Protoplast Fusion Hybrids of *Candida albicans* Sterol Mutants Differing in Nystatin Resistance

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Intraspecific protoplast fusion, induced by polyethylene glycol and Ca^{2+} , was carried out in various pairings between auxotrophic nystatin-sensitive, ergosterol-producing strains of *Candida albicans* and their nystatin-resistant, ergosterol-less mutants of different origins. Nutritionally-complemented stable heterozygous diploid hybrids were obtained, which proved to be sensitive, semi-resistant or resistant to nystatin as a consequence of complementation or non-complementation for ergosterol biosynthesis. Dominant mutation control of the resistance was not found.

INTRODUCTION

In *Candida albicans* with altered plasma membrane sterol and lipid compositions, phenotypic and genotypic resistance to polyene antibiotics have been studied extensively (for references, see Hamilton-Miller, 1973*b*; Gale *et al.*, 1978; Pierce *et al.*, 1978; Kerridge, 1980). However, investigations of the interactions between the genes responsible for the sensitivity and the resistance have not been possible so far, due to the lack of any sexual or parasexual cycles in this species.

Since the first attempt at the controlled protoplast fusion of *Geotrichum candidum* (Ferenczy *et al.*, 1974) and application of the most effective fusogenic agent, polyethylene glycol (PEG) (Kao & Michayluk, 1974) for fusion of fungal protoplasts (Ferenczy *et al.*, 1975; Anné & Peberdy, 1975), this method has also been used to study nutritional complementation in cases of intraspecific fusion of *Candida* species such as *C. tropicalis* (Fournier *et al.*, 1977; Vallin & Ferenczy, 1978), *C. utilis* (Delgado & Herrera, 1979) and *C. albicans* (Pesti *et al.*, 1979*a*). As a consequence, this simple but effective method for the high-frequency transfer of genetic material has led to advances in the genetic research of asexual fungi.

A number of genetic studies concerning the polyene antibiotic resistance of *Saccharomyces cerevisiae* and *Neurospora crassa* have been published, demonstrating that resistance to nystatin is due to both dominant and recessive gene mutations (Patel & Johnston, 1968; Ahmed & Woods, 1972; Molzahn & Woods, 1972; Bard, 1972; Karunakaran & Johnston, 1977; Grindle & Farrow, 1978).

In the present study, the possibility of protoplast fusion in *C. albicans* has been investigated not only for nutritional complementation but also for complementation relating to ergosterol biosynthesis in the fusion products, using various sterol mutants induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) (Pesti *et al.*, 1979*b*, 1981*b*).

METHODS

Strains and methods for their characterization. Various auxotrophic mutants were isolated after NTG treatment of a wild-type strain of *Candida albicans*. An adenine-requiring (*ade*), ergosterol-producing mutant,

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designated 33 *erg*⁺, and a similarly ergosterol-producing, methionine- and threonine-requiring (*met thr*) mutant, designated 35 *erg*⁺, were treated to obtain the nystatin-resistant sterol mutants (see Table 1). The 33 *erg*⁺ adenine-requiring mutant and its nystatin-resistant progenies formed pink colonies on medium containing 5 µg adenine ml⁻¹. All methods used in these experiments concerning the isolation and identification of these mutants, as well as the complete (YPG) and minimal (MM) media, culture conditions, determination of minimal inhibitory concentration (m.i.c.), cell volume, thin-layer chromatographic (t.l.c.) patterns of non-saponifiable sterol extracts of cells, and ultraviolet spectrophotometry of ergosterol content have been described previously (Pesti *et al.*, 1981a, b).

Protoplast formation. Overnight cultures of cells were grown with shaking in YPG liquid medium at 30 °C. The cells were harvested, washed with water by centrifugation and incubated in a solution containing 0.5% (v/v) 2-mercaptoethanol at 30 °C for 20 min. After repeated washing with 0.95 M-MgSO₄ solution, the cells were suspended at a density of 10⁷ cells ml⁻¹ in a solution containing 0.95 M-MgSO₄ or 1 M-mannitol and 2% (w/v) freeze-dried snail enzyme. After 60 min digestion, protoplasts were collected and washed by centrifugation (1000 g) in 0.35 M-CaCl₂, in order to prevent formation of crystals of CaSO₄ in the regeneration medium stabilized with 0.3 M-CaCl₂.

Protoplast fusion and regeneration. The methods described by Ferenczy & Maráz (1977) for *Saccharomyces cerevisiae* were used.

