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Segregation of 5-Fluorocytosine-Resistant Variants by *Candida albicans*

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Spontaneous production of 5-fluorocytosine-resistant variants by three *Candida albicans* isolates is due to segregation from a preexisting heterozygous state.

The fungistatic agent 5-fluorocytosine (5-FC) has repeatedly proven effective in the treatment of infections due to the pathogenic yeast *Candida albicans* (2, 3, 7). This agent is relatively nontoxic to humans and is a desirable antifungal agent for that reason. However, resistant strains occur at significant frequency (1, 8, 14) and limit the clinical usefulness of 5-FC. Although the mechanism of action of 5-FC on *C. albicans* has been studied extensively (4, 10, 12, 13), the genetic basis of resistance has not. Five genes determine 5-FC resistance in another yeast, *Saccharomyces cerevisiae* (6, 9).

Recent biochemical and genetic studies indicate that typical *C. albicans* isolates are diploid (11, 15, 16). The genetic studies (15, 16) showed, by induced mitotic segregation, that some clinical isolates are heterozygous for auxotrophic markers. The fact that 5-FC-resistant strains are common suggested to us that some or all *C. albicans* isolates might be heterozygous for 5-FC resistance and consequently capable of giving rise to resistant variants at high frequency by segregation. In the present report we show that some isolates give rise to resistant variants much more frequently than do other isolates and that three isolates of the former class are heterozygous for 5-FC resistance.

Seventeen clinical isolates, previously described (15), were studied. All were sensitive to 5-FC: growth on a defined minimal agar medium (MIN [5]) was inhibited by 5-FC at a clinically significant concentration (50 μg/ml). In a standard experiment, approximately 10^5 cells (from culture on MIN agar) were spread on MFC50 agar (MIN plus 5-FC at 50 μg/ml), and the resultant cultures were examined for growth after incubation at 37°C. Five isolates, which we have designated type C, were strongly but incompletely inhibited by 5-FC; the majority of cells spread on MFC50 gave rise to microcolonies, and a minority of cells gave rise to larger colonies (Fig. 1). Clones were isolated (on MIN agar) from twenty of the larger colonies produced by each type C isolate. All of the clones grew rapidly on MFC50 agar and produced uniformly large colonies; these results demonstrated that the larger colonies were composed of resistant variants. It is likely that resistant variants arose during growth on MIN before exposure to 5-FC and also during slow growth in the presence of 5-FC. The time course of colony formation on MFC50 agar by a type C isolate, MEN, is shown in Fig. 2. The remaining isolates, which we have designated type D, did not grow discernibly on MFC50 agar (Fig. 1f) and (with one exception) did not give rise to resistant variants, in duplicate experiments. An exceptional type D isolate (FC18) gave rise to a single resistant variant when approximately 2×10^5 cells were tested.

A segregational origin for resistant variants implies that a mixed (sectored) colony composed of susceptible and resistant cells will result on MIN agar if both segregants are viable and that the segregants are homozygous and thus genetically different from the heterozygous parent. The alternative origin, mutation, may also result in a sectored colony; however, in that case cells in the susceptible sector will not differ genetically from the parent. Sectored colonies were obtained from the type C isolate MEN by irradiating cells with ultraviolet light to stimulate sectored colony formation (16). Cells (100 to 250) were spread on each of a series of MIN agar plates and irradiated with a dose (200 ergs/mm^2) which did not result in detectable killing. The colonies formed were replica plated on MFC50 agar and examined for sectored colonies (i.e., half resistant to half sensitive) 1 day later. Six sectored colonies were found among 913 colonies on the irradiated plates, and no sectored colonies were found among 3,691 colonies on the unirradiated controls. A clone was isolated from the susceptible sector of each of four sectored colonies. The clones had the property expected of

