Arginine Auxotrophs of *Candida albicans* Deficient in Argininosuccinate Lyase

By GWENDOLYN F. GIBBONS AND DEXTER H. HOWARD*

Department of Microbiology and Immunology, UCLA School of Medicine, Los Angeles, California 90024, USA

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Auxotrophic mutants of *Candida albicans* FC18 were induced by a combination of treatments with nitrous acid and UV irradiation. Arginine (Arg⁻), histidine (His⁻) and methionine/cysteine (MetA⁻) auxotrophs were recovered by this means. The Arg⁻ auxotrophs lacked active argininosuccinate lyase (EC 4.3.2.1), the enzyme catalysing the final step in arginine biosynthesis. Thus the locus may be designated *arg-4*. The mutant strains bearing this mutation did not form germ tubes unless the germination medium contained arginine.

INTRODUCTION

Blastoconidia of the commensal fungus *Candida albicans* may initiate a hyphal form of growth by germination (Dabrowa & Howard, 1983). Germ tube formation is fostered by a variety of environmental factors but an incubation temperature of 37 °C is mandatory (Dabrowa & Howard, 1983; Odds, 1979). Among amino acids that induce germination, those that enter the metabolism by conversion to 2-oxoglutarate are generally the most active (Dabrowa *et al.*, 1976; Dabrowa & Howard, 1983). A study of the role of amino acids in germ tube formation by *C. albicans* would be assisted by a collection of biochemically defined mutants.

Until recently the amictic *C. albicans* was assumed to be haploid. However, auxotrophic mutants were not readily available (Howard, 1985; Whelan *et al.*, 1984) and several authors reported unsuccessful efforts to derive the customary diverse spectrum of such mutations by conventional techniques (Lingens & Oltmanns, 1964; Jacobson & Ayers, 1979; Olaiya & Sogin, 1979; Whelan & Magee, 1981; Whelan *et al.*, 1980). The reason for this intractability is now widely acknowledged to be that most available isolates of *C. albicans* are diploid (Hubbard *et al.*, 1985; Kakar & Magee, 1982; Kakar *et al.*, 1983; Olaiya & Sogin, 1979; Poulter *et al.*, 1981, 1982; Poulter & Rikkerink, 1983; Howard, 1985) and that the isolation of certain mutants from *C. albicans* stocks derives from the rather large extent of natural heterozygosity among those diploids (Whelan *et al.*, 1980; Whelan & Magee, 1981; Whelan & Soll, 1982).

In order to widen the spectrum of auxotrophs available for analysis, Kakar & Magee (1982) and Kakar *et al.* (1983) devised a method of two-step mutagenesis. The first step is the mutagenic agent acting on homozygous loci; the second step induces mitotic recombination. Alternatively, the two steps may be combined into a single mutagenic procedure of unusual intensity (Kakar *et al.*, 1983). We have adopted the technique of two-step mutagenesis in order to search for mutants of *C. albicans* that are auxotrophic for amino acids in the glutamate family (alanine, arginine, glutamic acid, histidine, ornithine and proline), which are among the most vigorous amino acid inducers of germination of the fungus (Dabrowa & Howard, 1983; Howard, 1985).

Abbreviations: GPA, glucose/peptone agar; MIN, yeast nitrogen base without amino acids or ammonium sulphate; YEPD, yeast extract/peptone/dextrose; YNBMM, yeast nitrogen base without amino acids.

The FC18 strain of *C. albicans* was chosen for our work because of the considerable amount of information about it already at hand (Kakar & Magee, 1982; Kakar *et al.*, 1983; Whelan *et al.*, 1980; Whelan & Magee, 1981). In the course of the study we obtained arginine, histidine and methionine/cysteine auxotrophs and identified the biochemical defect which determined arginine auxotrophy.

METHODS

Organism. *Candida albicans* FC18 was obtained from Dr P. T. Magee, Michigan State Laboratory, East Lansing, Mich., USA. Stock cultures were preserved in the Fungus Collection, University of California, Los Angeles, Calif., USA. Working cultures were maintained on glucose/peptone agar (GPA) in a refrigerator and were subcultured every month.

