Induction, Morphogenesis, and Germination of the Chlamydospore of *Candida albicans*

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Received for publication 30 July 1970

Early log phase yeast cells of *Candida albicans* transformed into suspensor cells and chlamydospores when streaked on washed agar without added nutrients. The transformation was apparently a result of endogenous metabolism since starved yeast cells did not form chlamydospores. Addition of glucose (5 mg/ml) to washed agar completely suppressed chlamydospore formation. Size of inoculum and age of inoculum markedly affected chlamydospore yield. Electron microscopy of thin sections revealed the chlamydospore wall to be double layered, the outer thin layer being continuous with the wall of the suspensor cell. A technique was devised to study germination of chlamydospores. Chlamydospores germinated by budding, and the fluorescent antibody technique was used to study the budding process.

Formation of the large, spherical, thick-walled structure termed chlamydospore is a characteristic of *Candida albicans*. This structure is of diagnostic importance in identification of *C. albicans*, and numerous culture media have been described aimed at promoting chlamydospore formation in isolates from clinical material. However, few studies of a biochemical nature have been attempted on the chlamydospore of *C. albicans*, and there appears to be nothing in the literature on the chemical composition of this structure. The absence of such studies may be attributed in part to the lack of a method for isolation of chlamydospores in quantity. This obstacle has been overcome to an acceptable degree in the work to be reported.

The word "chlamydospore" is derived from Greek "chlamys," meaning mantle or sheath. The term in mycology is defined as a thick-walled, non-deciduous, intercalary or terminal asexual spore made by the rounding up of a cell or cells (1). The chlamydospore of *C. albicans* was probably first noted by Robin (18), as can be judged from his drawings. Guilliermond (9) stated that, in *C. albicans*, chlamydospores form at the extremities of filaments, are gorged with glycogen, and are enveloped by a thick membrane formed of superimposed layers. The taxonomic significance of the chlamydospore of *C. albicans* was recognized by Langeron and Guerra (11) who also defined the chlamydospore of *C. albicans* as a large, spherical cell with a double wall, always terminal, and acid-fast when stained by the method of Kufferath.

**MATERIALS AND METHODS**

**Organism.** The organism used was *C. albicans* strain I.H.M. 582 (ATCC 10261) originally obtained from Juan E. Mackinnon, Montevideo, Uruguay.

**Media.** A defined liquid medium contained the following ingredients, per liter of distilled water: KH2PO4, 3.0 g; (NH4)2SO4, 3.0 g; CaCl2, 0.25 g; MgSO4·7H2O, 0.25 g; glucose, 20.0 g; monosodium glutamate, 1.0 g; ZnSO4·7H2O, 5.0 mg; Fe(NH4)2(SO4)2·6H2O, 15 mg; and biotin, 5 μg. A yeast extract-dextrose (YD) liquid medium was used in some experiments; 1 liter of this medium contained 10 g of glucose and 10 g of yeast extract (Difco). The polysaccharide medium of Nickerson and Mankowski (16) was also employed. Sterilization in all cases was achieved by autoclaving for 15 min at 121 C. Solidified media usually contained 1.5% agar (Difco); in some experiments described further on, biotin-free washed agar was used, purified by the method of Sentheshanmuganathan and Nickerson (20). Plain agar medium comprised 1.5% (w/v) agar (Difco or purified) and distilled water.

**Production and isolation of chlamydospores.** Unless otherwise indicated, chlamydospores were produced on pieces of single-thickness cellulose dialyzer tubing (obtained from Arthur H. Thomas Co., Philadelphia, Pa.) that had been placed on the surface of plain agar, either on slide cultures or in petri dishes. Moisture of condensation was allowed to evaporate by drying agar plates for 24 hr at 27 C prior to inoculation. For slide cultures, single-thickness cellulose dialyzer tubing was cut about 5 mm narrower and
about 10 mm shorter than a microscope slide and centered on the slide covered with plain agar. Slides were inoculated by a loop in four parallel streaks with use of standard inoculum. Standard inoculum was obtained as follows. *C. albicans* was grown on a rotary shaker for 24 hr in 250-ml Erlenmeyer flasks containing 50 ml of defined medium. At this time, 1 ml was transferred to fresh medium and incubated under identical conditions for 4 to 5 hr. Appropriate dilutions were made in the same medium to obtain 10^6 cells/ml. This inoculum was used routinely unless otherwise indicated. Cells grown on YD medium under similar conditions and processed as just described also gave excellent yields of chlamydo- spore-suspensory cell pairs under our standard condi- tions.

