JOURNAL OF BACTERIOLOGY, Sept. 1982, p. 1247–1252
0021-9193/82/091247-06$02.00/0

Vol. 151, No. 3

Genetic Analysis of *Candida albicans*: Identification of Different Isoleucine-Valine, Methionine, and Arginine Alleles by Complementation†

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Received 18 February 1982/Accepted 10 May 1982

By using the spheroplast fusion technique as a tool for genetic analysis, we have demonstrated complementation among three of four isoleucine-valine mutants, two of three methionine mutants, and two arginine mutants of independent origin from two different *Candida albicans* isolates. The two adenine mutants derived from the same parent strain did not complement. Complementation resulted predominantly from heterokaryon formation and, in some cases, from heterozygote formation. In either case, most fusion products were unstable and showed nuclear as well as chromosomal segregation, in a few cases resulting in recombination of parental auxotrophic markers. However, some fusion products were fairly stable.

The opportunistic pathogen *Candida albicans*, a dimorphic fungus, is an increasing medical problem in immunosuppressed patients. The success of genetic analysis in defining virulence factors in pathogenic bacteria makes such an approach to fungi promising. However, *C. albicans* has no known sexual cycle, and until recently, even its ploidy was a subject of controversy (3–5, 7). However, genetic experiments from this laboratory and others have shown conclusively that several clinical isolates are heterozygous for a number of recessive genes (11–13). At one stage, it was difficult even to isolate mutants (8, 10), but with the availability of potent mutagens, a diverse spectrum of auxotrophic mutants has been generated through either rendering homozygous naturally occurring heterozygous mutants or induced mutation followed by mitotic crossing over. Mutants have been generated to a level of 2% in the surviving population after a combination of mutagen treatments (S. N. Kakar, R. M. Partridge, and P. T. Magee, submitted for publication). Also, spheroplast fusion has made it possible to combine genetic information from two differing parents in this organism (5, 7) and to demonstrate complementation between auxotrophic mutants of independent origin, including histidine, lysine, and adenine (7; Kakar et al., submitted for publication). The present studies are an extension of the method to study complementation between auxotrophic mutants requiring isoleucine-valine, arginine, methionine, and adenine. The mutants were isolated independently and came from two different strains. We have identified at least three different *ilv*, two methionine, and two arginine complementation groups.

MATERIALS AND METHODS

***Candida* strains.** The mutants used in the present study were isolated by a two-step mutational process from two different isolates, FC-18 and MG-30, which have already been described (12, 13). The mutants were thus doubly auxotrophic (Table 1). The genetic designations are arbitrary and have no relation to similar markers identified in other laboratories.

Mutagen treatments and auxotroph isolation. The details of various types of media used for growth, irradiation by UV light, and selection and identification of auxotrophs have been described elsewhere (Kakar et al., submitted for publication). For nitrous acid treatment, cell suspensions containing 10^8 cells per ml were treated with 0.06 M sodium nitrite (2). Single-colony isolates were obtained, and their auxotrophic requirement was determined by replica plating and confirmed by the drop method.

Preparation of spheroplasts. A 30-ml amount of YEPD medium was inoculated with a loopful of cells, and the culture was grown overnight at 37°C in a shaker. Log-phase cells were harvested, washed, and suspended in spheroplast buffer (1.0 M sorbitol in 0.1 M potassium phosphate buffer, pH 7.5) at a concentration of 250 mg of cells (wet weight) per ml. β-Mercaptoethanol and Zymolase 60,000 were added to a concentration of 2.5 μl/ml and 0.125 mg/ml, respectively, and the mixture was incubated at 30°C with slow stirring for 2 to 3 h. The extent of spheroplast

† Michigan Agricultural Experiment Station journal article no. 10155.

