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Light Microscopy of Basidia, Basidiospores, and Nuclei in Spores and Hyphae of *Filobasidiella neoformans* (*Cryptococcus neoformans*)

KEITH H. ERKE

Department of Medical Research and Development, William Beaumont Army Medical Center, El Paso, Texas 79920

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Three hypha-forming strains of *Cryptococcus neoformans* were induced to form basidia and basidiospores. Light microscopy showed that basidia formed at the ends of terminal hyphal cells and were able to produce from a few to many basidiospores. The morphology of the sexual structures indicated that these strains belonged to the recently described perfect state of *C. neoformans*, *Filobasidiella neoformans*. The average dimensions of the basidiospores were 1.9 μm in width by 2.7 μm in length. Giemsa staining revealed that dikaryotic cells were formed in all three strains. Only one strain had both terminal and subterminal dikaryons, indicating functional clamp connections, whereas the two remaining strains had dikaryons restricted to the terminal cells. Basidiospores of two strains were mononucleate, and yeast cell clones derived from single basidiospores of these two strains were able to complete the sexual life cycle, thus indicating their primary homothallic nature.

In 1975 Kwon-Chung (7) described the sexual stage of *Cryptococcus neoformans*. She gave this yeast the perfect name of *Filobasidiella neoformans* and placed it in the *Filobasidiaceae* of the *Ustilaginales*. In the present study three hypha-forming strains of *C. neoformans* were found to form basidia and basidiospores. A light-microscopy study of these structures coincided with the observations of Kwon-Chung (7), thus confirming that they had a perfect stage belonging to the species *F. neoformans*. However, a sexual stage developed in each of the three individual strains without the necessity of mixing strains. Basidiospores were isolated by micromanipulation to observe their germination and to determine if they could complete the sexual life cycle. Finally, Giemsa staining was used to determine the nuclear patterns in hyphal filaments and basidiospores.

MATERIALS AND METHODS

Organisms and media. The source of *C. neoformans* hyphal strains Coward (9.5), Stanford (9.26), and C-145, all originally isolated from human cases of cryptococcosis and pathogenic for mice (13-15; T. G. Mitchell, Ph.D. thesis, Tulane Univ., New Orleans, La., 1971), was reported previously (4). All strains were maintained on Sabouraud dextrose agar at room temperature (22 to 24°C). Other media used in this study were Staib's nigerseed-creatinine agar (16), V-8 juice agar prepared from a filtrate diluted 1:2 (10), and minimal medium agar minus thiamine (MM − thi). Minimal medium (MM), initial pH 5.7, has the following ingredients per liter of distilled water: glucose, 10 g; $(NH_4)_2SO_4$, 5 g; KH_2PO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $CaCl_2 \cdot H_2O$, 0.1 g; NaCl, 0.1 g; thiamine-HCl, 0.5 mg; agar, 20 g; and 5 ml of a trace element solution (containing the following amounts in 100 ml: $CuSO_4 \cdot 5H_2O$, 0.8 mg; $MnSO_4 \cdot H_2O$, 8 mg; $FeCl_3 \cdot 6H_2O$, 4 mg; KI, 2 mg; $Na_2MoO_4 \cdot 2H_2O$, 4 mg; H_3BO_3, 10 mg; and $ZnSO_4 \cdot 7H_2O$, 8 mg).

Growth and sporulation. Hyphal growth was induced from stock cultures by making four point inoculations on V-8 or MM − thi agar and placing glass cover slips (22 mm) over the yeast cells. The plates were sealed with masking tape, placed in plastic bags, and incubated at room temperature (22 to 24°C). Optimum conditions for sporulation varied for each strain. Sporulation was best for the Stanford strain when cover slips were removed from a 40- to 70-day V-8 culture and squares containing the hyphal growth were cut out and transferred to the surface of fresh V-8 plates. For the Coward strain, squares containing hyphal growth of a 35- to 50-day V-8 culture were placed on the surface of fresh MM − thi agar. Strain C-145 gave optimum sporulation with hyphal cultures maintained serially on MM − thi agar. This sporulation occurred after cover slips were removed from 6 to 10 days of growth without transfer to fresh medium.

Micromanipulation. A deFonbrune micromanipulator, equipped with microneedles fashioned freehand (2) from 1-mm capillary tubes, was used to separate basidiospores for observing germination and obtaining monosporous yeast cell clones. A number of spores sticking to the microneedle from

an inidividual basidium were transported to agar blocks. The basidiospores were teased apart, and single spores adhering to the microneedle were carried to other blocks and positioned near glass beads so that they could be easily relocated (4). The agar blocks were then placed on the surface of Sabouraud dextrose agar plates for growth and microscopic observation.

Giemsa staining of nuclei. Small blocks of agar containing hyphal growth were placed on a glass slide with a few drops of egg white. Another slide was used to press the agar, and the slides were drawn apart to give a thin preparation. To obtain stained terminal cells, hyphal growth was sandwiched between dialysis tubing and glass cover slips in the following manner. Dialysis tubing squares (22 mm) were sterilized, placed on agar medium, and dried. Small blocks of agar containing hyphae were inoculated onto the center of the tubing and overlaid with 25-mm cover slips. After growth had occurred, both tubing and cover glass were removed from the agar and inverted, and the dialysis tubing was immediately peeled away, leaving hyphal cells adhering in place to the cover slip. To prepare basidiospores for staining, areas with abundant sporulation on the surface of agar blocks were touched to cover slips having thin smears of egg white. After drying, all preparations were fixed, hydrolyzed, and stained with Giemsa (1). All photomicroscopy and measurements of basidia and basidiospores were carried out with a Zeiss photomicroscope equipped with an eyepiece micrometer.

