A Complementation Analysis by Parasexual Recombination of *Candida albicans* Morphological Mutants

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Benomyl treatment (at 100 µg ml⁻¹) of *Candida albicans* 1001, and other strains derived from it, determined the appearance of morphological mutants similar to those derived from UV irradiation treatment. A permanent alteration in the morphogenesis of these mutant strains determined their inability to grow by budding, to form oval yeast cells or blastospores (Y⁻ phenotype) and their growth as long filamentous forms, mostly with the appearance of pseudomycelium, giving rise to rough colonies (R phenotype). In order to carry out a genetic complementation analysis, we isolated morphological mutants that carried other genetic markers (nutritional, conditional lethal) adequate for crosses by means of protoplast fusion. Wild-type hybrids of regular mononuclear oval yeast cells and smooth colonies were obtained by crossing pairs of complementing mutants, whereas hybrids from crosses of non-complementing mutants still retained their morphological alterations. Our results define two complementation groups, which represent two genes relevant for dimorphism, whose alteration interferes with the correct transition from blastospores to mycelium.

INTRODUCTION

The control of dimorphism is a key issue in research on the fungal pathogen *Candida albicans*. Different environmental conditions can determine a change in the budding pattern of growth of oval yeast cells, giving rise to filamentous forms of mycelial or pseudomycelial appearance. Conditions and inducers that determine mycelial growth are complex (Sevilla & Odds, 1986); a wide variety have been described and, not infrequently, data from different laboratories are contradictory (Odds, 1979). The extent to which dimorphism is controlled by specific genes whose expression would determine morphogenesis of either cellular form is controversial (Odds, 1985). As an alternative it has been suggested that metabolic regulation of permanently expressed genes could account for the formation of the major determinants of cell shape.

We believe that the characterization of mutants with permanent alterations in the functions that regulate dimorphism is one of the best approaches to clarify this problem. The feasibility of isolating several types of morphological mutants of *C. albicans* has been demonstrated. We have taken advantage of the correlation that exists in alterations of colony and cellular morphologies, to isolate, after UV-irradiation, morphological mutants of permanent pseudomycelial growth that can be genetically analysed by means of mitotic recombination (Pomés *et al.*, 1985). Our observations were followed by those of Slutsky *et al.* (1985), showing the spontaneous colonial switching that gave rise to colony phenotypes similar to that of our rough mutants, although no information was given about the cellular morphologies of these colonial variants. Hubbard *et al.* (1986) also described a mycelial mutant of *C. albicans*, and the feasibility of the genetic characterization of morphological mutants, by means of protoplast fusion has been demonstrated in our laboratory (Nombela *et al.*, 1987).

Abbreviations: Benomyl, methyl-benzimidazole-2-yl-carbamate; 5-FC, 5-fluorocytosine; phenotypes – Y⁻, filamentous form; R, rough colony.

In this communication we describe a set of morphological mutants, selected by growing *C. albicans* 1001 in the presence of methyl-benzimidazole-2-yl-carbamate (benomyl). A complementation analysis of these mutants was done by means of protoplast fusion.

METHODS

Organisms. *C. albicans* 1001, a wild-type strain originally isolated in a hospital, was obtained from the Spanish Type Culture Collection (Department of Microbiology, University of Valencia, Spain). *C. albicans* 29AW₂, a prototrophic heterozygous strain (*ADE/ade*), was spontaneously derived from the Ade⁻ auxotrophic strain *C. albicans* 29A (*ade/ade*), kindly supplied by W. L. Whelan. All the other strains used were derived from *C. albicans* 1001, by various treatments, as depicted in Fig. 1.

Media and culture conditions. YED agar, containing (w/v) 2% glucose, 1% yeast extract and 2% agar, was used as complete medium. Liquid cultures were grown in YED medium. Minimal medium (MM agar) contained (w/v) 0.67% Difco Yeast Nitrogen Base without amino acids, 0.5% glucose and 2% agar. MM agar was supplemented, when appropriate, with various amino acids or other growth factors at the concentrations specified by Sherman *et al.* (1979). 5-Fluorocytosine (5-FC), from Sigma, was also occasionally added to MM agar at a final concentration of 50 µg ml⁻¹. Except where indicated, cells were cultured at 28 °C. For long-term maintenance, strains were stored in sterile 50% (v/v) glycerol at -20 °C. For shorter periods, they were maintained on slants of YED agar at 4 °C.