After incubation for 60 hr, dialysis tubing was lifted carefully from agar with forceps, and the surface growth was rinsed off with distilled water and collected in a beaker. Yeast cells were separated from chlamydo- spore-bearing suspensors by filtration through a sintered glass filter, coarse porosity. Chlamydo- spore-suspensory pairs were rinsed off the sintered glass surface by inverting the funnel and applying a gentle stream of water from a wash bottle. After several washings by centrifugation, the chlamydo- spore-suspensory fraction was lyophilized and stored at \(-20^\circ C\).

For nutritional studies, ingredients added to the plain agar medium were sterilized by filtration through a membrane filter (Millipore Corp., Bedford, Mass.). In experiments evaluated on the basis of cell counts, inoculum was streaked on dialysis tubing. At the end of the incubation period, specified in individual experiments, the total growth was rinsed off into a beaker, and the suspension was homogenized by hand in a glass homogenizer. Cells were counted in a Neubauer hemacytometer.

**Induction of germination.** The following procedure was developed to permit observation of chlamydo- spore germination. Pieces (three or four) of capillary tubing (capillary tubes, alkali-free, Fisher Scientific, catalogue no. 2-668-40), 25 mm long, were spaced at 3- to 4-mm intervals in the middle of a microscope slide, at right angles to the long axis of the slide, and carefully covered with a layer of polysaccharide agar. Slides were inoculated as usual, but a cover slip was placed over the inoculum. When chlamydo- spores had developed, the glass tubing was removed, leaving troughs in the agar. These were filled with YD liquid medium and the preparation was sealed with melted petroleum jelly. This setup allowed continuous prolonged observation and recording of the mechanism of "germination."

**Microscopy.** Routine bright-field observations were made on a Leitz upright Pahnbolt microscope and recorded on Polaroid film. For time-lapse photography, the Sage Flash System was employed (Sage Instruments Inc., White Plains, N.Y.). Flash intensity settings were obtained empirically, utilizing Polmos type 42 film which, in given conditions, has an ASA of 120 (oral communication from manufacturers).

Maximal framing rate was employed at a given intensity setting, and changes in morphology were recorded on DuPont 930A and 931A 16-mm film. For fluorescence microscopy, a Reichert "Fluorex" unit was employed, consisting of a 200-w mercury vapor arc HBO-200 bulb and a reflecting dark-field condenser. A combination of exciting and ultraviolet excluding filters was selected, depending on the given conditions. Photographs were taken with Polaroid 3000 and Kodak high-speed Ectachrome EH 135-20 films.

For electron microscopy, sections (1 by 2 mm) were cut from 72-hr agar slide cultures and fixed either in osmic acid or KMnO₄. After fixation for 3 days in 3% (w/v) osmic acid, agar sections were dehydrated in three changes of ethanol (50, 70, and 80%, v/v). Fixation for 90 min in 5% (w/v) aqueous KMnO₄ was followed by washing in distilled water and dehydration in 95% ethanol. Fixed samples were embedded in Epon 812 (Shell Chemical Co.) following the procedure of Tafel (12). Sectioning was done on an LKB Ultratome equipped with a diamond knife. Sections were mounted on carbon-stabilized parlondion-coated grids and examined with an RCA-3D electron microscope.

**Fluorescent antibody procedure.** For production of antisera, *C. albicans* was grown for 20 hr in YD medium in agitated cultures at 27 C. Cells were collected, washed several times by centrifugation, and allowed to stand in 0.25% formaldehyde overnight at 5 C. After repeated washings in sterile saline, viability tests were done, whereupon the material was lyophilized. A suspension containing 1 mg of the lyophilized material per ml was administered intravenously to three young adult New Zealand rabbits by the following schedule: 0.5 ml on the first day of a week, followed by two 1.0-ml injections on the next 2 days. This series was repeated for three subsequent weeks; thus, each rabbit received a total of 12 injections. Rabbits were bled after 5 weeks, merthiolate (Lilly; 1/10,000) was added, and anti- sera were stored at 5 C until used.

