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Occurrence of Diploid Strains of *Cryptococcus neoformans*

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A mating between niacin and pantothenate auxotrophs of *Cryptococcus neoformans* gave a few prototrophic progeny that were self-fertile. These were uninuclear but contained twice as much DNA as the parental strains. Segregation of nutritional markers was observed upon sporulation. We conclude that these self-fertile strains are diploids.

The meningitis-causing pathogen *Cryptococcus neoformans* exists vegetatively as a haploid yeast. In its sexual cycle, two yeasts of opposite mating types fuse, followed by elongation of the dicaryotic cell into a hypha with clamp connections. The two nuclei merge in a distal cell. From this transient diploid four strings of haploid basidiospores emerge after meiosis and mitotic divisions (5). Kwon-Chung (6) noted occasional, transiently self-fertile colonies arising from basidiospores and suggested that this phenomenon might represent temporary diploidy. We also obtained preliminary evidence for the occurrence of heterozygous diploids (11). In the present report we confirm the spontaneous occurrence of diploids in this species.

Wild types B3501 (mating type α) and B3502 (mating type **a**) received from Kwon-Chung (5) were the background strains used for derivation of a niacinamide-requiring strain of mating type α (Nia31,α) and a pantothenic acid-requiring strain of mating type **a** (Pan39,**a**) (3). Brain heart glucose medium, consisting of 40 g of brain heart infusion and 20 g of glucose per liter of water, was used as a complete medium. Littman medium (7) or Fries salts medium (1) with thiamine (0.4 μg/ml) was used as minimal media. Niacinamide (1 μg/ml) and pantothenate (1 μg/ml) were added where indicated. Solid media contained 2% agar. Hay infusion agar (8) was used as a mating and sporulation medium. Hay (50 g) was autoclaved for 30 min with 250 ml of water and removed by filtration; the infusion was frozen for future use. The medium contained 25% infusion and 0.2% K_2HPO_4 adjusted to pH 6.2. Liquid cultures were grown at 37°C with agitation. Turbidity was monitored at 700 nm. Mating cultures were incubated at 25°C.

The DNA extraction method used was developed by P. R. Stewart (10) for *Saccharomyces cerevisiae*. A portion of a culture, the equivalent of 10 ml at an optical density of 1.0, was centrifuged at 5,000 × *g* for 5 min. The cells were washed twice with isotonic saline. They were then suspended in 2 ml of ice water, and an equal volume of cold 0.5 M $HClO_4$ was added. This disrupted the cells and precipitated proteins and nucleic acids. After 15 min at 0°C, the tubes were centrifuged at 10,000 × *g* for 15 min. This cold perchloric acid extraction was repeated. The nucleic acids in the precipitate were then extracted at 70°C for 15 min with 2 ml of 0.5 M $HClO_4$, then with 1 and 0.5 ml of $HClO_4$. DNA assays were performed on the combined extracts by using the Burton modification (2) of the Dische method. Cells were counted using a hemacytometer. The Slater method (9) was used for nuclear staining. Cells treated with mithramycin (Sigma Chemical Co., St. Louis, Mo.) were examined as a wet mount with a Zeiss fluorescence microscope with excitation at 450 to 490 nm.

Strains Nia31,α and Pan39,**a** were crossed. Random basidiospores were collected with a loop, suspended in 0.3 ml of water with agitation, and spread on minimal medium. After a few days, several hundred colonies were transferred to numbered squares on both minimal and mating media. Less than 1% of colonies growing on minimal medium produced spores on mating medium. Prototrophic sporulating colonies (possible heterozygous diploids) were streaked from minimal medium onto sporulation medium. The resulting colonies were all self-fertile.

Basidiospores from prototrophic, sporulating colonies were harvested, suspended, and spread on brain heart glucose plates. Single colonies, picked to brain heart glucose, were replica stamped to mating medium, minimal medium, minimal medium plus niacinamide, minimal medium plus pantothenate, minimal medium plus niacinamide and pantothenate, and complete medium. Wild-type, Nia^-, and Pan^- strains were replica stamped as controls (Table 1). To confirm that these putative diploids contained only one nucleus per cell, mithramycin-stained cells were examined under a fluorescent microscope. The stained cells clearly showed one nucleus per cell for putative diploids, just as for haploids. The DNA per cell determinations is shown in Table 2.

TABLE 1. Segregation of recessive markers and scoring of self-fertile trait in progeny of self-fertile colonies

Putative diploid strain[a]	No. of progeny with the following phenotype:				No. of self-fertile progeny
	Wild type	Nia^-	Pan^-	$Nia^- Pan^-$	
200					
Expt 1	4	4	10	8	0
Expt 2	2	3	4	10	1
204					
Expt 1	5	2	22	9	0
Expt 2	5	1	16	6	0

[a] 42 progeny in each of duplicate experiments for the two strains were tested. The sum of the numbers of the four phenotypes does not equal the number of offspring tested because many (33 to 55%) of the colonies replica stamped from brain heart glucose medium required complex medium (i.e., would not grow on minimal medium plus niacinamide and pantothenate).

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TABLE 2. DNA per cell in several strains of *C. neoformans*

Strain	Number of determinations	DNA/cell (fg)[a]
B3501[b]	10	41.6 ± 5.9
204[c]	8	70.8 ± 14.4
200[c]	9	71.3 ± 18.4

[a] Mean ± standard deviation.
[b] Wild type.
[c] Putative diploid.

The following criteria were used in establishing the authenticity of diploids. (i) A diploid should exhibit complementation between diverse auxotrophic markers. Our isolates grew on minimal medium; i.e., they were phenotypically wild type. The mutated genes were recessive in the diploid state. (ii) A diploid should be self-fertile. It is postulated that a diploid cell would have both α and **a** sex factors, resulting in self-fertility, and that the sex factors would segregate in formation of basidiospores, resulting in loss of the self-fertile trait. Only colonies capable of basidiospore formation on mating medium were selected as putative diploids. After many serial (vegetative) transfers on minimal medium, this characteristic was retained. Since self-fertility persisted through streaking, we conclude that it was a characteristic of single cells. (iii) A diploid should be heterozygous. This was ascertained by demonstrating that offspring of a self-fertile colony comprised all the phenotypes possible from recombination of nutritional markers of the parental haploids. In addition, the self-fertile trait is lost upon sporulation, as expected. The lone self-fertile colony may have been due to nonseparation of basidiospores (3). It is not clear why many offspring failed to grow on defined medium. The Nia^- and Pan^- strains were isolated after rigorous mutagenic treatment of the cells (4), and the treatment may have resulted in suppressors or secondary mutations. An attempt is currently being made to isolate cleaner auxotrophs. The important fact is that all four phenotypes expected were observed among the progeny. Our previous experience with heterozygous diploids (11) concerns two acapsular mutants. Analysis of a cross between these acapsular haploid strains has shown the markers to be linked. A small proportion of encapsulated progeny from the cross were self-fertile, suggesting complementation in heterozygous diploids in accord with a hypothesis by K. J. Kwon-Chung (personal communication). This hypothesis is supported by our finding that such putative diploids produce acapsular progeny upon sporulation (11). (iv) A diploid should have only one nucleus per cell. This was confirmed by mithramycin staining. (v) A diploid cell should contain approximately twice as much DNA as a normal (haploid) cell; this was shown. Our isolates are pseudowild, self-fertile, heterozygous, uninucleate, and double in DNA content. It is concluded that we have isolated diploid strains of *C. neoformans*.

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