Analysis of fusion products. From the upper layers of three separate colonies of each hybrid (formed after 5 d incubation in stabilized MM medium following protoplast fusion), cells were isolated for investigation of their heterokaryotic state by conventional colony segregation analysis and by determining the number of nuclei per cell using acridine orange staining (treatment for 5 min with a solution containing 100 µg acridine orange ml⁻¹ in iso-osmotic solution and viewed under a Zeiss Fluoval u.v. microscope). Three cell lines of each hybrid, obtained from fusion colonies by single cell isolation, were studied with regard to their ploidy by determining the DNA content per cell and by the isolation of auxotrophic partners segregated spontaneously as described by Fournier *et al.* (1977), the strains being cultured for 5 d on YPG medium without haploidizing agents.

For determination of the DNA content of the auxotrophic mutants and hybrids, cells grown for 4 d on YPG medium were used after washing four times in distilled water. Experiments were carried out by the method of Bostock (1970) and the DNA content was estimated with diphenylamine (Giles & Myers, 1965), using chicken erythrocyte DNA as a standard. The numbers of cells were counted in a haemocytometer.

RESULTS AND DISCUSSION

General characteristics of protoplast formation, regeneration and fusion

Formation of protoplasts from both nystatin-sensitive and nystatin-resistant cells was completed (>95%) within 60 min. The fact that there was no apparent difference in the kinetics of protoplast formation indicated that there was no basic difference in cell wall composition between the two types of cells. In agreement with earlier findings (Kerridge *et al.*, 1976), when stationary phase cells of both types of mutants were used, only 45% of them were converted into protoplasts after 3 h incubation. This was a consequence of cell wall alterations during ageing, which also proved to play an important role in the phenotypic resistance to polyene antibiotics. After embedding of the protoplasts in stabilized YPG agar medium, the frequency of regeneration was between 20.5 and 58.1%. Protoplast fusion was carried out between a double (*met thr*) and a single (*ade*) mutant in each case (Table 1). The frequencies of fusion obtained with PEG (mol. wt 4000; 35%, w/v) in the presence of 0.1 M-CaCl₂ were between 1.3×10^{-4} and 9.1×10^{-4} . Neither back-mutation nor cross-feeding occurred on control plates. Colonies formed on MM medium following incubation for 5 d contained mixtures of red (*ade*) and white (*met thr*, as well as prototrophic) cells. Fast-growing, white and nutritionally complemented sectors could also sometimes be seen; on later analysis these proved to be diploids.

Formation of unstable heterokaryons and stable diploids

Staining with acridine orange revealed that less than 1% of cells isolated from fusion colonies contained two or three nuclei. At the same time, both auxotrophic partners were frequently reisolated from each hybrid, which showed their heterokaryotic state. This state, however, was temporary. After the first recultivation of cells originating from fusion colonies on MM medium, stable uninucleated prototrophic progenies were obtained. From three

Table 1. Characteristics of auxotrophic and nystatin-sensitive or -resistant strains and diploids of *Candida albicans*

Strain	Genotype	M.l.c. nystatin (U ml ⁻¹)	Ergosterol content* [μ g (mg dry wt) ⁻¹]	T.l.c. pattern†	DNA content (fg DNA per cell)	Cell volume (μ m ³)
33 <i>erg</i> ⁺	<i>ade</i>	25	34.6	A	65.0 $\pm$ 0.7	59.54 $\pm$ 6.13
<i>erg-16</i>	<i>ade</i>	400	ND	B	65.5 $\pm$ 3.1	145.32 $\pm$ 10.98
35 <i>erg</i> ⁺	<i>met thr</i>	25	34.5	A	67.5 $\pm$ 3.6	55.25 $\pm$ 8.89
<i>erg-20</i>	<i>met thr</i>	400	ND	B	66.5 $\pm$ 1.3	87.62 $\pm$ 9.23
<i>erg-27</i>	<i>met thr</i>	200	ND	B	65.0 $\pm$ 2.9	93.16 $\pm$ 8.76
<i>erg-40</i>	<i>met thr</i>	400	ND	E	66.0 $\pm$ 8.3	97.92 $\pm$ 10.11
Diploid IX	33 <i>erg</i> ⁺ + 35 <i>erg</i> ⁺	25	37.8	—	115.0 $\pm$ 3.6	85.17 $\pm$ 13.12
Diploid XI	<i>erg-16</i> + <i>erg-40</i>	25	21.3	—	118.0 $\pm$ 2.2	89.57 $\pm$ 9.65
Diploid V	<i>erg-16</i> + <i>erg-20</i>	400	ND	—	120.0 $\pm$ 1.7	128.82 $\pm$ 10.13
Diploid VII	<i>erg-16</i> + <i>erg-27</i>	400	ND	—	110.0 $\pm$ 4.7	129.86 $\pm$ 9.96

* The maximum deviation from the mean was less than 2.5 %. ND, Not detected.