Media. GPA contained 2% (w/v) Bacto-Dextrose (Difco), 1% (w/v) Bacto-Peptone (Difco) and 2% (w/v) Bacto-Agar (Difco). Yeast extract/peptone/dextrose (YEPD) agar contained 1% (w/v) Bacto-Yeast Extract (Difco), 2% (w/v) Bacto-Peptone (Difco), 2% (w/v) Bacto-Dextrose (Difco) and 2% (w/v) Bacto-Agar (Difco). Yeast nitrogen base without amino acids (YNBMM) was prepared in accordance with the manufacturer's instructions (Difco) as was yeast nitrogen base without amino acids or ammonium sulphate (MIN). LBC synthetic medium (Lee *et al.*, 1975) consisted of $(\text{NH}_4)_2\text{SO}_4$, 5 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g; K_2HPO_4 , 2.5 g; NaCl, 5 g; glucose, 12.5 g; alanine, 0.5 g; leucine, 1.3 g; lysine, 1 g; methionine, 0.1 g; ornithine, 0.0714 g; phenylalanine, 0.5 g; proline, 0.5 g; threonine, 0.5 g; biotin (Nutritional Biochemicals Corp.) 1 mg; distilled water, 1 litre. The biotin was filter-sterilized and added to the other ingredients after they had been autoclaved at 110 °C for 20 min. All chemicals, including the amino acids used to define the auxotrophs, were purchased from Sigma, Calbiochem or Eastman Kodak.

Mutagenesis and growth conditions. A two-step mutagenesis procedure devised by Kakar *et al.* (1983) was used to derive the mutants. The procedure involved mutagenesis with nitrous acid (step 1) followed by UV irradiation (step 2) to induce mitotic crossing over.

Cells of *C. albicans* were grown at room temperature on YNBMM plates overnight. The cells were harvested in distilled water, washed once by centrifugation and resuspended in 0.2 M-sodium acetate buffer (pH 5.0) to a density of 2×10^7 cells ml^{-1} . Equal volumes of the suspended cells and 0.4 M-sodium nitrite made up in sodium acetate buffer were incubated at room temperature and the reaction was stopped at various time intervals by dilution (1:10) in 0.1 M-sodium phosphate buffer (pH 8.0). Cells were diluted further in distilled water and plated on GPA to determine the percentage of survivors. Preliminary experiments established that 15 min incubation of cells in sodium nitrite (0.2 M) under these conditions resulted in approximately 54% survival of cells and this time was chosen for the mutagenesis step.

The entire volume of cells exposed to sodium nitrite was poured into a plastic Petri dish and exposed for 15 s to UV light at a distance of 18 cm from the source (34% survivors; overall yield about 15% survivors). The light source was a GE-G875 UV tube (General Electric).

Isolation and testing of mutants. The cells from the nitrous acid-UV combination treatment were diluted in distilled water immediately after UV irradiation and plated on GPA. The plates were incubated at room temperature for 7 d. Colonies were replica plated onto GPA and YNBMM. Mutants were selected by preliminary testing for auxotrophy in accordance with the pattern method devised by Holliday (1956). The nutrient mixtures were incorporated into MIN agar plates. Amino acid auxotrophs were selected by subculture of indicated auxotrophs to MIN agar plates supplemented with single amino acids at a concentration of 0.2 mM.

Determination of the biochemical defect in the arginine mutants. The site of the lesions in selected arginine mutants was determined by growing subcultures of isolated clones on MIN agar plus the intermediates of the arginine biosynthetic pathway (Fig. 1).

The enzymes were analysed as follows. Exponential phase cells, grown in liquid YNBMM plus arginine at room temperature on a rotary shaker, were harvested by centrifugation, washed and resuspended in extraction buffer (0.02 M- KH_2PO_4 and 0.02 M- Na_2HPO_4 ; pH 7.5). Cell-free extracts were made by vortexing the cells in four 30 s pulses with 0.45 mm glass beads. All procedures were done on ice. Crude extracts were partially purified by $(\text{NH}_4)_2\text{SO}_4$ fractionation (Fincham & Boylen, 1957). The fractionated extracts were examined for argininosuccinate lyase (EC 4.3.2.1) activity by the method of Campanini *et al.* (1970). Protein was determined by the Lowry method with bovine serum albumin as the standard.