Isolation of mutants. Auxotrophic and thermosensitive mutations were induced by UV irradiation at the doses indicated (Fig. 1). Cell suspensions were spread onto YED agar, plated and irradiated as described (Pomés *et al.*, 1985). The irradiated plates were incubated at 28 °C for 48 h and replicated onto MM agar to identify auxotrophic mutants, or onto YED agar and incubated at 37 °C to select for conditional lethal strains not growing at 37 °C. The corresponding mutant phenotypes were confirmed after single-colony isolation and the nutritional deficiency of each auxotroph was established by the method of Sherman *et al.* (1979), using MM agar supplemented with 4–5 amino acids.

Morphological mutants appeared when *C. albicans* 1001, or other strains derived from it, were plated in media containing benomyl. These mutants were identified on the basis of their rough-colony morphology and usually grew as filamentous forms, oval yeast cells being absent or present in a minor proportion in their cultures. The morphological alteration (Y⁻ phenotype) was similar to that of morphological mutants obtained by UV treatment (Pomés *et al.*, 1985). To obtain a set of morphological mutants carrying genetic markers useful for protoplast fusion, we treated auxotrophs and thermosensitive strains with benomyl (purchased from Kromxpek, Barcelona)

Fig. 1. Origin of *C. albicans* morphological mutants used in this investigation. *C. albicans* 1001, a wild-type strain, and the rest of the strains derived from it, were subjected to UV irradiation (the numbers in parentheses indicate the dose in µJ mm⁻²) or to benomyl treatment (B) at a concentration of 100 µg ml⁻¹. His⁻ and Arg⁻ mutants were selected on the basis of their nutritional deficiencies; Ts (thermosensitive) strains were unable to grow at 37 °C; Y⁻ strains grew as filaments mostly of pseudomycelial appearance and were screened on the basis of their 'rough' colonial morphology (see Table 1 for some frequencies).

as indicated in Fig. 1. Cells grown for 48 h were suspended in sterile distilled water, and appropriate dilutions, to give a final count of 2×10^2 to 2×10^3 c.f.u. per plate, were plated on YED agar supplemented with 100 µg benomyl ml⁻¹. The plates were incubated for 2–7 d at 28 °C and screened for morphological variants.

Preparation of protoplasts. Exponentially growing cells were harvested, washed with sterile distilled water and pretreated by gentle shaking at 30 °C, for 45 min at a concentration of 1×10^8 cells ml⁻¹, in a solution containing 10 mM-Tris/HCl pH 9, 5 mM-EDTA and 1% 2-mercaptoethanol. After pretreatment, the cells were washed twice with 1 M-sorbitol, resuspended in 1 M-sorbitol to a concentration of 1×10^8 cells ml⁻¹ and the snail enzyme complex Glusulase (DuPont Pharmaceuticals) was added in a proportion of 30 µl ml⁻¹. This suspension was incubated at 30 °C with gentle shaking for 1–2 h, the extent of protoplast formation being tested by checking lysis in 5% sodium dodecyl sulphate under a phase-contrast microscope. When at least 95% of the cells were converted into protoplasts they were centrifuged, washed five times with protoplast buffer (0.1 M-potassium phosphate pH 7.5 containing 1.0 M-sorbitol), and finally suspended by very gentle shaking in a small amount of protoplast buffer and stored at 4 °C.

Fusion and regeneration of protoplasts. The method described by Kakar & Magee (1982) was essentially followed. Approximately 5×10^8 protoplasts from each parental strain, suspended in the corresponding amount of protoplast buffer, were mixed and maintained at room temperature for 15 min. Protoplasts were centrifuged and the pellet gently suspended in 5 ml of fusion buffer (40%, w/v, polyethylene glycol and 10 mM-CaCl₂ in 10 mM-Tris/HCl pH 7.5). After 45–60 min at room temperature, protoplasts were again pelleted and resuspended in 5 ml of protoplast buffer.