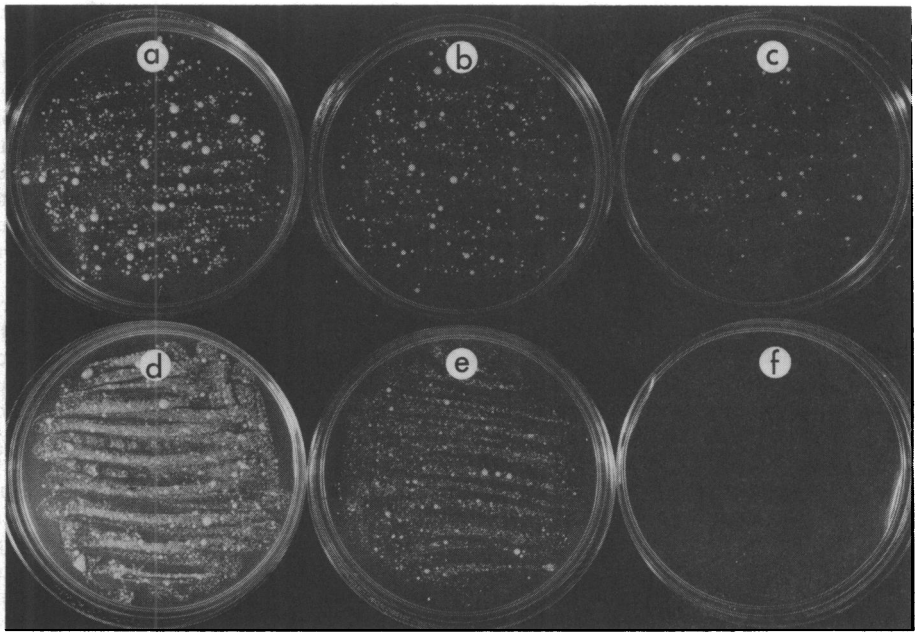

FIG. 1. *Type C isolates produce resistant variants, whereas type D isolates do not. Pure clones were grown on MIN agar for 24 or 48 h, suspended in saline (0.9% NaCl), and spread (ca. 10^5 cells) on MIN supplemented with filter-sterilized 5-FC (50 μg/ml final concentration). The cultures photographed after incubation for 5 days are MEN (a), MC56 (b), UT (c), M-N (d), and V1 (e), and the culture photographed after 7 days is FC18 (f). The result shown for isolate FC18 is typical of the type D isolates (FC18, MS24, FS56, MG30, FC15, MS42, FCU, BG, MA75, PL, S26, MS70). The temperature of incubation for all experiments was 37°C.*