For isolation of the gamma globulin fraction, 10 ml of whole yeast cell antiserum was fractionated by electrophoresis on potato starch in an E-C trough (E-C Apparatus Corp., Philadelphia, Pa.). Separation was allowed to proceed for 18 hr at 360 v with cooling; 0.05 M barbital buffer, pH 8.6, was employed. The position of gamma globulin in the starch block was localized by lightly pressing a filter-paper strip along the run, allowing it to dry, and staining with bromophenol blue. Gamma globulin was eluted by filtration through a sintered-glass filter of medium porosity, dialyzed against distilled water, and lyophil- ized.

**Celite-adsorbed fluorescein-isothiocyanate.** Calbiochem, Los Angeles, Calif.) was used to label gamma globulin. The technique outlined by Rinder- knecht (17) was used. The lyophilized gamma globu- lin was dissolved (5 mg/ml) in 1 ml 0.05 M sodium carbonate-bicarbonate buffer, pH 8.5. Celite-ad- sorbed fluorescein-isothiocyanate (10 mg) was suspended in the solution and allowed to react for 5 min with occasional agitation. The mixture was centrifuged, and the clear supernatant fluid was applied to a Sephadex G25 (Pharmacia) column...
(2.5 by 18 cm), prepared in 0.02 M sodium phosphate buffer, pH 6.5. While unreacted excess dye was retained by the column, a strong yellow band separated rapidly and was collected.

In the labeling of cells, 24-hr cultures of chlamydospores and yeastlike cells were collected and washed by centrifugation at ambient temperature and incubated for 1 and 12 hr at 3 C in the presence of fluorescein-labeled globulin. After incubation, the labeled cells were washed with phosphate-buffered 0.5% (w/v) NaCl (pH 6.5) to remove excess antibody, plated on YD agar, and allowed to grow for 30 min to 1 hr. Wet mounts were examined with a fluorescent microscope for the presence of budding structures; filter combinations employed were BG12 and OG1.

RESULTS

Induction of chlamydospores. Chlamydospores were produced abundantly when C. albicans was grown on the polysaccharide medium of Nickerson and Mankowski (16). However, such cultures also contained extensive filamentation. As the aim of this study was to obtain chlamydospore-containing cultures as free as possible from other forms of growth, attempts were made to reduce the extent of non-chlamydospore-bearing filamentation.

Plain agar medium was found to support chlamydospore formation in the absence of blastospore-bearing filaments. All filaments in a representative colony (Fig. 1) terminated with one or more chlamydospores. Marked differences in size permitted easy separation of the yeastlike growth by filtration. Chlamydospores were also formed on purified, biotin-free agar in the absence of any added nutrients, even when the inoculum consisted of cells washed several times in phosphate buffer. Development of chlamydospores was also obtained on streaking such washed cells on sterile cellulose dialysis membranes followed by incubation in a moist atmosphere. In routine use, chlamydospores were produced on dialysis tubing resting on plain agar contained in petri plates.

Many attempts were made to obtain chlamydospore development in liquid media with the hope of eventually achieving large-scale production. Among various conditions tested were the polysaccharide medium without agar; also, water- and acetone-soluble extracts of agar which were lyophilized and added to the defined medium in place of glucose. Cultures were incubated with or without agitation, and under air, CO₂, or N₂ atmospheres. However, all these attempts proved fruitless; only rarely has a chlamydospore been observed in any liquid medium.

Factors affecting chlamydospore yield. Three factors were found to influence the production of chlamydospores: age of culture, inoculum size, and inoculum age. Each of the variables was studied while the other two were kept constant. The optimal yield of chlamydospores [expressed as a ratio of chlamydospores to yeast cells (C:Y)] and the homogeneity of the culture were age dependent. Maximal chlamydospore development was observed between 48 and 60 hr of incubation. When incubation was prolonged, the number of blastospore-bearing filaments and budding yeast cells increased markedly.

