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Genetic Complementation in *Cryptococcus neoformans*

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A complementation test was devised for the fungus *Cryptococcus neoformans*. Complementation was signalled by the growth of prototrophic heterokaryons generated in crosses of the type aB × Ab, where a and b represent any two of the genetic markers *ilv1*, *cys1*, *cys2*, and *cys3*. The cloned complementing heterokaryons formed characteristic hyphal colonies that contained both hyphae and yeast cells. The heterokaryon-derived yeasts were of three kinds: parental haploids, recombinant haploids, and diploids.

Genetic studies of the pathogenic yeast *Cryptococcus neoformans* began with the discovery of its perfect state (*Filobasidiella neoformans*) by Kwon-Chung (1–3). Yeast cells of opposite mating type (**a** and α) conjugated when the clones were combined in culture. Dikaryotic hyphae with clamp connections grew from the zygotes thus produced, and this growth was followed by the formation of basidia at the hyphal tips. Karyogamy and meiosis completed the sexual phase, yielding haploid basidiospores that were arranged in chains (typically four chains per basidium), which resulted from repeated mitotic division of postmeiotic nuclei. This system provided a segregational test for allelism based on meiotic segregation.

White and Jacobson (5) obtained diploid *C. neoformans* yeast clones by selecting for prototrophs in suspensions of cells, predominantly basidiospores, derived from a cross in which the diplophase was expected to be prototrophic because of complementation of nutritional markers. That study may provide the basis for a functional test for allelism (a complementation test) based on the use of diploid-yeast-phase cells.

In the present study, we developed a method whereby complementation was signaled by the growth of heterokaryotic hyphae. The method was verified in a model system in which we used an *ilv* marker and three phenotypically similar *cys* markers. It was demonstrated that heterokaryotic hyphae could be cloned and that these clones gave rise to yeast cells, the majority of which were of a parental nuclear genotype. A minority of yeast cells, isolated from heterokaryons by selection, were of two kinds, recombinant haploid yeasts and diploid yeasts.

MATERIALS AND METHODS

Strains. The prototrophic 5-fluorocytosine (5-FC)-susceptible strains B-3501 and B-3502 (mating types α and **a**, respectively) have been described previously (2). The genetic markers *cys1*, *cys2*, *cys3*, and *ilv1* were induced by UV light. The genetic marker *fcy1* originated spontaneously.

Media. The undefined complete growth medium (YEPD) containing 10 g of yeast extract (Difco Laboratories, Detroit, Mich.), 20 g of peptone (Difco), 20 g of D-glucose, and 20 g of agar (Difco) per liter of distilled water. The defined minimal growth medium (MIN) contained 6.7 g of yeast nitrogen base without amino acids (Difco), 5 g of D-glucose, and 20 g of agar (Difco) per liter of distilled water. L-Cysteine, L-isoleucine, and L-valine were added to MIN as appropriate; each was added to a final concentration of 40 mg/liter. Resistance to 5-FC was determined on MIN supplemented with L-cysteine (40 mg/liter) and 5-FC (50 mg/liter); filter-sterilized 5-FC (Sigma Chemical Co., St. Louis, Mo.) was added to autoclaved MIN plus L-cysteine. The crossing medium contained 50 ml of V8 juice, 0.5 g of KH_2POH_4, and 40 g of agar (Difco) per liter of distilled water. The pH of the crossing medium was adjusted to 7.0 before being autoclaved.