For regeneration, 0.1 ml of the fusion mixture (containing $1-2 \times 10^7$ protoplasts) at appropriate dilutions was mixed with 10 ml of melted regeneration medium (YED or MM medium containing 1 M-sorbitol and 3% agar) at a temperature of 45–50 °C. These suspensions were poured onto Petri plates containing 15 ml of regeneration medium, and solidified at room temperature. After 2 h, the plates were transferred to either 28 °C or 37 °C. Colonies of regeneration products were visible after 4–5 d; they were characterized by transferring them to appropriate media (minimal or complete agar) as described below. Fusion frequencies were calculated as the proportion of colonies of regeneration products per pair of protoplasts plated in regeneration medium. Appropriate controls were run to show that protoplasts of each parental strain were unable to regenerate separately under the selective conditions (medium and temperature) used for regeneration of fusion products.

DNA determination. Stationary-phase cells were collected, washed and resuspended in distilled water. A sample was appropriately diluted to count the number of cells microscopically in a haemocytometer; at least 400 cells were counted, buds being counted as individual cells. Another sample was used for DNA determination by the method of Burton (1956) modified by Haber & Halvorson (1975), using the diphenylamine colour reaction with highly polymerized calf thymus DNA (Sigma) as standard.

RESULTS

Growth of *C. albicans* 1001, a wild-type strain, in YED agar containing 100 µg benomyl ml⁻¹ resulted in the appearance of rough-colony mutants (R) similar to those induced by UV treatment of the same strain (Pomés *et al.*, 1985). Although the colonial morphology of all R mutants was not absolutely uniform, they could be clearly distinguished from the smooth wild-type strains. Microscopic examination revealed that R mutants also displayed a permanent alteration in cell morphology, since they were unable to form the typical yeast cells (blastospores) but grew as long filamentous forms (Y⁻ phenotype). The appearance of these filaments was mostly pseudomycelial, although some of them might seem to display a hyphal morphology (Fig. 2*b, d, f*). Smooth segregants appeared spontaneously, either as sectors or whole colonies, when cells suspensions of R mutant strains were plated. UV irradiation not only induced the R phenotype but also increased the frequency of switching to a smooth one in various mutants (Table 1). It should be pointed out that the segregation of smooth colonies, observed in R mutants, did not mean reversion to a real wild-type phenotype. These smooth segregants never behaved as the original strain (*C. albicans* 1001) since they were rather unstable, with a high frequency of switching to the R phenotype, and their colonies contained a large number of aberrant cells.

The specificity of the benomyl treatment in leading to morphologically altered strains from *C. albicans* 1001 should be emphasized. Repeated screenings of benomyl-treated colonies did not reveal any other kind of mutant (auxotrophs or conditional lethal) whereas the indicated morphological alteration was always produced, at detectable frequency, when *C. albicans* 1001 or any other strain derived from it was treated with the fungicide. In order to gain an

Table 1. *Effect of UV irradiation and benomyl treatment on the segregation of morphological phenotypes from different strains of C. albicans*

Strain	Morphological phenotype	Treatment*	No. of colonies screened	Percentage of:†			Percentage survival
				R/S	S	R	
1001	Wild-type	—	12000	0	100	0	100
		Benomyl	3211	0	99.77	0.23	97
A14 (His ⁻)	Wild-type	—	2328	0	100	0	100
		UV (64)	12780	0.17	99.39	0.44	54.8
		Benomyl	5481	0	99.96	0.04	16.2
T22 (Ts)	Wild-type	—	10000	0	100	0	100
		UV (64)	1022	0	99	1	21.4
		Benomyl	12560	0	99.98	0.02	80.7
B17	R, Y ⁻	—	1434	0.55	1.32	98.13	100
		UV (64)	1317	0.53	3.11	96.36	73.6
B14	R, Y ⁻	—	7940	0.012	0.012	99.76	100
		UV (64)	6040	0.017	0.08	99.88	76
B22	R, Y ⁻	—	2117	0	0	100	100
		UV (64)	1847	0.16	1.19	98.65	78.7

* The numbers in parentheses indicate the dose of UV irradiation in $\mu\text{J mm}^{-2}$.