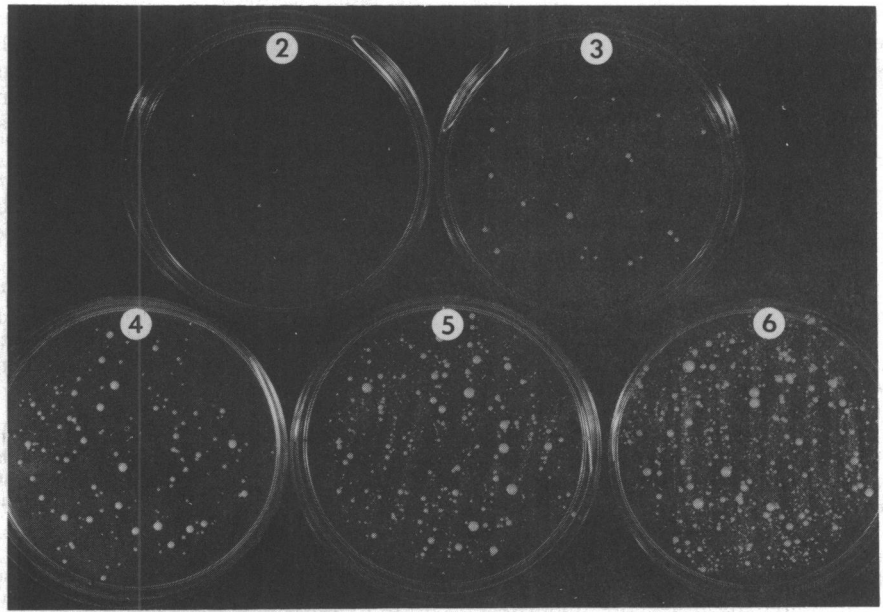

FIG. 2. *Progressive background growth and colony formation by isolate MEN. A clone (grown for 49 h on MIN agar) was suspended in saline, and 0.1 ml (1.6×10^5 cells by viable count) was spread on each of five MIN plus 5-FC (50 μg/ml) plates. The seeded plates were incubated at 37°C for the times indicated in the figure (2 to 6 days), refrigerated, and photographed.*

the homozygous segregant: they failed to give rise to resistant variants and differed significantly from the parent strain in that respect. When tested by the method described in Fig. 1, each gave a result identical to that shown in Fig. 1f. These results indicate origin of resistant variants by segregation, rather than by mutation.

Isolate MEN was partially resistant to 5-FC, as shown by its slow growth on MFC50 agar (discussed above). In contrast, the clones from the four susceptible sectors seemed to be susceptible rather than partially resistant in that they did not grow discernibly on MFC50 agar. Support for the apparent difference in resistance was obtained by determining the minimum 5-FC concentration which prevented growth when added to liquid MIN (minimum inhibitory concentration (MIC); Table 1). It was found that isolate MEN was intermediate in resistance (MIC = 10 or 50 (μg/ml) in different experiments) between susceptible segregants (MIC = 1 μg/ml) and resistant segregants (MIC > 500 μg/ml). These results, taken with those obtained with MFC50 agar, indicated that resistance was partially expressed by the heterozygote.

We have also obtained a sectored colony from each of two other type C isolates (MC56, UT), and we have found that those colonies are also the result of segregation. Thus, the characteristic behavior of at least three of the five type C isolates is due to heterozygosity for 5-FC resistance. The type D isolates (with one exception) resemble the homozygous sensitive segregants of MEN in their failure to produce resistant variants under standard conditions and their susceptibility, as measured by MIC and growth on MFC50 agar. We take those properties to indicate that type D isolates (with one exception) are homozygous sensitive at the 5-FC resistance gene which is heterozygous in MEN. The exceptional type D isolate (FC18) was slightly resistant (Table 1) and was the only type D isolate to yield a resistant variant (cited above); the genetic basis of its behavior is unknown.

TABLE 1. *MICs of 5-FC for clinical isolates and segregants*[a]

Strain	MIC (μg/ml)
MEN	10, 50[b]
S1[c]	1
R1[c]	>500
S2	1
R2	>500
Type D[d]	1
FC18	10

[a] Approximately 2,000 cells were introduced into (1 ml) liquid MIN supplemented with filter-sterilized 5-FC (0, 0.1, 1.0, 10, 50, and 500 μg/ml), and growth was assessed visually after 4 days of standing incubation. MIC is the lowest 5-FC concentration which prevented visible growth.

[b] Isolate MEN gave variable values for MIC, probably due to clonal variation in numbers of resistant variants in the inocula.

[c] S1 and R1 are pure clones from the sensitive and resistant sectors, respectively, of a sectored colony from MEN, S2 and R2 are pure clones from the sensitive and resistant sectors of another sectored colony from MEN.

[d] All type D isolates, except FC18.

It is possible that the frequent occurrence of 5-FC-resistant *C. albicans* isolates noted by several workers is due to selection for homozygous resistant segregants produced by heterozygotes which are themselves partially resistant and therefore selectable by 5-FC, particularly by low 5-FC concentrations. Isolates which yield a high frequency of resistant variants were common (5 of 17) among the isolates examined in the present study; examination of a larger sample will provide a better estimate of their frequency in the general *C. albicans* population. Other workers (1, 8, 10, 14) have briefly mentioned results which suggest to us that some of their isolates were heterozygous for resistance. Zimmerman and Kern (17) have considered the possible occurrence of heterozygosity in a general discussion of drug resistance in pathogenic fungi.