Genetic analysis. Haploid parent strains and self-fertile diploid strains were each grown as patches on YEPD agar at room temperature for 2 days. Culture samples of the former were mixed on crossing medium, and samples of the latter strains, after 2-day growth, were individually spread on crossing medium. After the incubation of paired haploids or self-fertile diploids on crossing medium for 5 to 14 days, samples of basidiospores were obtained by scraping the agar surface in areas that were seen to be rich in hyphae; in situ microscopic examination was used to choose areas that were rich in hyphae and basidiospores. The sample of cells, predominantly basidiospores but typically containing hyphal fragments and blastospores, was spread along one edge of a slab of YEPD agar mounted on a microscope slide. On this slab, random basidiospores were isolated with a micromanipulator (Lawrence Precision Machine, Hayward, Calif.). Care was taken to isolate only small basidiospores having characteristic morphology. The isolated basidiospores were incubated at 30°C for 3 days; 62% of the isolated basidiospores grew to form colonies. The basidiospore clones were transferred to YEPD agar for the determination of phenotype. We replica plated 2-day YEPD cultures on MIN, MIN plus L-cysteine, or MIN plus L-cysteine plus 5-FC to score each culture for the requirement for L-cysteine and resistance to 5-FC. All basidiospore clones grew on MIN plus L-cysteine. The phenotypes (the requirement of the clones for L-cysteine to grow and resistance to 5-FC) were unambiguous after incubation of the replicas for 2 days at 30°C. The mating types of basidiospore clones were determined by crosses to tester strains B-3501α and B-3502**a**.

Complementation test. Parent strains were grown as patches on YEPD agar at room temperature for 2 days. Samples of these cultures were mixed on crossing medium, and the mixture (with parallel samples of the individual

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TABLE 1. Segregational tests for allelism[a]

Cross	No. of spores that were:	
	Auxotrophic[b]	Prototrophic
cys1 CYS2 × *CYS1 cys2*	17	7
cys1 CYS3 × *CYS1 cys3*[c]	34	14
cys2 CYS3 × *CYS2 cys3*	12	5

[a] Parent strains were crossed, and basidiospores were isolated and characterized as detailed in Materials and Methods.
[b] Spores requiring cysteine.
[c] The results of this cross (XW89) are shown in summary form here and in detail in Table 4.

parent strains) was incubated overnight at 30°C. Samples of the confluent cultures were suspended in water, and samples containing ~10^5 cells were spread on MIN. These cultures were incubated at 30°C and examined daily for the presence of hyphal colonies. Cultures in which growth of these colonies occurred were scored as complementing; the hyphal nature of putatively hyphal colonies was confirmed by microscopic examination. Hyphal colonies were evident in complementing cultures after incubation for 3 to 6 days. Cultures in which these colonies were not seen were scored as noncomplementing; these cultures were discarded after incubation for 8 days.

Isolation of hyphal fragments. Samples from 4-day-old hyphal colonies were spread along one edge of a slab of MIN mounted on a microscope slide, and fragments containing one to three hyphal cells were isolated by micromanipulation.

RESULTS

Markers. Complementation was studied with four genetic markers: an *ilv* marker, which determined a requirement for isoleucine plus valine, and three independent *cys* markers, each of which determined a requirement for L-cysteine. It was supposed that the *ilv* marker would complement each *cys* marker in crosses of genotype *ilv CYS* × *ILV cys*, where *CYS* and *ILV* denote the wild-type alleles. However, it was not known whether the *cys* markers represented mutations in different genes and should therefore be expected to exhibit intergenic complementation (e.g., *cys1 CYS2* × *CYS1 cys2*) or whether they represented mutations in one gene.

The segregational test for allelism is based on phenotypic ratios determined in random samples of basidiospores isolated from appropriate crosses. In this study, the test indicated that the *cys* markers were not allelic (Table 1). Both parents in each of the crosses required cysteine for growth, but cysteine-independent recombinants were found among the haploid products of meiosis at a frequency close to that expected if the markers assorted independently. These results defined three genes (*CYS1*, *CYS2*, and *CYS3*) and their corresponding mutant alleles (*cys1*, *cys2*, and *cys3*). The *ilv* marker and the *cys* markers provided a useful model system for the detection of complementation because complementation was expected in heterokaryons or diploids derived from crosses (aB × Ab) that used any two of these markers.

Complementation. For complementation testing, auxotrophic haploid yeast strains were mixed, and the culture was then incubated to permit conjugation and outgrowth of hyphae. Preliminary evidence for complementation was provided by the observation that certain paired parents, but not the individual parents, formed derivatives that grew on MIN to form dry white (H) colonies which were readily distinguished from the glistening cream-colored (Y) colonies typical of encapsulated yeast phase colonies (Fig. 1). Microscopic examination revealed that typical H colonies contained abundant hyphae bearing clamp connections (Fig. 2), whereas typical Y colonies were composed of encapsulated yeast cells (not shown).