RESULTS

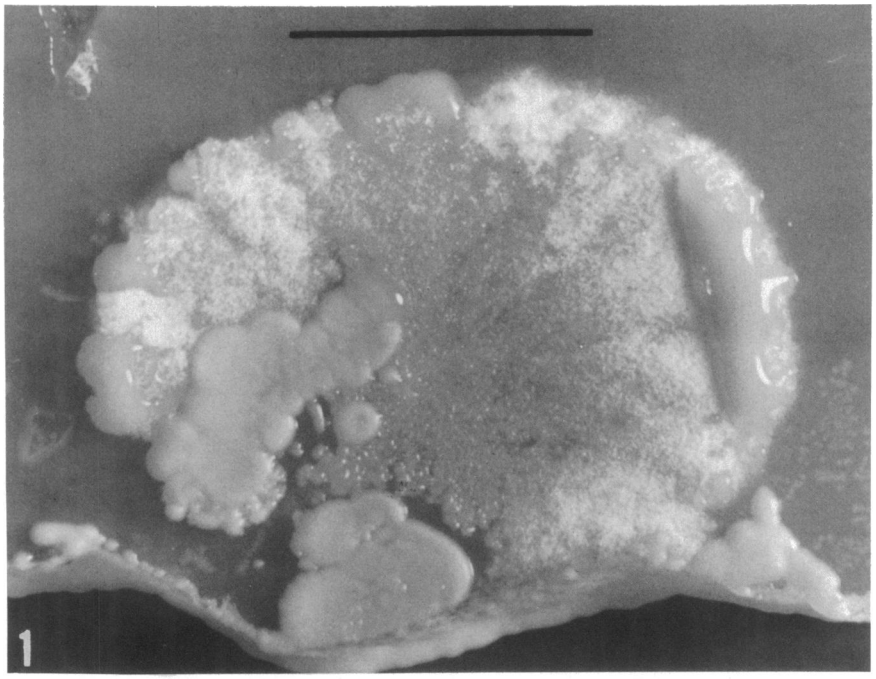
The hyphal growth of all three strains could be maintained serially on MM − thi agar under cover slips. After removing the cover slips, yeast growth was limited on the agar surface when grown on this medium. When cover slips were removed from hyphal growth in MM agar the surface area was overgrown with yeast cells. The surface area of similar cultures in MM − thi agar became covered with short, thick, white aerial hyphae and with noticeably less yeast growth.

Although some sporulation of all three strains has been seen under a variety of different conditions on both MM − thi and V-8 agar, the conditions described in Materials and Methods gave repeatable and the most abundant production of basidia and basidiospores for each of the three strains. The Stanford strain showed the best sporulation of all three strains. Four days after cover slips had been removed and hyphal growth had been transferred to fresh V-8 agar, much of the surface area was covered with white aerial hyphae (Fig. 1) and had abundant sporulation. Basidia and basidiospores of the Coward strain appeared within 2 days after V-8 agar blocks had been transferred to MM − thi agar and were most abundant on the periphery of the hyphal colony. Strain C-145 sporulated in 2 to 3 days after removal of cover glasses from a 6- to 10-day culture in MM − thi agar. After removal of the cover slips, each strain was also capable of producing yeast cells from short branches or from sessile positions along the hyphae.

The observations made on the sexual structures concurred with those made by Kwon-Chung (7). Figures 2, 3, and 4 show basidia and basidiospores of the Stanford, Coward, and C-145 strains, respectively. Each basidium developed as a swelling at the end of a terminal hyphal cell and was capable of producing from a few to many basidiospores. Spores appeared on basidia in chains (Fig. 2 and 3) or in clusters (Fig. 4). Basidiospores were formed in the majority of cases from four separate points on a basidium and resulted in four chains on most basidia. Basidia with more than four chains have never been observed, and in only occasional instances have one, two, or three chains been seen. In many cases where long chains were formed, the ends were fused together, apparently giving more support to the individual chains (Fig. 5). All sporulation of the Stanford and Coward strains and most sporulation of C-145 occurred on aerial hyphae above the surface of the agar. In some cases, C-145 had basidia with small clusters of basidiospores that formed beneath the surface of the agar.

The enlarged ends of the basidia were generally cylindrical or clavate in shape (Fig. 6), with the dimensions of the inflated area for the Stanford and Coward strains ranging in width from 4.7 to 7.7 μm and in length from 6.6 to 15.3 μm. Basidiospores were formed successively on the basidium, with the older spores being pushed outward. Sterigmata were not present. Basidiospores were either oval, round, cylindrical, or teardrop in shape, with the distal end from the basidium being rounded and the proximal end generally truncate (Fig. 7). Fifty-seven spores from the Stanford and Coward strains varied in width from 1.3 to 2.2 μm (average, 1.9 μm) and in length from 2.1 to 3.4 μm (average, 2.7 μm). Giemsa-stained preparations of Stanford and Coward basidiospores revealed the presence of a single nucleus in each spore (Fig. 8). Only rarely were spores seen with two or three nuclei, and these spores were considerably larger than spores with a single nucleus.