† Definitions of the different patterns obtained by t.l.c. of non-saponifiable sterol extracts are given by Pesti *et al.* (1981*a*).

isolates of each hybrid, both auxotrophic partners could be reisolated after a 2 week cultivation on YPG medium. Haploidization with chloral hydrate was tested, but this agent was not used because the nystatin-resistant auxotrophs and hybrids proved to be twice as susceptible as the sensitive ones. This was probably the result of an increased uptake of chloral hydrate by the altered plasma membrane of the resistant cells. Quantitative DNA analysis confirmed the diploid state of the hybrids and showed that their DNA content was about twice that of the parental strains (Table 1). Isolation of stable diploids of *C. albicans* via protoplast fusion was consistent with earlier findings (cf. Peberdy, 1979; Ferenczy, 1980). Haploid recombinant progenies were not obtained spontaneously, in contrast to the results of Fournier *et al.* (1977) with *C. tropicalis*.

Sensitivity and resistance of diploids to nystatin

Various types of nystatin-resistant sterol mutants were isolated from the two auxotrophic strains, *33 erg*⁺ and *35 erg*⁺; these were divided into groups on the basis of the t.l.c. patterns of their non-saponifiable sterol extracts (Pesti *et al.*, 1981*a*). Diploids were created between two sensitive strains (diploid IX), between a sensitive and a resistant strain (diploid I), between two different types of resistant strains (diploid XI) and between resistant strains belonging to the same t.l.c. group (diploids V, VII, VIII). Sensitivity to nystatin, ergosterol content, sterol pattern, DNA content, and cell volume of the parental strains and the diploids are shown in Table 1.

Diploid IX, originating from two nystatin-sensitive, ergosterol-producing strains, proved to be sensitive to nystatin and gave a 10% higher ergosterol yield than its auxotrophic parents.

Diploid I was a fusion product of a nystatin-sensitive, ergosterol-producing strain (27/4 strain, requiring histidine, methionine and threonine) and a nystatin-resistant, ergosterol-less mutant (*erg-2* strain, requiring adenine). This diploid was also sensitive to nystatin and produced as much ergosterol as did diploid IX. Thus, the mutation responsible for the resistance could be considered to be recessive (Pesti *et al.*, 1979*a*).

Diploid XI was the fusion product of two nystatin-resistant, ergosterol-less strains belonging to different t.l.c. groups. It was also sensitive and produced ergosterol, but about 40% less than diploid IX. This result could be a consequence of the partial complementation for ergosterol synthesis. None of these mutants displayed dominant mutation for nystatin resistance.

Diploids V and VII were nystatin-resistant, and they did not produce any ergosterol. The parents of these diploids belonged to the same t.l.c. group. Two possible explanations were considered. First, one of the sterol mutants of each diploid had a dominant mutation which resulted in no ergosterol production. Second, mutations in both parents of the diploid occurred in the same locus concerned with ergosterol synthesis. To decide between these possibilities, each parent of heterozygous diploids V and VII was fused separately with the appropriate ergosterol-producing strains (*33 erg*⁺, *ade* and *35 erg*⁺, *met thr*). The hybrids of these fusions (*35 erg*⁺ + *erg-16*, *33 erg*⁺ + *erg-20* and *33 erg*⁺ + *erg-27*) were nystatin-sensitive (25 U ml⁻¹) and produced ergosterol. These results suggest that the non-complementation for ergosterol synthesis in diploids V and VII was a consequence of an allelic mutation and not a dominant one.

It is interesting that diploid VIII, *erg-16* + *erg-48*, whose parents were resistant to nystatin (400 U ml⁻¹) and seemed to belong to the same t.l.c. group, produced ergosterol. The resistance of this hybrid (50 U ml⁻¹) was much lower than that of its parents, but it was twice as high as that of the sensitive strains. The genes responsible for such a relatively small increase in resistance were referred to as 'modifier genes' by Ahmed & Woods (1972), who presumed that various processes controlling the biosynthesis and assembly of membrane components might be responsible for such a result.

The cell volumes of the nystatin-resistant *C. albicans* mutants were significantly higher than those of the sensitive ones (Table 1), in agreement with Hamilton-Miller's (1973*a*)

finding. The cell volumes of the nystatin-sensitive, ergosterol-producing diploids were considerably higher than those of the sensitive auxotrophs but lower than those of the resistant diploids. These results confirm the hypothesis that the lack of ergosterol in the sterol mutants of yeasts results in an increased cell volume.

The application of protoplast fusion to strains for which a parasexual cell cycle is not known could be promising for the study of the genetic background of resistance to fungicides. The loss or decrease of pathogenicity and alterations in certain morphological or biochemical characteristics in nystatin-resistant strains have been observed but not investigated further (Hamilton-Miller, 1973*b*; Merz & Sandford, 1979). On the other hand, isolation of heterozygous diploids of pathogenic species could provide information about the pathogenicity in different types of mutants.

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