Complementation was detected in all crosses in which it was predicted from the genotypes of the parents (Table 2). In the two crosses in which a *cys* marker was homozygous, no complementation was detected. In addition, we could detect no complementation when potentially complementing strains with the genotypes α *cys1 CYS2* × α *CYS1 cys2* and **a** *cys1 CYS2* × **a** *CYS1 cys2* did not mate because they were identical in mating type (data not shown).

No evidence was obtained to support the possibility that conjugation led directly to complementing diploid yeasts. Prototrophic Y colonies were seen in some complementation tests, but in those experiments, similar colonies were also seen in parallel cultures of one or both parent strains; these Y colonies were assumed to result from reversion of a parental marker and were not studied.

As a test for prototrophy of the heterokaryons, MIN was used to grow clones from hyphal fragments isolated by micromanipulation. Altogether, 62 hyphal fragments were isolated from H colonies formed in the seven positive complementation tests listed in Table 2; 34 fragments (55%) grew to form colonies. These colonies were typical H colonies. At least one colony-forming hyphal fragment was obtained from each positive complementation test. These experiments confirmed the preliminary complementation results by showing that complementation was a property of isolated heterokaryons.

Heterokaryon instability. Microscopic examination of suspensions of H colonies grown from isolated hyphal fragments showed that these colonies contained both hyphae and yeast cells. The ratio of yeast cells to hyphal cells was not determined precisely; the ratio seemed to be age-dependent and strain-dependent.

It was supposed that yeast cells might originate from heterokaryons by at least four processes. The simple budding of yeast cells from the dikaryon would presumably have yielded haploid yeasts of the parental (nuclear) genotype. Morphologic evidence for budding from hyphae had already been provided (2). Meiosis, had it occurred during the growth of the heterokaryon clone, would have yielded haploid yeasts of parental and recombinant genotypes. The outcome of parasexual nuclear interaction in the heterokaryon was not readily predictable; either diploid yeasts or recombinant haploid yeasts could have resulted. Diploid yeasts might also have originated by apomixis or by postmeiotic fusion of haploid nuclei.

To determine the genotype of yeast cells derived from heterokaryons, cloned heterokaryons were suspended in water, and the suspensions were then spread on YEPD and MIN to permit the growth of yeast subclones recognized by their characteristic (Y) colony morphology. YEPD supported the growth of heterokaryons, all parental strains, and all predicted recombinants. MIN selected for growth of the heterokaryon and prototrophic yeast derivatives. The majority of yeasts derived from heterokaryons were auxotrophs; this was inferred from the higher counts (approximately 1,000-fold) of yeast colonies obtained on YEPD when the suspensions were spread in parallel on both media. Yeast colonies obtained from cultures on both media were purified for genetic analysis. Two crosses (XW89 and XW86) were studied in detail.

