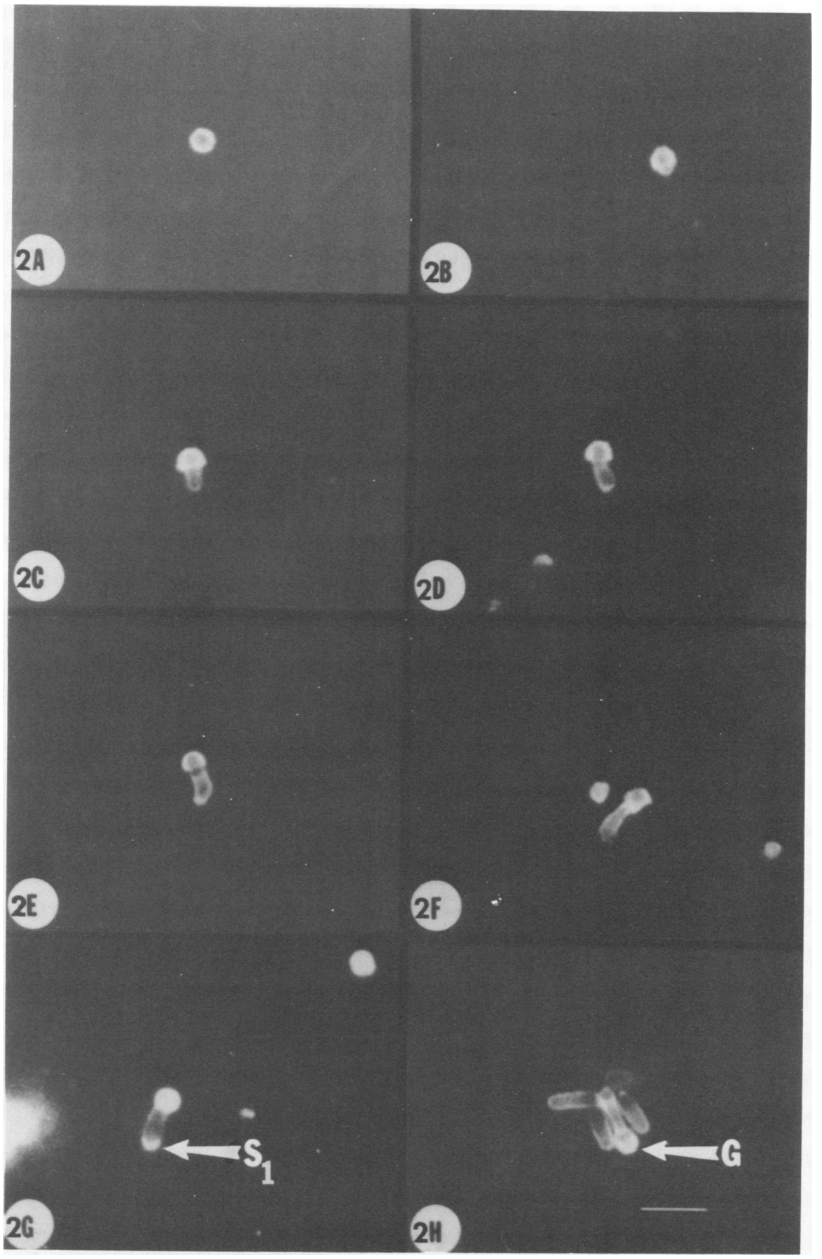

FIG. 2. *FA-stained germinating M. trichosporium exospores. A through F show exospores in progressing stages of germination. Figure 2G shows a germinating exospore which displays polar fluorescence, indicating that the newly formed germ tube is beginning the process of sporulation. Figure 2H shows a germinating exospore which is part of a rosette of M. trichosporium cells.* S_1, *Early stage of sporulation; G, germinating exospore.*

the polar tip of FA-stained sporulating cells. If exospore-specific antigens are present on the capsule, the differential FA staining of *M. trichosporium* would be due to the presence of antibody specific for the vegetative cell plus antibody specific for the exospores in the anti-*M. trichosporium* IgG.

To resolve the nature of the FA staining character of *M. trichosporium*, the anti-*M. trichosporium* IgG was cross-absorbed with *M. tri-*

chosporium exospores. The resulting solution was used to stain *M. trichosporium* (Table 1, method 1). Reagent 2 is fluorescein isothiocyanate-conjugated goat anti-rabbit IgG. The results of the staining procedure (Fig. 3A and B) indicate that all reactivity of the anti-*M. trichosporium* IgG for exospores was removed by cross-absorption with exospores, whereas reactivity for the vegetative cells was unaffected. Figure 3A is a phase-contrast photomicrograph of an *M. trichosporium* rosette and a free exospore which had been stained using method 1, Table 1. Figure 3B is the identical field photographed under ultraviolet illumination.

Lack of spore fluorescence could be a disadvantage in performing developmental studies. If the percentage of spores were to be determined in an early-stationary-phase culture, it would be necessary to search for and count round, nondescript, nonfluorescing spores in the midst of large, fluorescing vegetative cells which would far outnumber the spores. Therefore, a second staining technique was developed which would permit the spores to fluoresce, but with a different color.