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TABLE 1. Isolation of double mutants

Isolate	Step 1[a]	Single mutant	Step 2	Double mutant
FC-18	25 min HNO_2 →	A86 (*his2/his2*)	25 min HNO_2 →	A623 (*his2/his2 met2/met2*)
FC-18	25 min HNO_2 + 45 s UV →	A50 (*trp1/trp1*)	25 min HNO_2 →	A537 (*trp1/trp1, ilv2/ilv2*)
			25 min HNO_2 →	A558 (*trp1/trp1, met3/met3*)
FC-18	15 min HNO_2 →	A146 (*arg1/arg1*)	25 min HNO_2 →	A641 (*arg1/arg1, met3/met3*)
			25 min HNO_2 →	A642 (*arg1/agr1, his4/his4*)
			25 min HNO_2 →	A658 (*arg1/arg1, ilv3/ilv3*)
MG-30	25 min HNO_2 →	B24 (*arg2/arg2*)	25 min HNO_2 →	B520 (*arg2/arg2, ilv4/ilv4*)
MG-30	25 min HNO_2 →	B26 (*ade1/ade1*)	25 min HNO_2 →	B523 (*arg2/arg2, ade1/ade1*)
MG-30	25 min HNO_2 →	B23 (*ilv1/ilv1*)	25 min HNO_2 →	B537 (*ade1/ade1, ilv5/ilv5*)

[a] HNO_2, Nitrous acid treatment; UV, UV light treatment.

formation was tested at intervals by checking lysis in 5% sodium dodecyl sulfate. When the cells were 95% spheroplasts, 6 ml of spheroplast buffer was added for every 4 ml of mixture. Cells were centrifuged at 1,500 rpm for 5 min and washed five times in 10 ml of spheroplasting buffer each time. Spheroplasts were finally suspended by very gentle shaking in 10 ml of spheroplast buffer and stored overnight at 4°C.

Fusion. Approximately 5×10^8 spheroplasts from each parent strain were mixed and left at room temperature for 15 min. They were then centrifuged at 1,500 rpm, and the pellet was gently suspended in 5 ml of fusion mix (40% [wt/vol] polyethylene glycol 4,000, 10 mM $CaCl_2$, and 10 mM Tris [pH 7.5]). After 45 to 60 min, the cells were pelleted by centrifugation at 600 × *g* and resuspended in 5 ml of spheroplast buffer. A viable cell count in the absence of osmotic stabilizers showed that the fusion mix contained 0.01 to 0.34% viable cells.

Regeneration. A 0.1-ml sample of the appropriate dilution of the fusion aggregate suspension (1×10^7 to 2×10^7 spheroplasts) was mixed with 10 ml of appropriate regeneration medium (1 M sorbitol, 3% agar, and either YEPD or minimal medium supplemented with the appropriate amino acids and tetracycline [10μg/ml]) maintained at 45 to 50°C. The mixture was immediately poured into the corresponding regeneration medium petri plates, allowed to solidify, left for 1 to 2 days at room temperature, and then shifted to a 37°C incubator. Regeneration was highly efficient (>90%).

Regenerating colonies started appearing after 4 to 5 days and were picked onto YEPD master plates for replica plating onto appropriate media to determine the auxotrophic requirement. Some of the mixed-sectored colonies were tested to determine the type of segregants they yielded.

RESULTS

Complementation among isoleucine-valine auxotrophs. In the laboratory strains used to isolate mutants, only mutants which concomitantly required isoleucine and valine were obtained. The results are in accordance with the classical biosynthetic pathway for these amino acids (2), since all but one of the enzymes in the pathway serve to catalyze the synthesis of precursors for both amino acids. The extent of complementation through spheroplast fusion between various independently isolated isoleucine-valine mutants was scored on minimal regeneration medium supplemented with isoleucine and valine (Table 2). The mutant strains were doubly auxotrophic; thus, they required some other factor in addition to isoleucine-valine for growth. The choice of regeneration medium ensured that none of the parents would grow, as the medium lacked the second factor(s) required by each of the parents, but allowed fusion products of two parents, which apparently complemented for the outside markers, to grow irrespective of complementation between isoleucine-valine mutant markers. Among such regenerating colonies, a fairly large number is expected to be prototrophic for isoleucine-valine if the mutations in the two parents complement. In the absence of complementation, the number of such colonies will be very small. In general, two kinds of colonies could be visually characterized: a very small fraction of large-sized colonies (3 to 4 mm in diameter) in a background of a large number of small-sized colonies (1 to 2 mm in diameter). In one cross (A658 × B520), colonies were medium (2 to 3 mm in diameter) to small in size.