Germination assays. The parental FC18 strain was grown on YNBMM and each of the auxotrophs or fusion products (see next section) was grown on MIN agar supplemented with the required amino acid. The cultures were incubated at room temperature overnight. Cells were harvested in distilled water, washed once by centrifugation and resuspended to a concentration of 1×10^7 cells ml^{-1} . The media used to foster germination included: (i) single inducers (L-arginine, L-glutamic acid, L-proline, L-leucine and *N*-acetylglucosamine) at a concentration of 0.2 mM in 0.033 M-sodium phosphate buffer (pH 7.0); (ii) LBC medium; and (iii) LBC medium supplemented with 0.2 mM-L-arginine. About 1×10^6 cells ml^{-1} were incubated in 1 ml of each medium for 3 h at 37 °C. The tubes

were agitated gently on a Multipurpose Rotator, model 150 (Scientific Industries). A loopful of the cells was removed from each of the media, placed on a glass slide and examined microscopically. The percentage of cells with germ tubes was determined by counting 100 cells.

Preparation of spheroplasts. Cells of the auxotrophs were grown in YEPD broth overnight at 37 °C. The exponential phase cells were harvested, washed in distilled water and resuspended in spheroplast buffer (1.0 M-sorbitol in 0.1 M-potassium phosphate buffer, pH 7.5) at a concentration of 250 mg cells (wet wt) ml⁻¹. β -Mercaptoethanol and Zymolyase 5000 (Miles Laboratories) were added to a concentration of 2.5 μ l (ml mixture)⁻¹ and 2.5 mg (g wet wt cells)⁻¹, respectively, and the mixtures were incubated at 37 °C with slow stirring for 1 to 2 h. The extent of spheroplast formation was tested at intervals by checking lysis in 5% (w/v) SDS. When 95% of the cells had converted into spheroplasts, 1.5 ml spheroplast buffer was added for every 1 ml of mixture. Spheroplasts were centrifuged at 620 g and gently washed five times in 10 ml spheroplast buffer. Spheroplasts were finally suspended by very gentle shaking in 10 ml spheroplast buffer and stored overnight at 4 °C.

Spheroplast fusion and regeneration. Complementation was studied by parasexual methods involving spheroplast fusion (Fournier *et al.*, 1977; Evans *et al.*, 1982; Pesti & Ferenczy, 1982; Poulter *et al.*, 1981). Approximately 5×10^8 spheroplasts from each type were mixed and left at room temperature for 15 min. The mixtures were centrifuged at 620 g and the pellets were resuspended gently in 10 ml fusion mixture [40% (w/v) polyethylene glycol 3350 and 10 mM-CaCl₂ in 10 mM-Tris/HCl (pH 7.5)]. After 60 min incubation at room temperature, the cells were pelleted by centrifugation at 600 g and the pellets resuspended in 10 ml spheroplast buffer.

A 1 ml sample of the diluted fusion aggregate ($1-2 \times 10^7$ spheroplasts) was mixed with 100 ml of a regeneration medium (MIN with 1.0 M-sorbitol, 0.2 mM-arginine, 10 μ g tetracycline ml⁻¹ and 3% agar) kept at 45–50 °C. The mixtures were immediately poured into Petri dishes and left at room temperature for 1 to 2 d. The plates were then shifted to 37 °C for an additional incubation for 1 to 2 d. Regeneration was efficient (82%). Colonies were picked to YEPD agar plates for replica plating onto appropriate media to determine the auxotrophic requirements.

RESULTS

Isolation of auxotrophs

Initial experiments were done to determine the optimum concentration of sodium nitrite together with the time of exposure which would yield 50% survival and the time of exposure to UV irradiation which resulted in 50% survival (Kakar & Magee, 1982; Kakar *et al.*, 1983; P. T. Magee, personal communication). Approximately 1.5% of the population that survived the two treatments were mutants. Survivors grew slowly and required a minimum of 7 d to grow out. Auxotrophs were identified as those colonies that failed to grow on YNBMM, but grew normally on GPA. Of the slow-growing survivors, three classes of auxotrophs were identified: arginine (Arg⁻), histidine (His⁻) and methionine/cysteine (MetA⁻) auxotrophs. Auxotrophy was confirmed by determining growth on minimal media supplemented with individual amino acids at a concentration of 0.2 mM (Holliday, 1956).