Table 1 shows the results of a second FA staining method. Reagent 1 upon application bound only to vegetative cells. Reagent 3 (rhodamine isothiocyanate-conjugated goat anti-rabbit IgG) reacted with the already bound reagent 1 to render the vegetative cells orange. Reagent 4 reacted with exospores and the areas of vegetative cells which were not already blocked by reagents 1 and 2. Reagent 2 bound to the previously bound reagent 4, producing green fluorescing exospores (Fig. 4). Under ultraviolet illumination, the intense green fluorescence of the exospores could be easily distinguished from the light orange fluorescence of the vegetative cells. However, this technique did have one disadvantage. The working concentration of each of the two indicator FA solutions

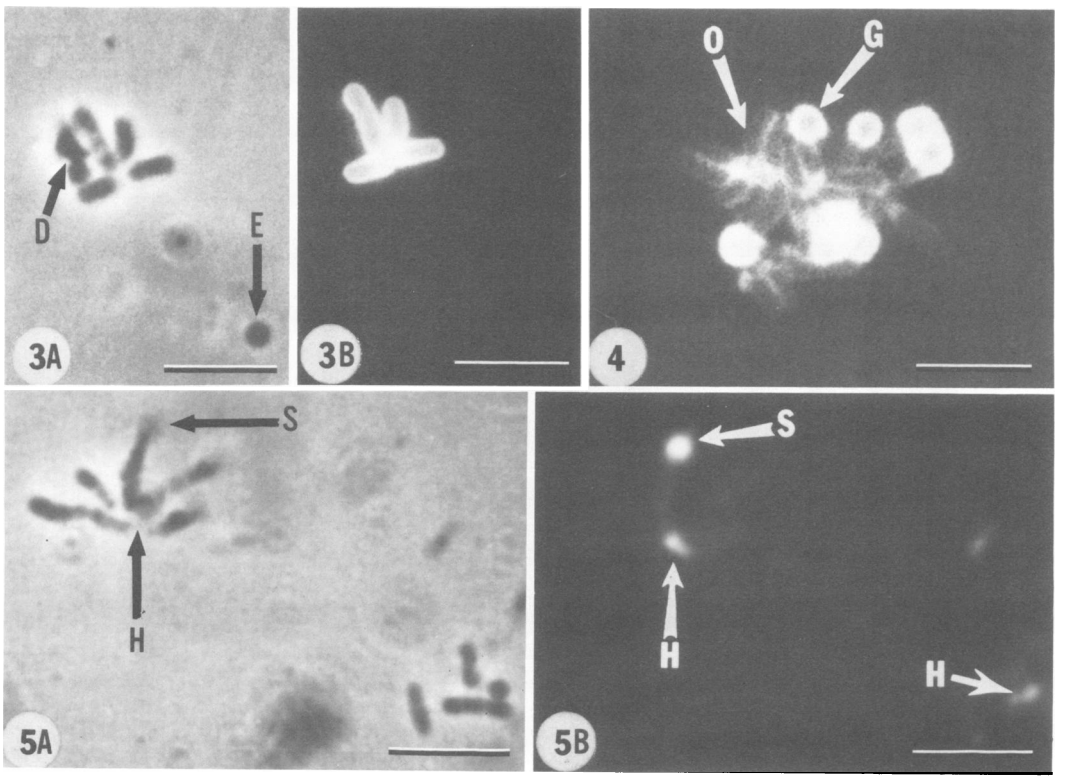

FIG. 3. *(A) Phase-contrast photomicrograph showing a rosette of M. trichosporium cells and an exospore stained by using FA method 1, Table 1. D, Debris; E, exospore. (B) Same field as 3A under ultraviolet illumination.*

FIG. 4. *M. trichosporium stained by using FA method 2, Table 1. G, Green fluorescing exospores; O, orange fluorescing vegetative cells.*

FIG. 5. *(A) Phase-contrast photomicrograph of M. trichosporium stained by using FA method 3, Table 1. H, Holdfast; S, sporulating cell. (B) Same field as Fig. 5A under ultraviolet illumination. H, Holdfast; S, sporulating cell.*

(fluorescein isothiocyanate-conjugated goat anti-rabbit IgG and rhodamine isothiocyanate-conjugated goat anti-rabbit IgG) was critical and had to be determined two or three times weekly. Both FA solutions contain anti-rabbit IgG and as such bind with the same antigens (the already bound reagents, 1 and 4, both of which are rabbit IgG). If the concentration of either FA solution was not correct, a poor color contrast resulted due to the competitive binding of the fluorescent IgG molecules.

A third FA staining technique was investigated as shown in Table 1. Reagent 1 is the same as that used in methods 1 and 2. Reagent 5, goat anti-rabbit IgG, was used to block the antivegetative cell IgG molecules so that they could not react with reagent 2 which is fluorescein isothiocyanate-conjugated goat anti-rabbit IgG. This procedure resulted in +4 fluorescence of exospores and no fluorescence of vegetative cells (Fig. 5). Figure 5A is a phase-contrast photomicrograph of an *M. trichosporium* rosette, and Fig. 5B is the identical field as 5A photographed under ultraviolet illumination. Although the vegetative cells were blanked by using this technique, the holdfast material of the cells still fluoresced. This reflects the somewhat nonspecific FA staining character of the holdfast material as observed throughout this study. It was also observed that the holdfast material of the organism was the last part of the cell to lose its fluorescence when increasingly greater dilutions of specific IgG were used to stain the organism.

In summary, it was found that spore-specific antigens are present on the exospores, the exospore portions of sporulating vegetative cells and germinating exospores. Three FA staining techniques were developed, each of which had advantages and disadvantages when compared to the other two methods but any of which can be used to study the life cycle of *M. trichosporium*.

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