Parental yeasts from heterokaryons. Heterokaryon clones

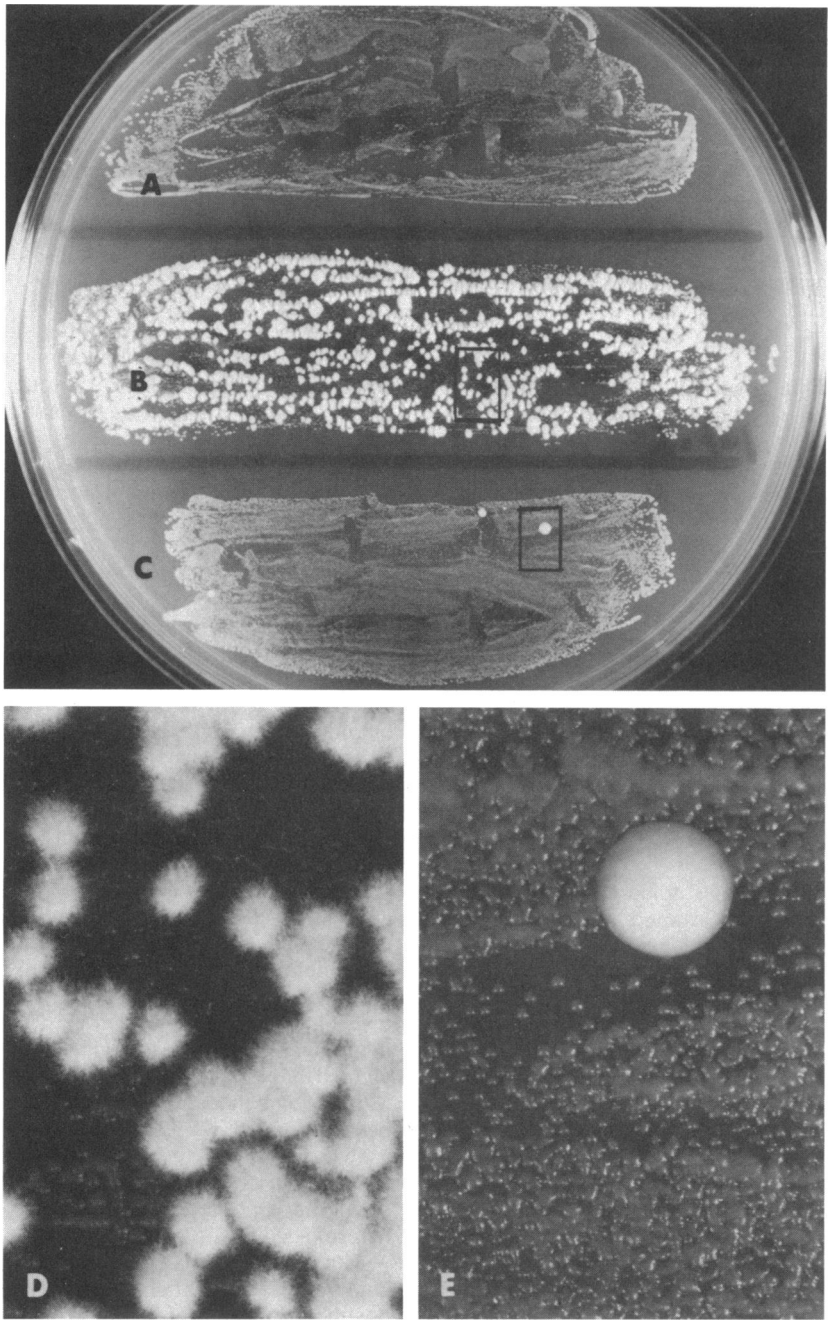

FIG. 1. Complementation in the cross XW89. Parent strains XW33c18 (**a** *CYS1 cys3 fcy1*) and M102 (α *cys1 CYS3 FCY1*) were grown for 2 days at room temperature as patches on YEPD. Samples of these cultures were mixed on the crossing medium. After overnight incubation at 30°C, samples of the mating mixture and parallel parental cultures were suspended in water, and samples that contained ~10^5 cells were spread on MIN. The cultures were photographed after incubation (30°C) for 4 days. Shown are parent strain XW33c18 (a) the XW33c18 × M102 mating mixture (b), and parent strain M102 (c). Also shown are an enlargement (d) of the indicated portion of b to show the characteristic H colony morphology and an enlargement (e) of the indicated portion of c to show a large Y colony (a prototrophic revertant) against a background of slow-growing parental Y colonies.

were grown on MIN. The yeasts that were isolated by spreading suspensions of cloned heterokaryons on YEPD were indistinguishable from the parent yeasts. Six independent heterokaryon clones were grown from the cross XW89 (**a** *CYS1 cys3 fcy1* × α *cys1 CYS3 FCY1*), and yeasts of one parental genotype (α *cys1 CYS3 FCY1*) were isolated from five clones. Both parental genotypes were represented among the yeasts isolated from the sixth heterokaryon clone.