The first step in the germination process was a swelling of the basidiospore. It became rounded and enlarged approximately to the size of a *C. neoformans* yeast cell. The next step resembled typical yeast budding with the formation of a daughter cell. The first daughter



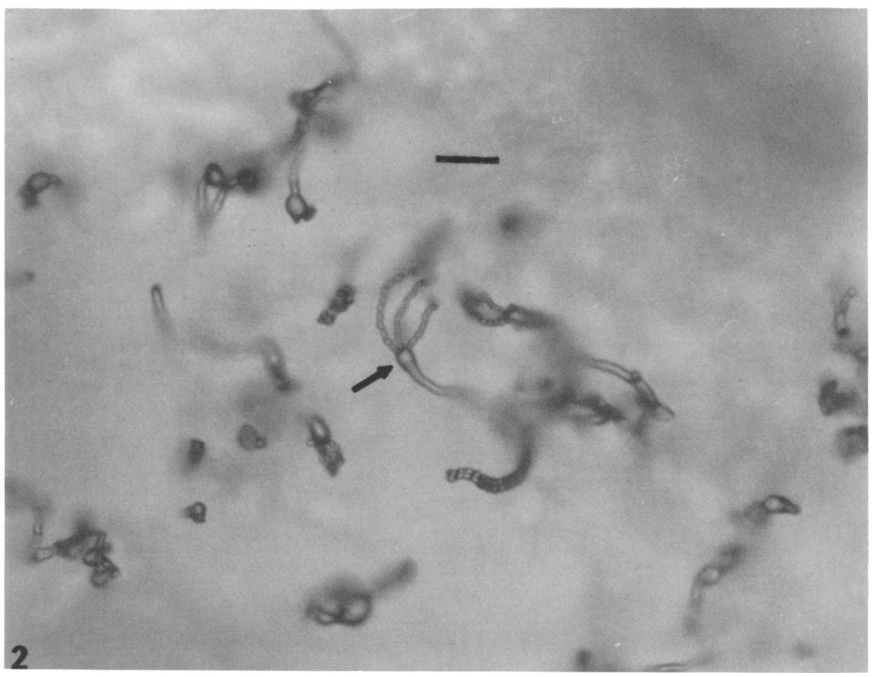
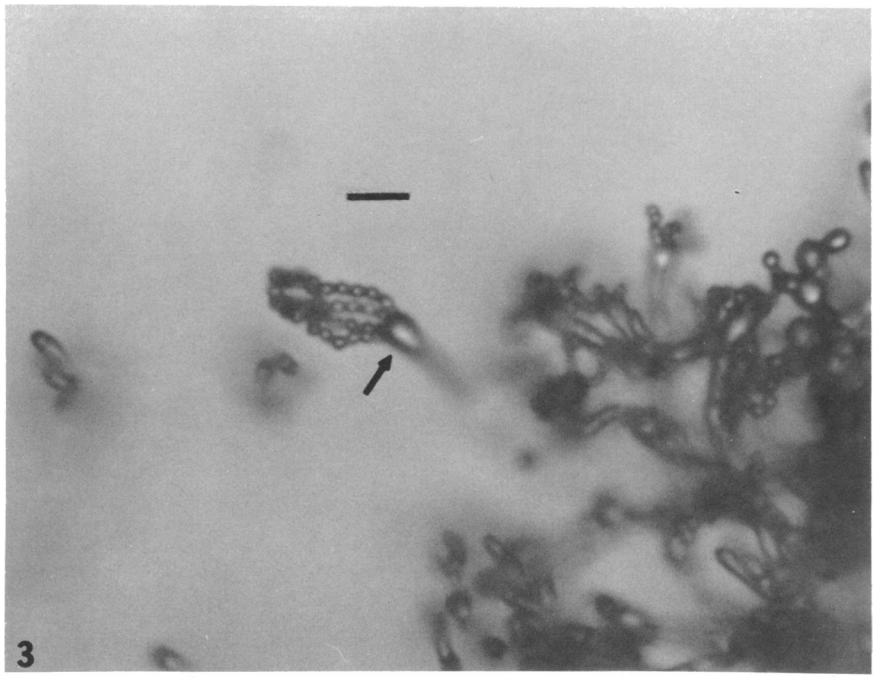


FIG. 1. *Colony of C. neoformans Stanford strain producing basidia and basidiospores among the areas of white aerial hyphae. Bar, 1 cm.*

FIG. 2. *Basidium (arrow) of the Stanford strain with four chains of basidiospores (only three chains are partially in focus). Other basidia and/or basidiospores are seen in the surrounding area. Bar, 30 μm.*