The effect of inoculum size on chlamydospore development was studied by using different densities of washed cells. The initial concentration was 10⁷ cells/ml; this suspension was diluted 1/10, 1/100, and 1/500. Use of the undiluted suspension and the 1/10 dilution resulted in the appearance of chlamydospores as early as 18 hr. These cultures also contained extensive filamentation which was absent from slides inoculated with 1/100 and 1/500 dilutions of the cell suspension. After 48 hr of incubation with the more dilute inocula, cultures exhibited colonies containing suspensors terminated with chlamydospores. Thus, a dilution which contained 10⁴ cells/ml was used routinely as a standard inoculum.

The effect of inoculum age was studied by using washed standard inoculum; slides were examined after 48 hr. A growth curve for C. albicans in the defined liquid medium was established, and inocula representing four different points on the curve were assayed for efficiency of chlamydospore production: 1 hr (lag phase), 5 and 8 hr (log phase), and 24 hr (early stationary phase). At the time of inspection (48 hr), slides inoculated with either lag- or stationary-phase cells had hardly any macroscopically visible growth. On the other hand, cultures inoculated with 5- or 8-hr cells had produced visible colonies, rich in chlamydospores. As there was no observable difference between the latter two, the inoculum age was set at 4 to 5 hr for routine work.

Effect of nutrients on chlamydospore development. The effect of amino acids on the development of chlamydospores is summarized in Table 1. Although an effort has been made to quantitate the C:Y ratio, the figures have only an illustrative significance. C:Y would vary from slide to slide and from colony to colony on the same slide. However, proline and glutamic acid consistently caused complete inhibition of chlamydospore development. On the other hand, L-methionine and L-serine seemed to increase chlamydospore number by promoting filamen-
tation generally and by decreasing the number of yeast cells in a colony. The results obtained with amino acids did not vary appreciably when ammonium or nitrate nitrogen were added.

The effect of various carbohydrates on the production of chlamydospores has been reported many times in the literature (see review by Hayes, 10). Generally, chlamydospores are produced in the absence of easily utilizable carbon source. However, it was of interest to determine the amount of glucose needed to suppress chlamydospore development. Figure 2 illustrates the effect of adding increasing concentrations of glucose to plain agar medium.
Table 1. Effect of amino acids on the development of chlamydospores

<table>
<thead>
<tr>
<th>Amino acid added</th>
<th>Chlamydospores per colony</th>
<th>C:Y \times 10^4</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>20</td>
<td>12</td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>Glycine</td>
<td>90</td>
<td>64</td>
</tr>
<tr>
<td>DL-Lysine</td>
<td>33</td>
<td>18</td>
</tr>
<tr>
<td>DL-Histidine</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>40</td>
<td>21</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>484</td>
<td>480</td>
</tr>
<tr>
<td>L-Serine</td>
<td>156</td>
<td>520</td>
</tr>
<tr>
<td>L-Proline</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>L-Aspartagine</td>
<td>37</td>
<td>20</td>
</tr>
</tbody>
</table>

a Chlamydospores were counted directly on the slide after incubation for 60 hr. Yeast cell count was obtained by rinsing a colony into 1 ml of water and counting on a Neubauer hemacytometer.

Chlamydospore yield decreased with increasing concentration of glucose. There were no chlamydospores present at a glucose concentration of 5 mg/ml. The inhibitory effect of glucose was enhanced in the presence of 2,4-dinitrophenol. Only 1 mg of glucose per ml was needed to suppress chlamydospore formation entirely when dinitrophenol was added to the medium at a final concentration of 5 \times 10^{-5} M. At 10^{-4} M, the same effect was achieved with 0.5 mg of glucose/ml (Fig. 2).

Effect of inoculum starvation on chlamydospore development. Cells grown in YD medium for 12 hr with continuous agitation were collected by centrifugation, washed three times in 0.2 M phosphate buffer, pH 5.5, at ambient temperature, and then suspended in a volume of buffer equivalent to that of the growth medium and returned to the rotary shaker at 27 C. At certain time intervals, a sample of cells containing 2.5 mg (dry weight)/ml was assayed for oxygen uptake in a Warburg apparatus. Both endogenous and exogenous respiration rates were measured, the latter in the presence of glucose (5 \mu moles/vessel). After 10 hr of starvation, the endogenous respiration rate was very low. The QO2 was less than 5, whereas the nonstarved control had a QO2 of 37. At this point, cells were stained with iodine and compared to nonstarved controls. A dark-orange granulation due to glycogen was prominent in the control and absent in the starved cells. Portions were also diluted and inoculated for chlamydospore development. The "spidery" growth pattern depicted in Fig. 3 was not seen and, although filamentation was present, no chlamydospores were observed.