† R, rough colony mutants; S, smooth segregants.

understanding of the nature of the morphological alterations induced by benomyl we isolated a number of benomyl-induced mutant clones carrying different genetic markers (auxotrophy, thermosensitivity), that would enable us to carry out crosses by means of protoplast fusion. The process used for this isolation is depicted in Fig. 1. Occasionally the order of treatments was reversed and resistance to 5-FC was used as another marker.

To test the effect of benomyl in a strain known to be heterozygous we did control experiments with the Ade⁺ strain 29AW₂ (*ADE/ade*). Out of 7755 colonies grown in media containing 100 μg benomyl ml^{-1} 3 (0.04%) were Ade⁻ and 14 (0.18%) were sectorial (Ade⁺/Ade⁻). UV irradiation also determined the appearance of Ade⁻ auxotrophs with higher frequencies: 0.39% of Ade⁻ colonies and 0.68% of sectorial colonies when a UV dose of 32 $\mu\text{J mm}^{-2}$ was used. No auxotrophs were detected among 8271 control (non-treated) colonies examined.

Five independent R, Y⁻ benomyl-induced clones, carrying other markers, were obtained in order to study complementation between pairs of mutants in an attempt to differentiate between genetic determinants responsible for the altered morphological phenotype. Six pairs of mutants were crossed by fusion of the corresponding protoplasts and regeneration of fusion products under selective conditions (Table 2). Appropriate controls showed that protoplasts of neither of the two mutants of each pair could regenerate separately under the selective conditions that allowed regeneration of the products of their fusion. Fusion frequencies (Table 2) were in the order of those observed in other studies (Kakar *et al.*, 1983).

Fusion products regenerating in selective plates are usually heterokaryons of irregular appearance. Ten fusion products from each cross were transferred to fresh selective plates, and the plates incubated under the selective conditions required in each case. Under these conditions, colonies of more regular appearance and faster growth were recovered; these were hybrids resulting from the combination of both parental genomes (Sarachek *et al.*, 1981), that grew under the selective conditions as a result of complementation of the selective markers. On the other hand, the transfer of either fusion products or hybrids to complete (non-selective) medium led to the segregation of parental phenotypes. We carefully examined hybrids from each cross to explore the possibility of complementation of determinants of the morphological alteration.

As expected the cross B7 $\times$ B711 gave rise to R, Y⁻ hybrids since both parental strains carried the same mutation determining the rough-filamentous phenotype (Fig. 1). Similar observations were made in three other crosses, namely B14 $\times$ B211, B14 $\times$ B213 and B14 $\times$ B71, in which the

Table 2. *Characteristics of hybrids from crosses of different morphological mutant strains of C. albicans, done by means of protoplast fusion*

Strains crossed	Selection conditions*	Fusion frequency	Hybrids (phenotype† and other properties)
B14 (Y ⁻ , His ⁻) × B211 (Y ⁻ , Arg ⁻)	MM, 28 °C	8×10^{-3}	Rough, filamentous (Y ⁻)
B14 (Y ⁻ , His ⁻) × B22 (Y ⁻ , Ts)	MM, 37 °C	4×10^{-4}	Thermostable, smooth, Y ⁺ ; 87.5–91.5 fg DNA per cell (see Fig. 2)
B14 (Y ⁻ , His ⁻) × B213 (Y ⁻ , Arg ⁻)	MM, 28 °C	5×10^{-4}	Rough, filamentous (Y ⁻)
B14 (Y ⁻ , His ⁻) × B71 (Y ⁻ , Ts)	MM, 37 °C	4×10^{-3}	Thermostable, rough, filamentous (Y ⁻) (see Fig. 2)
B22 (Y ⁻ , Ts) × B71 (Y ⁻ , Ts)	YED agar, 37 °C	6×10^{-3}	Thermostable, smooth, Y ⁺
B7 (Y ⁻) × B711 (Y ⁻ , Ts, 5-FC ^R)	MM, +5-FC, 37 °C	6×10^{-4}	Rough, filamentous (Y ⁻)

* MM, minimal medium.