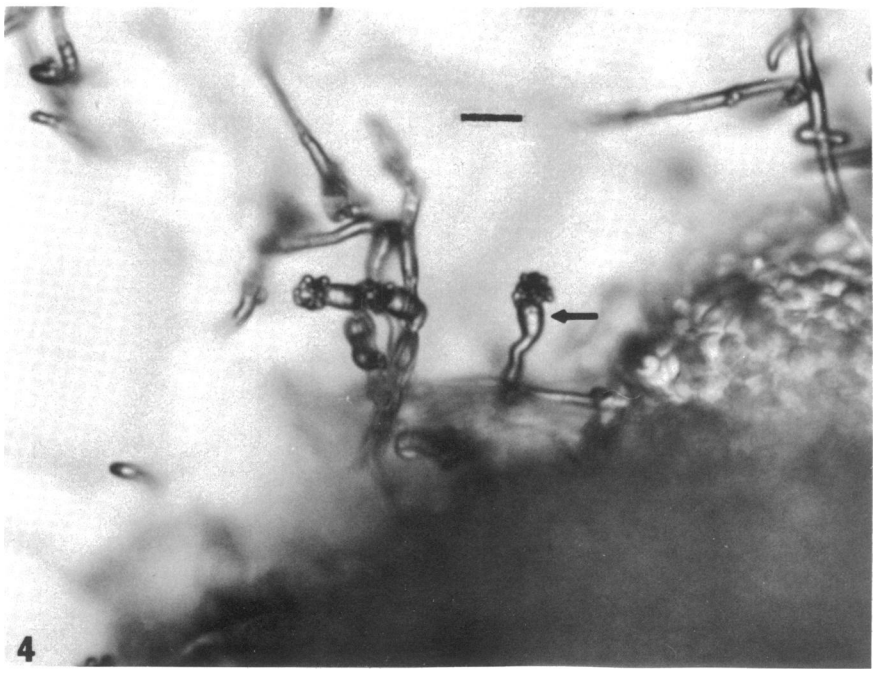
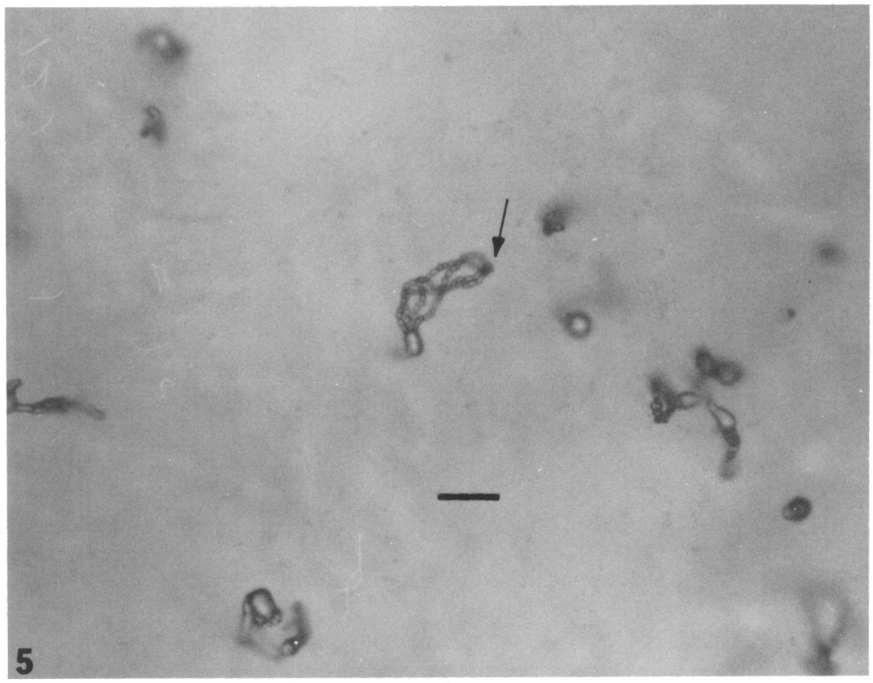


FIG. 3. *Basidium (arrow) of the Coward strain with four chains of basidiospores. Bar, 16 μm.*
FIG. 4. *Basidium (arrow) of strain C-145 bearing clusters of basidiospores. Two basidia directly to the left have similar spore clusters. Bar, 20 μm.*

cell very often grew until it equaled the size of the mother cell before either cell initiated new budding. Thereafter, new cells arose by a budding process from either mother cell, daughter cell, or both. The germination sequence is illustrated in steps A through E in Fig. 11.

Table 1 indicates the ability of 25 yeast cell clones derived from single spores of the Stan-