In similar experiments, seven independent heterokaryon clones were grown from the cross XW86 (**a** *CYS2 cys3 fcy1* × α *cys2 CYS3 FCY1*). Yeasts of one parental genotype (α *cys2 CYS3 FCY1*) were obtained from six clones, and both parental genotypes were represented among the yeasts isolated from the other heterokaryon. These experiments demonstrated that yeasts which were indistinguishable from the parent strains (in terms of mating type, genotype at *CYS*

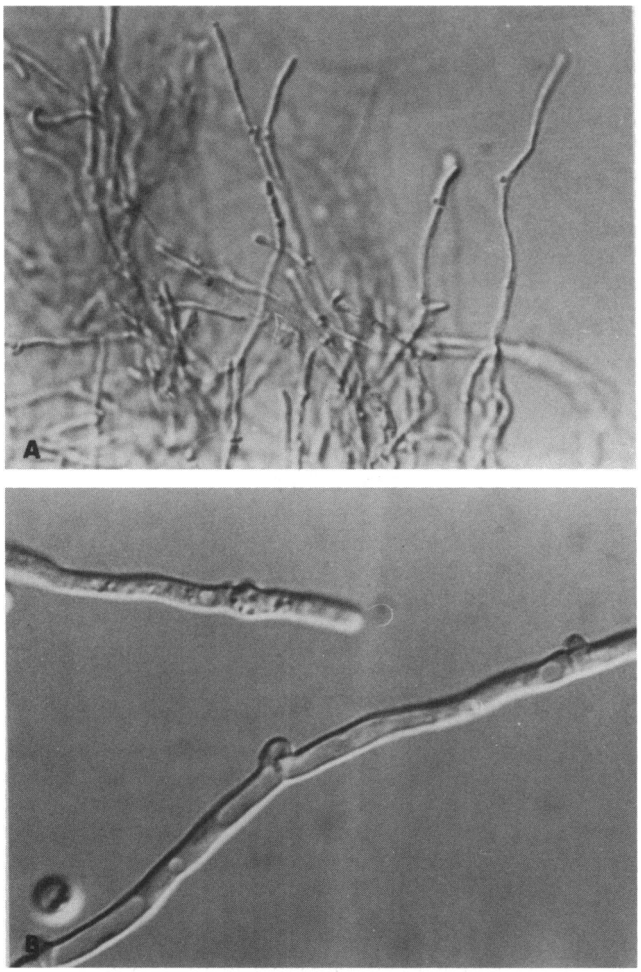

FIG. 2. Microscopic appearance of hyphae in an H colony (the cross XW89) grown from an isolated hyphal fragment. A sample of the colony was suspended in water for examination.

genes as determined by the complementation test, and 5-FC resistance phenotype) were readily obtained from heterokaryon clones and probably constituted the large majority of yeasts derived from heterokaryons (see above). It seems likely that these yeasts of parental genotypes originated from heterokaryons in a process which did not include karyogamy and meiosis.

In these experiments, we did not address the question of whether yeasts of both parental types were equally likely to arise from the heterokaryon. The observed poor recovery of one parental type (**a** *CYS1 CYS2 cys3 fcy1*), which was the genotype of the parent common to strains XW86 and XW89, may have been due to some unknown property of those nuclei that resulted in their inefficient inclusion in yeast cells. However, it is also possible that yeasts of both parental types were produced with equal probability, but that a secondary factor, such as a difference in growth rates on MIN after their origins from the heterokaryons, may have resulted in unequal recovery. It should be noted that these yeasts were produced by heterokaryons growing on MIN, so that differential selection against one parental type may have occurred before sampling. A more direct experiment, in which yeasts are recovered soon after they originate, is required to resolve this problem.

TABLE 2. Complementation in *C. neoformans* heterokaryons[a]

Marker of parent	Growth[b] after cross with:		
	a *cys1*	**a** *cys2*	**a** *cys3*
α *cys1*	−	+	+
α *cys2*	+	−	+
α *ilv1*	+	+	+

[a] Complementation was indicated by growth of hyphal colonies after samples of mating mixtures were spread on MIN and incubated for 5 days. Mating was verified in all crosses; observation of parallel cultures on V8 medium revealed hyphal elements and (after incubation for 7 to 14 dyas) basidia and basidiospores.