The MetA⁻ auxotrophs are commonly encountered in the FC18 strain of *C. albicans* and are considered to arise from UV-induced segregation of a heterozygous locus (Whelan *et al.*, 1980; Whelan & Magee, 1981). Our isolate was not characterized further. The two Arg⁻ auxotrophs, labelled FC18-1 and FC18-6, were purified by several passages on MIN + arginine to ensure that a single auxotrophic type was being studied. The isolates grew in whole colonies only on complete media or on plates supplemented with arginine. The His⁻ auxotroph, labelled FC18-3, was used in complementation studies but the site of the lesion was not identified.

The spontaneous reversion rate was established by plating 10⁹ cells on YNBMM and counting the number of colonies that grew. Each revertant was retested and confirmed by streaking on YNBMM. The spontaneous backward rate of mutation for the arginine auxotroph FC18-1 was four per 10⁹ cells and that for FC18-6 was one per 10⁹ cells. The back mutation rates for the histidine and methionine/cysteine mutants were not determined. This low reversion rate makes these Arg⁻ mutants suitable subjects for studies on transformation of *C. albicans*.

Identification of the lesion in the Arg⁻ isolates

The arginine biosynthetic pathway described for fungi (Rhodes & Howard, 1980) is shown in Fig. 1. The intermediates shown were used to determine the site of the mutation in the Arg⁻ isolates. The two mutants (FC18-1 and FC18-6) and their parent FC18 were inoculated on YNBMM with and without arginine, and onto YNBMM supplemented with the intermediates ornithine, citrulline and argininosuccinate. The arginine requirement of the two mutant strains

Fig. 1. Pathway of arginine biosynthesis. The numbers denote enzymes: 1, ornithine carbamoyltransferase (carbamoyl-phosphate:L-ornithine carbamoyltransferase; EC 2.1.3.3); 2, argininosuccinate synthase (L-citrulline:L-aspartate ligase, AMP-forming; EC 6.3.4.5); 3, argininosuccinate lyase (L-argininosuccinate arginine-lyase; EC 4.3.2.1).

could not be fulfilled by any of the said precursors, indicating that the probable site of the lesion was the terminal step in the pathway, which is catalysed by argininosuccinate lyase.

The site of the lesion was confirmed by assay for argininosuccinate lyase. Crude cell-free extracts of both mutants and the parent were prepared from exponential phase cells in YNBMM supplemented with arginine. The extracts were partially purified by the $(\text{NH}_4)_2\text{SO}_4$ precipitation protocol (Fincham & Boylen, 1957). This method removes >80% of the arginase, as well as free arginine, from the extracts and allows a colorimetric procedure which measures arginine production from argininosuccinate to be used for the enzyme assay (Campanini *et al.*, 1970). The extracts from the parent FC18 strain contained active argininosuccinate lyase [$6700 \text{ U (mg protein)}^{-1}$; 1 unit forms $1 \mu\text{mol}$ arginine from argininosuccinate h^{-1}] whilst no such activity was detectable in the FC18-1 or FC18-6 mutants. These results confirm that the auxotrophy of the Arg^- mutants was due to their failure to produce active argininosuccinate lyase. Thus the locus affected in FC18-1 and FC18-6 should be designated *arg-4*, in accordance with the suggestion of Poulter & Rikkerink (1983) that the designation of loci in *Candida* be made to correspond to those of loci in *Saccharomyces*.

Germination of auxotrophs

Amino acids that enter metabolism by conversion to glutamate promote more filamentation, on average, than other groups of amino acids (Dabrowa & Howard, 1981, 1983). Among the glutamate family arginine, glutamic acid and proline are the most active (Dabrowa *et al.*, 1976). The Arg^- auxotrophs along with the His^- auxotroph were chosen for germination studies in various media that are conducive to germ tube formation (Table 1). The parental strain germinated well in LBC medium and reasonably well in buffer with single amino acids or *N*-acetylglucosamine. However, the Arg^- strains would germinate only in media in which the specific defect was redressed. Thus in spite of the fact that LBC medium contains proline, a vigorous inducer of germination, the medium would not support germination of the Arg^- or His^- mutants.