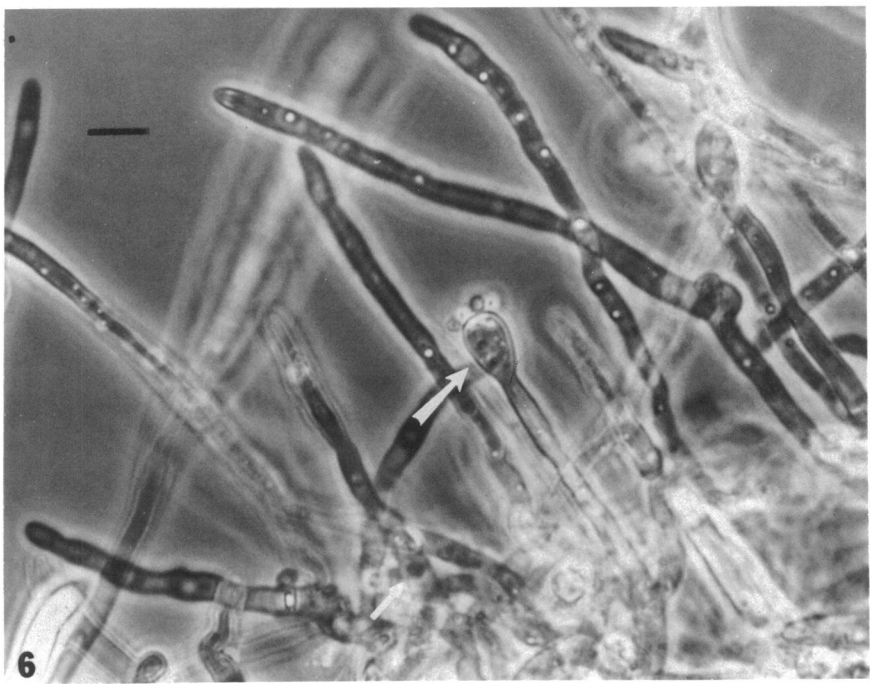


FIG. 5. *Four basidiospore chains with ends (arrow) fused together; Stanford strain. Bar, 30 μm.*

FIG. 6. *Tease preparation of Coward hyphae showing basidium (arrow) with four basidiospores still attached. A clamp connection can be seen four bar lengths to the right of the basidiospores. Phase contrast. Bar, 12 μm.*

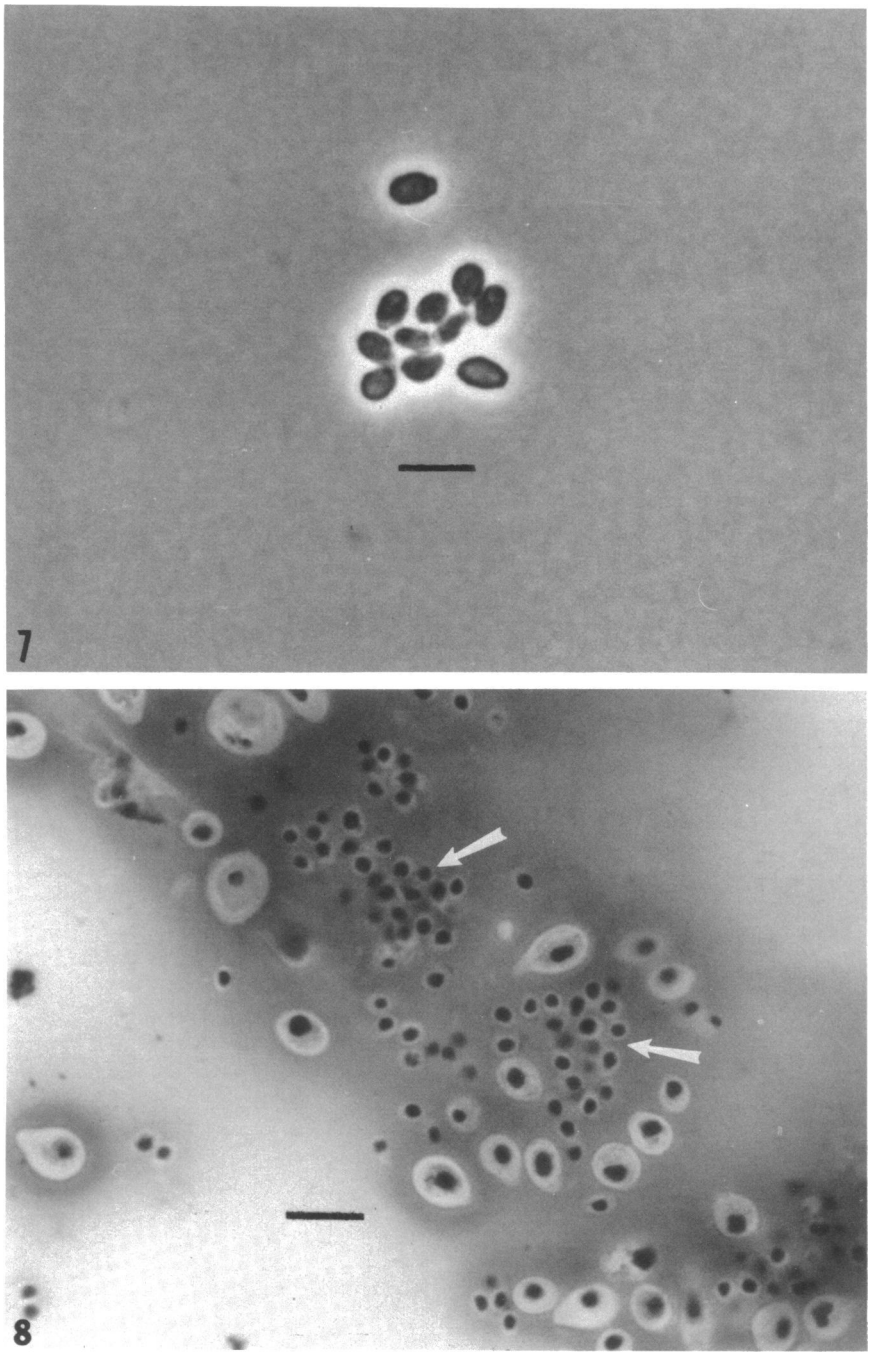


FIG. 7. *Basidiospores of Stanford strain micromanipulated to agar surface. Phase contrast. Bar, 6 μm.*
FIG. 8. *Giemsa stain illustrating nuclei of Stanford strain basidiospores. Spores are scattered throughout field with clusters at end of arrows. Larger yeast cells can also be seen in the field. Bar, 10 μm.*

ford and Coward strains to form hyphae, basidia, and basidiospores when induced to sporulate under the same conditions as the parental strains. All of the single-spore cultures were able to form basidia and basidiospores, although three cultures originating from spores of a single basidium of the Coward strain showed markedly reduced sporulation when