[b] +, Growth; −, no growth.

Recombinant yeasts from heterokaryons. Prototrophic yeast clones were selected from cloned heterokaryons derived from the cross XW89. For this selection, the colonies were isolated on MIN from suspensions of heterokaryon clones. Three clones of the five independent yeast clones isolated were found to be capable of mating. The other two clones were self-fertile (see below). The genotypes of the mating-competent clones were inferred from analyses of crosses to the wild-type (Table 3).

The genotype of prototroph XW89h1P might be accounted for by recombination (*cys1 CYS3* × *CYS1 cys3* → *CYS1 CYS3*) of the *cys* markers, with maintenance of the parental α *FCY1* genotype, but it might also be accounted for by the reversion of the *cys1* marker (**a** *cys1 CYS3 FCY1* → α *CYS1 CYS3 FCY1*), independent of recombination. The data do not permit a distinction between the possibilities.

The genotypes of two prototrophs (XW89h2P and XW89h3P) were most readily explained by meiotic recombination. Although the *CYS1 CYS3* genotype might have been explained by either reversion or recombination, reversion was rendered less likely by the finding that these prototrophs were recombinant (α *fcy1*) for other markers. Generalized (meiotic) recombination accounted for these genotypes, whereas reversion did not.

The foregoing experiments suggest that at least two of the three mating-competent prototrophs resulted from karyogamy and meiosis during the growth of the heterokaryon.

Diploid yeasts derived from heterokaryons. Two prototrophic yeast clones, isolated from XW89 heterokaryons (see above), were taken to be self-fertile because, in pure cultures on the crossing medium, they grew as hyphae that developed basidia and basidiospores. The strains clearly

TABLE 3. Genetic analysis of mating-competent prototrophic yeasts isolated from heterokaryons[a]

Prototroph	No. and phenotype[b] of basidiospores				Prototroph genotype[c]
	aR	α**S**	**a**S	α**R**	
XW89h1P	0	4	7	0	α *CYS1 CYS3 FCY1*
XW89h2P	5	2	5	2	α *CYS1 CYS3 fcy1*
XW89h3P	2	1	4	2	α *CYS1 CYS3 fcy1*

[a] Prototrophic yeast clones that were isolated from hyphal fragments (h1, h2, h3) that were derived from cross XW89 were crossed with wild-type strain B3502a (genotype **a** *CYS1 CYS3 FCY1*), and the phenotypes of random basidiospores were determined.

[b] **a** or α, Mating type; R, 5-FC resistance; S, 5-FC susceptibility. No cysteine-requiring basidiospores were found.

[c] Inferred from the analysis.

TABLE 4. Genetic analysis of cross XW89 and derivative self-fertile prototrophs[a]

Mating types	Basidiospore genotype[a]			No. of basidiospores isolated[b]		
	CYS1	*CYS3*	*5-FC*	XW89	XW89h5P	XW89h4P
a	−	−	F	5	3	0
a	−	−	f	1	1	0
α	−	−	F	3	6	0
α	−	−	f	0	3	0
a	−	+	F	6	1	9
a	−	+	f	4	4	2
(P) α	−	+	F	3	0	2
α	−	+	f	1	3	2
a	+	−	F	2	3	0
(P) **a**	+	−	f	1	1	0
α	+	−	F	1	2	0
α	+	−	f	5	0	0
a	+	+	F	3	1	6
a	+	+	f	4	3	7
α	+	+	F	4	3	8
α	+	+	f	3	1	4

[a] Shown are the 16 genotypes derivable from all possible postmeiotic combinations of the markers of cross XW89. Genotypes: Mating type (**a** or α), genotype at *CYS1* (−, *cys1*; +, *CYS1*), genotype at CYS3 (−, *cys3*; +, for *CYS3*), resistance to 5-FC (F, *FCY1*; f, *fcy1* [the resistance allele]). P, parental.