Complementation between arginine and histidine auxotrophs and germination of fusion products

The optimum time for spheroplast formation with Zymolyase 5000 varied from 1 to 2 h for strain FC18 and for each of the auxotrophs. Because of these variations, incubation times were monitored during each experiment.

One Arg^- and the His^- auxotroph were selected for complementation studies. Three examples of prototrophic fusion products (arbitrarily labelled FP10, FP16 and FP18) derived from regenerated spheroplasts were selected for germination studies. The ability of cells of the fusion products to germinate was assessed in the same media as those used to test the germination of the auxotrophs. The prototrophic fusion products from the cross FC18-3 (His^-) and FC18-6 (Arg^-) germinated to about the same degree in the various media as did the parent FC18 (Table 1).

Table 1. Germination of strain FC18, and arginine and histidine auxotrophs and certain auxotrophic derivatives

Germination medium	Percentage germination						
	Parent FC18	Auxotrophs			Fusion products		
		FC18-1	FC18-6	FC18-3	FP10	FP16	FP18
L-Arginine*	58.6	31.2	38.6	6.0	40.5	55.0	66.0
L-Glutamic acid*	40.8	1.0	1.0	5.5	ND	73.0	68.0
L-Proline*	64.5	2.0	0.0	3.0	75.5	65.5	57.5
N-Acetylglucosamine*	70.5	1.5	0.6	1.3	96.0	75.0	72.0
LBC medium	84.9	2.2	0.2	6.0	89.5	71.5	76.0
LBC plus L-arginine	89.3	82.0	77.8	6.5	92.0	89.5	85.0

ND, Not determined.

* In 0.033 M-sodium phosphate buffer, pH 7.0.

DISCUSSION

Recognition of the fact that most clinical isolates and many laboratory stock cultures of *C. albicans* are diploid has led to techniques designed to render homozygous certain naturally occurring heterozygous mutants or to induce mutations that are revealed by mitotic crossing over (Kakar & Magee, 1982; Kakar *et al.*, 1983). In keeping with the experience of Whelan *et al.* (1980), short term exposure (15 s) of strain FC18 to UV irradiation resulted in isolation of methionine/cysteine mutants (MetA⁻). But the two-step mutagenesis procedure with nitrous acid and UV irradiation provided several Arg⁻ and His⁻ mutants which were among those sought for our studies on germination of blastoconidia of *C. albicans* (see Introduction).

The FC18-1 and FC18-6 mutants could not process the intermediates in arginine biosynthesis to the final product arginine. The defect was confirmed by showing that both mutants lacked active argininosuccinate lyase, the enzyme catalysing the final step in arginine biosynthesis. Such a mutation is analogous to the *arg-10* mutation in *Neurospora crassa* (Esser & Kuenen, 1967) and to the *argA* mutation in *Aspergillus nidulans* (Cybis *et al.*, 1972). The locus should be designated *arg-4* in *C. albicans* in accordance with the suggestion of Poulter & Rikkerink (1983) that designation of loci in *Candida* be made to correspond to those of loci in *Saccharomyces*.

The auxotrophic mutants did not germinate even in the presence of strong inducers of germination such as N-acetylglucosamine or proline. The Arg⁻ auxotrophs could be induced to germinate only on media containing arginine (Table 1). As expected, the prototrophic complementation products germinated in the same sorts of media as did the parental FC18 strain. In *Neurospora* a large number of auxotrophic mutants have been tested for the ability of their conidia to germinate (Schmit & Brody, 1976). None of the auxotrophs formed colonies on minimal media but most of the strains did form germ tubes. Interestingly, those auxotrophs that failed to germinate had defects in the synthesis of aromatic amino acids or basic amino acids (arginine, lysine and histidine). In addition, sporulation defects have been described in arginine auxotrophs of *A. nidulans* (Serlupi-Crescenzi *et al.*, 1983).

The results from the complementation studies affirmed that the rate of occurrence of prototrophs far exceeded the rate of spontaneous back mutation. In these studies singly auxotrophic mutants were used in the spheroplast fusions. The use of doubly auxotrophic strains allows for more detailed sorts of genetic analyses (Kakar *et al.*, 1983) but our goal was simple restoration of germination capacity by complementation of the arginine marker.

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