When two strains, both auxotrophic for isoleucine and valine but differing in requirements for other growth factors, were crossed by spheroplast fusion, a few large and many small

TABLE 2. Complementation between isoleucine-valine mutants on isoleucine-valine-supplemented regeneration plates

Cross	Extent of regeneration (no. of large/small colonies per plate)	Large colonies		Small colonies		
		No. tested	No. (%) of prototrophs	No. tested	No. (%) of prototrophs	
					Full	Sectored
A537 × A653 (*trp1/trp1 ilv2/ilv2* × *ilv3/ilv3 arg1/arg1*)	3/1,500–4/1,500	8	4 (50)	45	2 (4.4)	0
A537 × B520 (*ilv2/ilv2 trp1/trp1* × *ilv4/ilv4 arg2/arg2*)	5/1,500–6/1,500	2	2 (100)	90	14 (15.6)	42 (46.6)
A537 × B537 (*ilv2/ilv2 trp1/trp1* × *ilv5/ilv5 ade1/ade1*)	20/1,000–25/1,000	40	39 (97.5)	64	12 (18.8)	26 (40.6)
A658 × B537 (*ilv3/ilv3 arg1/arg1* × *ilv5/ilv5 ade1/ade1*)	20/1,000–25/1,000	55	55 (100)	45	3 (6.7)	11 (24.4)
B520 × B537 (*ilv4/ilv4 arg2/arg2* × *ilv5/ilv5 ade1/ade1*)	20/800–30/800	51	51 (100)	45	12 (26.7)	7 (15.5)
A658 × B520 (*ilv3/ilv3 arg1/arg1* × *ilv4/ilv4 arg2/arg2*)	5/1,500–6/1,000	45 (Medium)	36[a] (80)	45	17 (37.8)	18 (40.0)

[a] Eighteen of these prototrophs were mixtures of Ilv^+ and Ilv^- segregants.

colonies grew on minimal medium supplemented with isoleucine-valine (Table 2). We chose to analyze them separately. In the case of the crosses involving Ilv^- strains, the large colonies were predominantly or exclusively prototrophic for isoleucine-valine, except in the fusion involving A537 × A658, in which only four of the eight large colonies were prototrophic. About half of the large prototrophic colonies in cross A658 × B520 were sectored or mixed prototrophs and contained isoleucine-valine-requiring segregants. The large colonies that were fully prototrophic probably represented stable fusion products. The rest of the colonies were isoleucine-valine auxotrophs which were apparently complementing for outside markers but had lost chromosomes carrying wild-type *ILV* alleles. In addition, one-third of the large-colony auxotrophs from cross A658 × B520 had segregants with the requirements of one parent or the other.

The small colonies were of three kinds, fully prototrophic, mixed prototrophic containing auxotrophic segregants, and fully auxotrophic. The extent of prototrophy (full plus mixed) varied from cross to cross. It was low for the cross A537 × A658 (4.4%), but fairly good for all of the other crosses (31.1 to 77.8%).

The types of segregants found among mixed prototrophs of small colonies are shown in Table 3. The most common type of segregant was either of one or the other parental type or a mixture of both. There were also several segregants which required isoleucine-valine only. Interestingly, there were two cases in which the segregants showed auxotrophic requirements derived from both parents and were thus recombinant in that sense. These were a mixed Ilv^-/Ilv^- Ade^-/Ade^- Arg^- colony from A658 × B537 and an Ilv^-/Trp^- Ade^- colony from A537 × B537.