[b] Genotypes: XW89, **a** *CYS1 cys3 fcy1*, × α *cys1 CYS3 FCY1* (known); XW89h5P, $\frac{\mathbf{a}\ \underline{CYS1}\ \underline{cys3}\ \underline{fcy1}}{\alpha\ cys1\ \ CYS3\ FCY1}$ (inferred); XW89h4P, $\frac{\mathbf{a}\ \underline{CYS1}\ \underline{CYS3}\ \underline{fcy1}}{\alpha\ cys1\ \ CYS3\ FCY1}$ (inferred). XW89h5P and XW89h4P were derived from cross XW89.

differed from haploid strains, such as the parent strains for cross XW89, which grew individually as yeasts on crossing medium but underwent conjugation and the remainder of the sexual cycle when grown in mixed cultures. It seemed likely that the self-fertile prototrophs were diploid.

The results of genetic analysis of the cross XW89 and the two self-fertile prototrophs derived from it are shown in Table 4. Cross XW89 served as a basis for comparison because the genotypes of the parent strains were known and Mendelian segregation theory predicted a 1:1 ratio for all allele pairs among the haploid products of meiosis (**a**:α, *cys1*:*CYS1*, *cys3*:*CYS3*, and *fcy1*:*FCY1*) among the products of meiosis. Results consistent with Mendelian segregation were found for all marked genes of cross XW89; the largest observed deviation from expectation (18 *cys3*:28 *CYS3*) was not sufficient to invalidate Mendelian segregation ($\chi^2 = 2.2$; $0.2 > P > 0.1$).

Analysis of self-fertile strain XW89h5P indicated that it underwent Mendelian segregation for the four markers of cross XW89. The largest deviation from expectation (21 *cys1*:14 *CYS1*) did not invalidate Mendelian segregation ($\chi^2 = 1.4$; $0.3 > P < 0.2$). These results were consistent with the hypothesis that strain XW89h5P was diploid. The inferred genotype of this strain (Table 4) accounted for its prototrophic phenotype by complementation.

Analysis of self-fertile strain XW89h4P indicated that it underwent Mendelian segregation for three markers (**a**:α, *cys1*:*CYS1*, and *fcy1*:*FCY1*) of the four present in cross XW89. The largest deviation from expectation among these three markers (25 *cys1*:15 *CYS1*) was not sufficient to invalidate Mendelian segregation ($\chi^2 = 2.5$; $0.2 > P > 0.1$). Segregation of the *cys3* marker was not observed. That result could not reasonably be explained by sampling error, and the systematic absence of the marker was taken to mean that strain XW89h4P was a diploid which was homozygous *CYS3*/*CYS3* (genotype shown in Table 4).

Linkage relationships can be deduced from the data shown in Table 4 by computing the ratio of parental to nonparental genotypes for each of the pairwise combinations of markers and by comparing the observed ratios with the ratio of one parental to one nonparental genotype predicted by independent assortment. Linkage would have been indicated by a significant excess of parental genotypes. No convincing evidence of linkage was obtained for any of the six pairwise combinations of markers in cross XW89, although the data were consistent with a weak linkage of *cys1* to *fcy1* (30 parental to 16 nonparental genotype; $\chi^2 = 4.3$; $0.05 > P > 0.02$). A similar analysis of the results obtained for strains XW89h4P and XW89h5P indicated independent assortment of all markers.

Thus, with the use of these genetic analyses, we established that the self-fertile strains XW89h4P and XW89h5P were diploid.

DISCUSSION

The present study was undertaken to devise a simple test for complementation that is suitable for genetic studies with *C. neoformans*. Although in principle a complementation test might be based on heterokaryons or on diploid yeasts in this species, heterokaryons are immediately derived from the zygote in the life cycle and were chosen for that reason. It was readily shown that complementation was signaled by the growth of prototrophic heterokaryons in certain crosses in which complementation was expected because the markers were independently determined to be nonallelic. Growth of prototrophic heterokaryons was not observed in other crosses in which the markers were homoallelic. The method was unambiguous because complementing heterokaryon clones were readily distinguished from adventitious prototrophic yeast clones.