† All hybrids were prototrophic.

Fig. 2. Colonial and cell morphologies of *C. albicans* B14 (a, b), B22 (c, d), B71 (e, f) and two hybrids from the crosses B14 $\times$ B22 (g, h) and B14 $\times$ B71 (i, j) obtained by protoplast fusions. Bar, 40 μ m.

parental strains were independent isolates. The corresponding hybrids displayed a rough colonial morphology and consisted of filamentous forms, indicating that no complementation of the morphological phenotypes of parental strains had occurred. This is illustrated in Fig. 2 where the colonial and cell morphologies of the hybrid B14 $\times$ B71 (i, j) and of the parental strains (a, b, e, f) are shown.

However, the situation was completely different when the hybrids obtained from the B14 $\times$ B22 cross were examined. As imposed by the selection conditions all the clones were prototrophic and thermostable, but in addition their colonies were clearly of a smooth wild-type appearance (Fig. 2g), indicating that complementation of the morphological alterations of the parental strains had occurred. These colonies consisted only of the typical oval yeast cells with the characteristics of *C. albicans* blastospores (Fig. 2h) and contained only one nucleus per cell as revealed by chromomycin nuclear staining (data not shown). Moreover, their DNA content (87.5–91.5 fg per cell) was in the order of twice the DNA content of the original strain (*C. albicans* 1001, 41 fg per cell). The possibility of recovering parental phenotypes from clones of the hybrids was clearly demonstrated by growing them under non-selective conditions (complete medium at 28 °C), the frequency of segregation of parental markers being increased by low doses of UV irradiation (32 $\mu\text{J mm}^{-2}$). In a typical experiment 5212 colonies from a hybrid clone were examined after the UV treatment; 35 (0.67%) R, Y⁻ segregants were recovered, 31 of them being prototrophic and thermostable, one His⁻, two Ts and one His⁻, Ts. Similar results were obtained in the cross B22 $\times$ B71 that led to hybrids displaying wild-type colonial and cell morphologies. In this case the hybrids displayed a higher instability and a more detailed analysis was not done.

DISCUSSION

Dimorphism in *C. albicans* is a key issue in research on this organism. The diversity of environmental conditions that can determine the transition (Odds, 1979) from blastospores to mycelium in this species has made it difficult to study. However, the use of a clearly defined regime to induce the morphological change, when analysing various cytological, physiological and biochemical aspects of this transition, has enabled Soll (1986) to formulate a model emphasizing the importance of subtle temporal, spatial and quantitative differences in various programmes of events that run in parallel in either type of growth, as being basic for the genesis of one or other of the morphological phenotypes. The demonstration that the majority of gene products expressed during bud and hyphal growth are the same (Manning & Mitchell, 1980), justifies this emphasis. However, differential expression of dimorphism-specific genes, an important control for morphogenesis, cannot be ruled out.

New approaches in the isolation and the characterization of various types of mutants from *C. albicans* (Whelan *et al.*, 1980; Whelan & Soll, 1982) have allowed for the utilization of genetic approaches to study different aspects of the biology of this yeast. This also applies to the problem of dimorphism; mutants altered in these functions are being sought in order to answer the question of genetic control of dimorphism. Morphological mutants that are induced by UV irradiation (Pomés *et al.*, 1985) may represent the type of mutants that can contribute to an understanding of the basis of dimorphism. The evidence reported here clearly shows that growth of *C. albicans* 1001 derived strains in the presence of benomyl resulted in the appearance of this type of mutant with high frequency. This fungicide is believed to interfere with chromosome disjunction in fungi, leading to chromosomal loss with the corresponding segregation of heterozygous markers (Morris & Oakley, 1979; Wood, 1982). The minimal inhibitory concentration (MIC) of benomyl for *C. albicans* 1001 was approximately 500 $\mu\text{g ml}^{-1}$, well above the values of the MICs for filamentous fungi, and also much higher than the concentration of 100 $\mu\text{g ml}^{-1}$ used for inducing morphological mutants. The evidence we present with a prototrophic heterozygous *C. albicans* strain that carried a recessive adenine deficiency is also suggestive of an effect of benomyl interfering with chromosome disjunction, thus leading to the unmasking of recessive alleles, through chromosome loss. However, other possibilities cannot be ruled out without a more detailed examination of the problem. If the action of benomyl that led to the appearance of our morphological mutants was to cause chromosome loss, one should conclude that the strains from which they derived carried recessive mutant alleles of genes controlling morphogenesis. These mutations could either be present in the original strain 1001 or be introduced by the UV irradiation used to select for markers (auxotrophic, thermosensitive) appropriate for protoplast fusion (Fig. 1). In any case the potential of using benomyl for genetic analysis in *C. albicans* seems important and will be investigated further.