The use of 5-FC in treating infections due to strains heterozygous for resistance seems inadvisable because of the possibility of selection for the heterozygote and the strong likelihood of rapid overgrowth of resistant segregants. The simple screening procedure described in this paper (Fig. 1) may provide a routine presumptive method for detecting heterozygotes.

Conventional genetic analysis of *Candida albicans* is precluded by the apparent absence of a sexual phase. We have recently provided a limited alternative method of genetic analysis (15, 16). We can now, because 5-FC resistance is recessive and selectable, select for the products of meiosis, partial meiosis, or haploidization. That selective ability may be useful in discovering or devising a genetic system.

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LITERATURE CITED

1. **Auger, P., C. Dumas, and J. Joly.** 1979. A study of 666 strains of *Candida albicans*; correlation between serotype and susceptibility of 5-fluorocytosine. J. Infect. Dis. **139**:590–594.
2. **Bennett, J. E.** 1974. Chemotherapy of systemic mycoses. N. Engl. J. Med. **289**:30–32.
3. **Bennett, J. E.** 1974. Chemotherapy of systemic mycoses. N. Engl. J. Med. **290**:320–324.

4. **Diasio, R. B., J. E. Bennett, and C. E. Meyers.** 1978. Mode of action of 5-fluorocytosine. Biochem. Pharmacol. **27**:703–707.
5. **Fink, G. R.** 1970. The biochemical genetics of yeast. Methods Enzymol. **17A**:59–78.
6. **Grenson, M.** 1969. The utilization of exogenous pyrimidines and the recycling of uridine-5′-phosphate derivatives in *Sacchacomyces cerevisiae*, as studied by means of mutants affected in pyrimidine uptake and metabolism. Eur. J. Biochem. **11**:249–260.
7. **Hoeprich, P. D.** 1978. Chemotherapy of systemic fungal diseases. Annu. Rev. Pharmacol. Toxicol. **18**:205–231.
8. **Holt, R. J.** 1978. Clinical problems with 5-fluorocytosine. Mykosen **21**:363–369.
9. **Jund, R., and F. Lacroute.** 1970. Genetic and physiological aspects of resistance to 5-fluoropyrimidines in *Saccharomyces cerevisiae*. J. Bacteriol. **102**:607–615.
10. **Montplaisir, S., E. Drouchet, and L. Mercier-Soucy.** 1975. Sensitivity and resistance of pathogenic yeasts to 5-fluoropyrimidines. II. Mechanisms of resistance to 5-fluorocytosine and 5-fluorouracil. Ann. Microbiol. **126B**:41–49.
11. **Olaiya, A. F., and S. J. Sogin.** 1979. Ploidy determination of *Candida albicans*. J. Bacteriol. **140**:1043–1049.
12. **Polak, A.** 1977. 5-Fluorocytosine—current status with special references to mode of action and drug resistance. Contrib. Microbiol. Immunol. **4**:158–167.
13. **Polak, A., and W. H. Wain.** 1977. The influence of 5-fluorocytosine on nucleic acid synthesis in *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigata*. Chemotherapy **23**:243–259.
14. **Schönbeck, J., and S. Ansehn.** 1973. 5-Fluorocytosine resistance in *Candida* spp. and *Torulopsis glabrata*. Sabouraudia **11**:10–20.
15. **Whelan, W. L., and P. T. Magee.** 1981. Natural heterozygosity in *Candida albicans*. J. Bacteriol. **145**:896–903.
16. **Whelan, W. L., R. M. Partridge, and P. T. Magee.** 1980. Heterozygosity and segregation in *Candida albicans*. Mol. Gen. Genet. **180**:107–113.
17. **Zimmermann, F. K., and R. Kern.** 1978. Genetic aspects of medical mycology. Mykosen **21**(Suppl. 1):24–26.