TABLE 1. *Hyphal growth pattern of Stanford and Coward strain single-spore isolates and their ability to form basidia, basidiospores, and brown pigment*

Strain	Source of single-spore clones		Brown pigment on Staib's nigerseed-creatinine agar	Hyphal growth pattern	Basidia and basidiospores[a]
	Basidium	Spore			
Stanford strain	1	1	+	Normal	++
	2	1	+	Normal	++
	3	1	+	Normal	++
		2	+	Normal	++
		3	+	Normal	++
	4	1	+	Normal	++
		2	+	Normal	++
		3	+	Normal	++
		4	+	Normal	++
	5	1	+	Normal	++
		2	+	Normal	++
		3	+	Normal	++
		4	+	Normal	++
		5	+	Normal	++
Coward strain	1	1	+	Normal	++
		2	+	Normal	++
	2	1	+	Normal	++
		2	+	Stunted	+
		3	+	Normal	++
		4	+	Stunted	+
		5	+	Normal	++
		6	+	Stunted	+
	3	1	+	Normal	++
		2	+	Normal	++
	4	1	+	Normal	++

[a] +, Basidia and basidiospores present but very few as compared to the parental strain; ++, basidia and basidiospores formed as well or better than the parental strain.

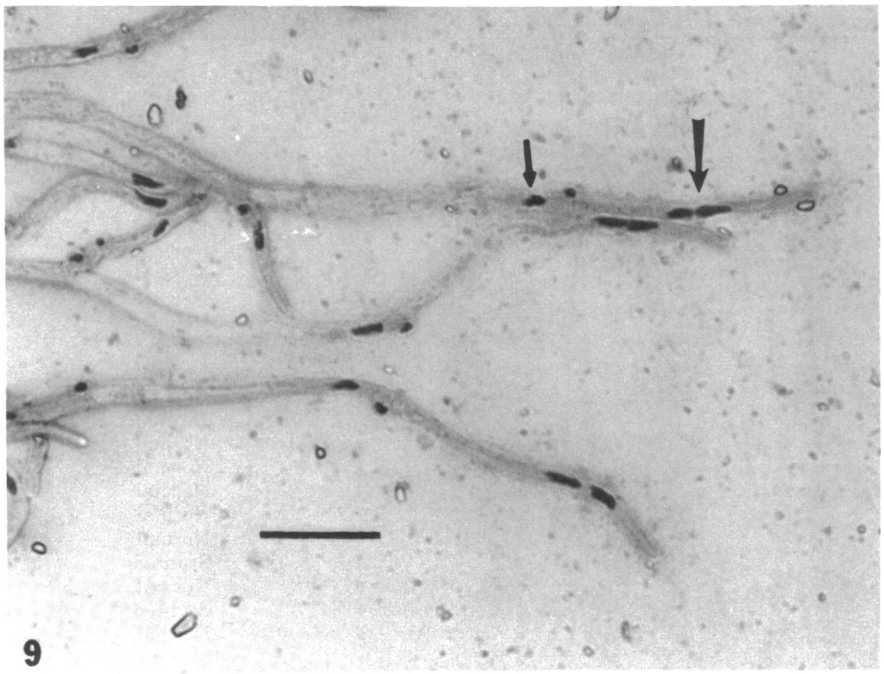
compared with that of the parental strain. These three isolates also showed reduced growth in both hyphal and yeast cultures, the square area of growth reaching only about one-half the size of other single-spore isolates. All of the cultures formed a brown pigment when grown on Staib's nigerseed-creatinine agar.

Giemsa staining revealed the presence of dikaryotic cells in all three hyphal strains grown on V-8 agar; however, dikaryons in the Stanford and C-145 hyphae were restricted to terminal cells. Figure 9 shows the predominent terminal cell and first subterminal cell nuclear pattern for the Stanford strain. From 70 to 90% of the terminal cells were dikaryotic. The first subterminal cell was monokaryotic, and a very high percentage of the clamps, whether between the terminal and first subterminal cells or farther down the hyphae, contained nuclei. The majority of the remaining terminal cells were monokaryotic, with occasional anucleate or trinucleate cells. The nuclear pattern for strain C-145 was similar to that of the Stanford strain but with fewer dikaryotic terminal cells (25 to 30%). The number of dikaryons in the Coward hyphae was difficult to estimate since they varied significantly from one preparation to another. Dikaryons tended to occur in sectors that generally had large numbers of such cells. In the Coward strain dikaryons appeared in both terminal and first subterminal cells (Fig. 10), and only a few nuclei were seen in the clamp connections. The majority of the remaining cells were monokaryotic. Clamp connections were found at the septa of adjacent cells in all three strains regardless of whether the cells were monokaryotic or dikaryotic. Figure 11 summarizes the sexual and asexual life cycles of *C. neoformans* based on the information that is presently known.