Effect of excess moisture on chlamydospore development. The presence of condensation liquid on cultures inhibited chlamydospore development. Figure 3 shows the yeastlike colony formed upon inoculation of freshly poured agar. In practice, the moisture of slide cultures was controlled by employing 0.5 ml of 26.5% (w/v) CaCl2 per ml (4), and petri plates were dried for 24 hr at 27 C before inoculation.

Morphogenesis of chlamydospores. Time-lapse recording of the development of chlamydospores was conducted after incubation of slide cultures for 24 hr. The permanence of the formed spores and the process of development could be observed. The four frames shown in Fig. 4 represent 12-min intervals. It can be seen that every branch from the main filament terminates in a chlamydospore. Chlamydospores are borne on rather elongated suspensor cells. It has also been observed that a chlamydospore may rest directly on a long filament, giving rise to so-called sessile spore formation. The structure that will become a chlamydospore itself or support one is initiated by budding. A round cell is formed as opposed to the elongated cells composing the pseudomycelium. As the bud grows, material is deposited inside, giving rise gradually to the thick-walled structure characteristic of chlamydospores. It was also observed that a round cell which, judging from its appearance might become a chlamydospore, budded once more and gave rise to a second round cell which did become a chlamydospore. This phenomenon was probably observed by Roux and Linossier (19). They described structures termed "protochlamydospores" which concentrated glycogen and other cellular material; as the "true" chlamydospore was formed, all this material would migrate into the chlamydospore, leaving the protochlamydospore...
seemingly empty. The resolution of the film employed for time-lapse photography does not allow one to differentiate between the inner structure of old and young chlamydospores. Whereas the center of a young spore is granular in appearance, in older cultures (72 hr) coalescence has taken place.

Examination of electron micrographs of thin sections of chlamydospores failed to reveal any structurally distinguishable cytoplasmic constituents. Similar findings reported by Baker-spiegel (2) were attributed by him to poor fixation. The chlamydospore wall is double layered, consisting of a thin, electron-transparent outer layer surrounding a thick electron-dense inner layer (Fig. 5). The thickness of the inner layer increases with age of the chlamydospore. The thickness of the wall of mature spores is approximately 400 nm. It can be seen (Fig. 5) that the outer layer is a continuum of the suspensor wall. A membrane seems to separate the inner layer from the cytoplasm (Fig. 5).

Germination of chlamydospores. Chlamydospores were observed to germinate when the
environment was changed. Lyophilized chlamydospores and filaments would start to bud and gradually convert into yeast-phase growth when placed into YD medium. However, not all chlamydospores would bud. Best results were obtained when the change of environment was achieved without disturbing preformed growth. Age of the culture was also important; only
relatively young chlamydospores (24 to 40 hr) could be induced to germinate by budding. About 3 to 4 hr elapsed between the change of the medium and observable germination. During this time, the center of the chlamydospore developed a granular appearance, and rapidly moving particles could be observed in the suspensor filaments (Fig. 6). The wall of the spore also lost some of its thick appearance. As the germination of chlamydospores proceeded, giving rise to pseudomycelium, blastospore-bearing filaments also arose. Within 12 to 16 hr, the predominant appearance of the culture was yeast-like with occasional filaments. Miwatani et al. (14) observed several round structures emanating from a chlamydospore. However, the structures they identified as buds did not increase in size nor did they multiply.

The sequence of steps in the budding process is presented in Fig. 7. The elapsed time from Fig. 7A to 7C was 3 min. Figure 7D was taken 30 min later to show the enlargement of the daughter cell. A bulging of the cell initiates the appearance of a bud. It would seem that the thick chlamydospore wall is ruptured while some inner membrane covers the bud as it first appears.