The action of benomyl on *C. albicans* 1001 enabled us to obtain a set of morphological mutants characterized by permanent growth as long, filamentous, pleomorphic forms, many of them with a pseudohyphal aspect. The cells lost their capacity to express the normal functions that determine growth by budding and adopted a mode of growth closer to that of pseudomycelium. Although it has not been clearly demonstrated, it seems feasible that the formation of pseudomycelia represents an intermediate stage in the process leading to hyphae. We therefore believe that the alterations in our morphological mutants affect the controls that operate in wild-type cells, enabling them to behave as dimorphic. The instability observed with the reversion to smooth colonies or sectors did not represent true reversion to a wild-type phenotype, but rather switching to a different colony phenotype that, in turn, could also switch to still other morphologies, in a similar manner to that reported by Slutsky *et al.* (1985, 1987). A preliminary account of this type of variability that seems to be activated in benomyl-induced mutants, has been presented (Pomés *et al.*, 1987) and further details will be reported elsewhere.

These observations clearly indicate that the morphological mutants selected in the presence of benomyl carried permanent heritable alterations that must affect some gene(s) of relevance for dimorphism. The nature of the morphological alteration, namely a rough colony morphology correlated with growth as filamentous forms, that contrasted with the glossy smooth appearance of wild-type colonies consisting of oval cells, was similar in all mutants. But the question arose of whether the genetic determinants altered in all mutants were the same or different. The availability of well-established methods for carrying out complementation analysis in *C. albicans* using a parasexual approach (Poulter *et al.*, 1981; Saracheck *et al.*, 1981; Pesti & Ferenczy, 1982; Kakar & Magee, 1982; Whelan *et al.*, 1986; Nombela *et al.*, 1987) enabled us to compare pairs of mutants with respect to their morphological genotype.

Our evidence clearly shows that strains B14 and B22, both of them of R, Y⁻ phenotype, gave rise to mononuclear hybrids of smooth and glossy colonies, that consisted of oval yeast cells, the typical blastospores. The combination of both parental genomes in these hybrids was confirmed by their capacity to segregate parental markers under non-selective conditions. It follows that the altered genetic determinants, responsible for the morphological phenotype, must be different in these two mutants. The same conclusion was obtained when the pair B22 × B71 were crossed. On the other hand, B14 did not complement when crossed to B211, B213 and B71, suggesting that they carried the same genetic alteration leading to their aberrant morphology.

We conclude that several genes are involved in the control of the functions that determine a correct transition from blastospores to mycelium in *C. albicans*. Mutants representative of, at least, two of those genes have been described in this paper. This multiplicity of genes is not unexpected in view of the complexity of the phenomenon of dimorphism. Our demonstration of the feasibility of characterizing morphological mutants genetically, by parasexual recombination, shows that it could be possible to identify mutants altered in the relevant genes by the procedures of classical genetics. In view of the possibility of non-integrative transformation in *C. albicans* (Kurtz *et al.*, 1987) an obvious extension of this work, which we are exploring, is the use of these mutants for transformation with gene banks to clone and characterize the corresponding genes, and to establish their putative role in dimorphism.

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