DISCUSSION

The limited yeast growth on the surface of MM–thi agar when cover slips were removed made it possible to observe more abundant sporulation on the hyphae of the Coward and C-145 strains. The ability to maintain serial hyphae cultures of all three strains in MM–thi agar under cover slips was most likely due to the less than absolute requirement of *C. neoformans* for thiamine (9).

The capacity of the Stanford and Coward strain single-spore clones to complete the life cycle coupled with the mononuclear condition of



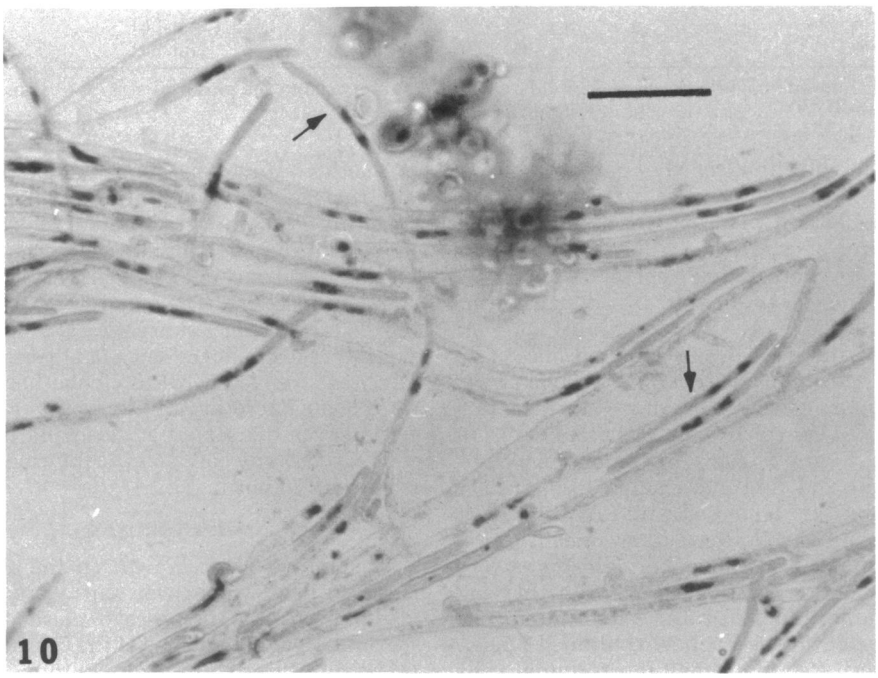


FIG. 9. *Three Giemsa-stained hyphal filaments of the Stanford strain illustrating the dikaryotic terminal cells (large arrow), nuclei trapped in clamp structures (between arrows), and monokaryotic first subterminal cells (small arrow). Bar, 40 μm.*

FIG. 10. *Giemsa-stained sector of Coward strain hyphae showing several dikaryotic cells. Arrows indicate hyphal strands having both terminal and first subterminal dikaryons. Bar, 40 μm.*