The present complementation test was restricted to phenotypes expressed by the heterokaryon. Isolation of diploid yeasts from the heterokaryon should permit complementation tests for other phenotypes, such as capsule formation, that are not expressed by the heterokaryon. Still and Jacobson took this approach when they isolated encapsulated self-fertile yeasts from crosses in which both parents were acapsular mutants (4).

The finding that complementing heterokaryons could be cloned from yeast-free hyphal fragments permitted a study of heterokaryon stability. It was readily shown that heterokaryons gave rise to yeasts during the growth of the clone, and it was also shown that a large majority of these yeasts were auxotrophs that were phenotypically similar to the parents. Such a result suggested that most heterokaryon-derived yeasts originated in a vegetative process, such as budding, which yields haploid yeasts corresponding to the two parental genotypes. Strong support for a vegetative origin of yeasts from heterokaryons was obtained by showing that all yeasts (15 in all) in a sample were indistinguishable from one or the other parent strain in genotype. In the example of cross XW89, there were 2 parental genotypes among 16 genotypes derivable from random assortment of the four markers present (Table 4). Then, the probability of finding only parental genotypes in the sample examined (seven heterokaryon-derived yeasts) was low (0.125^7) if all yeasts derived from heterokaryons resulted from meiosis. Our results were not consistent with the hypothesis that meiosis is the general origin for heterokaryon-derived yeasts, but instead indicate that a great majority of such yeasts originate in a vegetative process.

The demonstrated presence of yeasts in heterokaryon

clones is relevant to mapping experiments. The presence of yeast cells of parental genotypes in samples of basidiospores obtained from heterokaryons would lead to erroneous linkage assignments unless care is taken to eliminate the yeast cells from the analysis.

The finding that the majority of heterokaryon-derived yeasts were of a parental genotype for nuclear markers may be of use in studies of cytoplasmic inheritance in *C. neoformans*. It seems likely, subject to verification, that cytoplasmic determinants borne by the parent yeast cells would have been mixed at random in the cytoplasm of the zygote and the resultant heterokaryon. Then, recovery of the parental nuclei in heterokaryon-derived yeast cells would permit distinction between nuclear and cytoplasmic inheritance.

The experimental scheme used in the present study was designed to select for prototrophic heterokaryon-derived yeasts. These might originate in several ways (see above). Among the five independent prototrophic yeasts that were characterized, two were most readily explained as results of meiosis because they were haploids that were recombinant for markers (mating type and 5-FC resistance) that were presumably independent of the selection for prototrophy. Another prototrophic haploid yeast may have resulted from meiotic recombination, although the possibility could not be excluded that it resulted from reversion of a heterokaryon-derived parental yeast to prototrophy.

Such considerations suggest that meiosis occurred during growth of the heterokaryon on MIN. Whether basidiospores were produced is not known. It seems likely that meiosis was infrequent in the present experiments because a high frequency of meiosis would have been signaled by a high frequency of prototrophic recombinants, and this was not observed. It should be noted, however, that other (unpublished) experiments indicate that heterokaryons generated in other crosses undergo meiosis at high frequency on MIN.

The isolation of diploid yeasts in the present study confirmed the previous isolations of diploid yeasts by White and Jacobson (5). However, definitive conclusions concerning how diploid yeasts originated from heterokaryons cannot be drawn from either study. It would be interesting to know whether diploid yeasts result from premeiotic or postmeiotic karoyogamy. The diploid XW89h5P may be explained as the direct result of premeiotic karyogamy followed by inclusion of the diploid nucleus in a yeast cell. However, this diploid strain may also be explained by the karyogamy of certain postmeiotic nuclei. The other diploid, XW89h4P, was homozygous *CYS3/CYC3*; that genotype is most readily explained by postmeiotic karyogamy. Further studies of a larger sample of well-marked diploids are required before firm conclusions can be drawn. The present study demonstrated that the heterokaryon stage of the *C. neoformans* life cycle was accessible to genetic study, and this study may provide a useful tool for studies of complementation, cytoplasmic inheritance, and diploidy.

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