The fluorescent-antibody technique was employed to study the budding process. A higher frequency of budding was observed in mounts prepared from cultures which had been exposed to antibody for 12 hr at 5 C before plating; some degree of synchronization was probably achieved. Figure 8 shows the initiation of a bud from a cell, and the enlargement of buds. Note that the fluorescence of the mother cell wall is discontinued. This can be interpreted as being due to either the presence of newly synthesized cellular material or the presence of only a very thin layer of the old wall, exhibiting low fluorescence.

**DISCUSSION**

The development of chlamydospores was observed when washed inocula were plated on a plain agar-distilled water medium. Since chlamydospores were not produced by a starved inoculum under similar conditions, it may be assumed that differentiation is accomplished through endogenous metabolism. Although it cannot be ex-
cluded that the process of starvation had some effect other than the mere exhaustion of metabolizable reserves, the cumulative effect of glucose and dinitrophenol in suppressing chlamydospore formation strongly supports the indication that chlamydospore development is a result of endogenous metabolism. It is known that *C. albicans* has a high rate of endogenous respiration that declines rather rapidly with time (15). Van Niel and Cohen (21) showed that only one-third of a glucose substrate is oxidized by starved suspensions of *C. albicans*, with two-thirds of the substrate being converted to a polymerized form. The effect of dinitrophenol on chlamydospore formation may be interpreted in this light. Dinitrophenol promotes the complete oxidation of the glucose substrate (7) and less glucose is required in the presence of dinitrophenol to suppress chlamydospore formation.

Another example of differentiation occurring in the absence of uptake of nutrients is found in the cellular slime mold *Dictyostelium discoideum* (5). During the development of the fruiting body, the organism sequentially utilizes endogenous

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**Fig. 6. Germination of chlamydospores.** A, Early stage, characterized by fragmentation of the central globule; B, increasing fragmentation, coupled with movement of globules into suspensor cells; C, budding of chlamydospores. All photographs, X750.
amino acids and proteins; the amino acid pool drops to approximately 30% of its initial value, the ethanol-soluble protein fraction to 60%, and the ethanol-insoluble protein to about 80% of its initial value (23). Similarly, yeastlike cells of _Mucor rouxii_ (grown under CO₂) develop into sporangiophores and sporangia in nutrient-free conditions on exposure to oxygen (3).

The fluorescent-antibody technique was employed by Goos and Summers (8) in a study of cell wall replication in _C. albicans_. There are differences between the experimental conditions described by these workers and those employed in the present study. Goos and Summers described the fluorescence of cells incubated for 24 hr after exposure to fluorescent antibody. Their conclusion that parent cell wall material was incorporated in newly formed cells was not confirmed in the present work in which the incubation time was 30 min to 1 hr. Chung, Hawirko, and Isaacs (6) studied cell wall growth and bud formation in _Saccharomyces cerevisiae_ by the fluorescent-antibody technique. Cells were examined at 15-min intervals after labeling. The cell wall of a bud was almost entirely newly synthesized and contained very little, if any, old cell wall material. Yeast cells labeled with anti-yeast fluorescent antibody remained as discrete
fluorescent structures throughout the process of budding. However, the buds formed after removal of free fluorescent antibody were very largely nonfluorescent. They concluded that bud formation does not involve incorporation of segments of cell wall from the mother cell. Our findings agree with those of Chung et al. and underline the importance of examining preparations shortly after they have been labeled.

The chlamydospore-suspensor cell pair may be regarded as a unit since (i) the outer wall layer of the chlamydospore is continuous with the suspensor cell wall, (ii) much of the content of the suspensor cell appears to be concentrated in the chlamydospore, and (iii) during germination of the chlamydospore there is a return of cytoplasmic activity in the suspensor cell. Matile (13) showed that the lysosomal apparatus of yeasts and filamentous fungi is localized in vacuoles. In the basidiomycete Coprinus lagopus, autolysis of the fruiting body is brought about by chitinase which is localized in vacuoles in gill cells and is released therefrom to cause dissolution of the chitinous cell walls (W. Iten, Thesis, Eidgenossische Technische Hochschule, Zurich, Switzerland, 1969). In view of the changes in both suspensor cell and chlamydospore on germination of the latter, it is likely that some lysosomal activity may be contained in the vacuole of the suspensor cell and play a role during germination of the chlamydospore.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grants AI-00251 and TI AI-43 from the National Institute of Allergy and Infectious Disease.
LITERATURE CITED