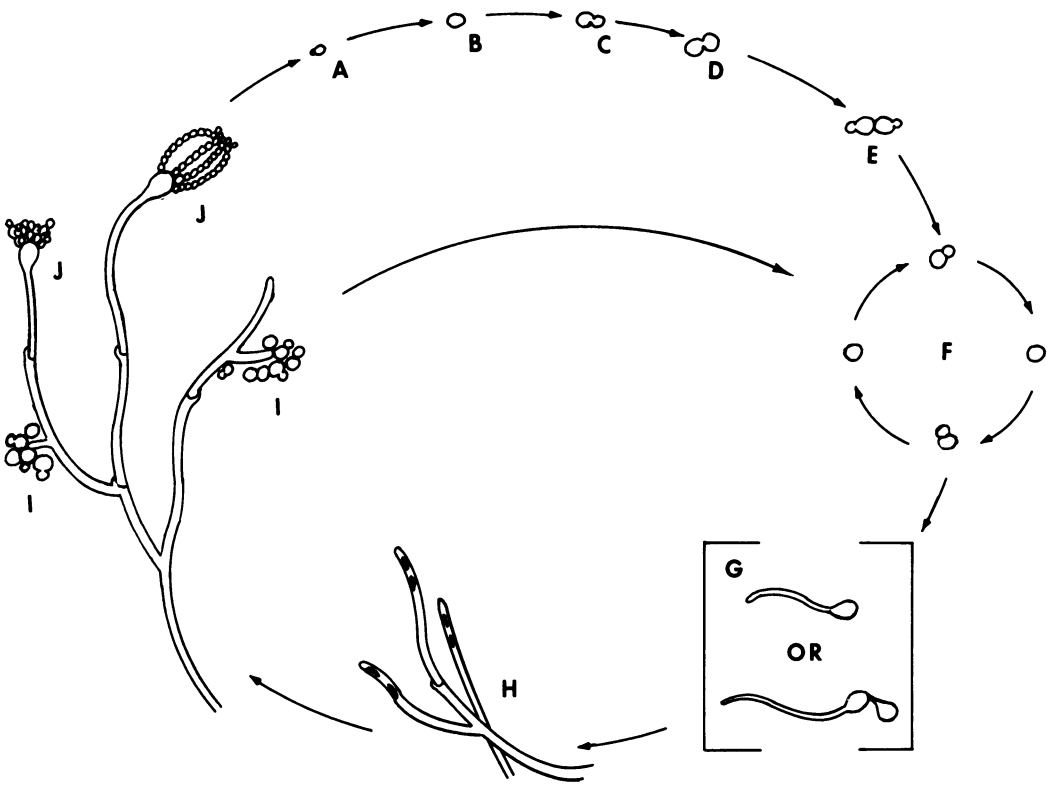


FIG. 11. *Life cycle of C. neoformans illustrating both asexual and sexual phases. (A-E) Sequence represents swelling of a basidiospore and subsequent budding. (F) Asexual yeast cell cycle. (G) Brackets indicate as yet unknown alternatives to initiate hyphal formation by either germination of individual yeast cells or previously conjugated yeast cells. (H) Development of hyphae having clamp connections and dikaryotic cells. (I) Budding yeast cells formed along hyphal strands. (J) Basidia and basidiospores formed at ends of terminal hyphal cells.*

the basidiospores strongly suggest primary homothallism (as defined by Whitehouse [18]) for these two strains of *C. neoformans*. There still remains the possibility that spore nuclei were diploid and formed from haploid nuclei of opposite mating type. The small size and the considerable number of spores that many basidia were capable of forming, together with the fact that sporulation generally occurred on each basidium at four separate points, argues against diploidy. Kwon-Chung (7) reported her strains to be heterothallic but did not specify the type of heterothallism, bipolar or tetrapolar. The presence of both homothallic and heterothallic forms in *C. neoformans* would seem to be akin to the situation for the wood-rotting basidiomycete *Sistotrema brinkmannii*, which consists of an aggregate of biological forms having homothallic, bipolar heterothallic, and tetrapolar heterothallic patterns of sexuality (17). However, it seems safe to say at this point that more *C. neoformans* strains would have to be studied and genetic analysis carried out with several strains before any conclusions could be reached. Such matings and subsequent genetic analysis could perhaps elucidate the mating types present in the heterothallic forms. Additionally, such studies may shed light on whether the homothallic behavior of the strains reported in this study are due to mutations at mating loci, as is the case for homothallic behavior that sometimes occurs in heterothallic species (*Schizophyllum commune* and *Coprinus lagopus*; 12).

Among the three hyphal strains, only the Coward strain had both terminal and subterminal dikaryotic cells, indicating the presence of fused clamp connections. A recent electron microscopic study by Kwon-Chung and Popkin (8) revealed the presence of fused clamp connections in the hyphae of two compatible strains of *C. neoformans*. The restriction of dikaryons to the terminal cells of the Stanford and C-145 strains and the many nuclei trapped in the

clamps showed that the final fusion of the hook cells to subterminal cells did not occur. Evidence for the failure of clamp fusions in the hyphae of the Stanford strain has been demonstrated in an earlier electron microscopic study (3). Clamps that fail to fuse have been termed pseudoclamps and have unequivocally been shown to be characteristic of common B matings among tetrapolar heterothallic species of the higher basidiomycetes, including *S. commune* and *Coprinus* species (12). What factors are responsible for such nuclear behavior in these apparently homothallic strains of *C. neoformans* remain unknown. The restriction of dikaryons to terminal cells did not inhibit the ability to fruit, since the Stanford strain formed the most abundant basidia and basidiospores among the three strains. Kwon-Chung (7) indicated that the sexual stage was initiated by conjugation of two yeast cells. In the present study it was not determined at what point dikaryons were established among the strains of *C. neoformans*. As was indicated in step G of Fig. 11, it was unclear whether the fusion of yeast cells was a prerequisite for hyphal formation and dikaryotization or whether other mechanisms cause yeast cells to form hyphae that can subsequently anastamose to establish dikaryons.

The formation of basidia and basidiospores among hyphal strains of *C. neoformans* prompts interesting speculation on three questions that might have ecological and epidemiological significance for this pathogenic yeast. First, although only a relatively few laboratory strains have demonstrated ability to form hyphae and therefore may have sporulating potential, it remains unknown as to what extent this stage might exist in the natural environment of *C. neoformans*. Second, if such a stage does exist in nature, what role do the basidiospores play in the dispersal of this organism in the environment? Powell et al. (11) have shown that a small percentage of *C. neoformans* airborne particles from pigeon excreta exist with diameters of less than 5.5 μm. Both basidiospores and small yeast cells (9) have dimensions less than this, although the basidiospores would seem to be better designed aerodynamically than yeast cells to become airborne. Finally, what possible role might the basidiospores play as infective agents? In order for *C. neoformans* to become deposited in the lung alveoli, Powell et al. (11) and Farhi et al. (5) have postulated that the infective particle of *C. neoformans* exists in a small form, less than 5.0 μm or approximately 3.0 μm, respectively. Gutierrez et al. (6) have recently reported a granulomata of the lung due to a small form of *C. neoformans*. Measurements of the nonbudding organisms in the lung averaged 2.0 by 2.6 μm, which approximates the average size (1.9 by 2.7 μm) of basidiospores formed by the Stanford and Coward strains. Whether such an infection could result from exposure to basidiospores remains an interesting but unanswered question.

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