Most of the auxotrophs from various crosses were either of one or the other parental type or mixtures of the two. In some cases, the colonies were only isoleucine-valine requiring, either alone or in mixture with parental marker segregants.

Complementation among arginine requirers. In the cross A658 × B520, both parents required arginine as well as isoleucine-valine. The fact that 87% of the regenerated colonies had no arginine requirement is indicative of complementation between the two arginine mutants. This view was confirmed when another cross

TABLE 3. Segregants among mixed prototrophs of fusion products of isoleucine-valine mutants from isoleucine-valine-supplemented regeneration medium (small colonies)

Genotype segregant	No. of segregants from following cross:				
	A537 × B520	A537 × B537	A658 × B537	B520 × B537	A658 × B520
ilv	21	4	—[a]	1	18
ilv trp	1	12	—	—	—
ilv trp/ilv arg	10	—	—	—	—
ilv arg	10	—	—	—	0
ilv trp/ilv ade	—	5	—	—	—
ilv ade	—	4	5	0	—
ilv/trp ade	—	1	—	—	—
ilv arg/ilv ade	—	—	3	4	—
ilv arg	—	—	1	2	—
ilv/ilv ade/ade arg	—	—	1	—	—
ilv/ade	—	—	1	—	—

[a] —, Not tested.

TABLE 4. Complementation among methionine, arginine, and adenine mutants

Cross	Regeneration plates[a]	Extent of regeneration (size of colonies)	No. of colonies tested	No. (%) of prototrophs	
				Full	Sectored
Arginine mutants					
A642 × B523	Min + Arg	1,000 (Small)	90	45 (50)	12 (13.3)
Methionine mutants					
A623 + A558	Min + Meth	1,500 (Small)	90	24 (26.7)	63 (70)
A623 + A641	Min + Meth	1,500 (Small)	90	45 (50)	10 (11.1)
A558 × A641	Min + Meth	1,500 (Small)	90	0	1 (1.1)
Adenine mutants					
B523 × B537	Min + Ade	8–10 (Medium, 1,500 small)	45	0	1 (2.2)

[a] Min, Minimal medium; Arg, arginine; Meth, methionine; Ade, adenine.

involving these two arginine mutations was regenerated on arginine-supplemented minimal medium plates and a large number of small colonies appeared (Table 4). Most (63.3%) of the colonies that were prototrophic for arginine were either full (50%) or mixed prototrophs (13.3%). Again, most of the segregants among mixed prototrophs were either of one or the other parental type or mixtures of both (Table 5). Among mixed colonies, there were single-colony cases of all sorts of segregation combinations. Importantly, there were two cases of a reassortment of outside markers. One was a +/His^- Ade^-/Arg^- mixed colony; the other was a +/His^- Ade^-/Arg^- Ade^- mix. Thus, the double auxotroph recombinant class was obtained in this cross also, although rarely.

Complementation among Ade^- and Met^- cells. Similar complementation patterns for three methionine and two adenine mutants are also shown in Table 4, and the type of segregants in the mixed prototrophs is shown in Table 5. Although there was no difference in the number of small to very small regenerating colonies produced by the various fusion mixtures of Met^- cells, only two of the three fusion combinations had prototrophic components as either full or sectored-mixed prototrophs. In the A558 × A641 cross, only one colony had a methionine prototrophic component among 90 colonies tested. In the A623 × A558 cross, the segregants were predominantly either histidine requiring or had a requirement other than *his*, *met*, or *trp* which was not ascertained. In this case also

TABLE 5. Segregants from among mixed prototrophs of fusion products of methionine, arginine, and adenine mutants from supplemented regeneration medium (small colonies)

Cross segregants	No. of segregants in following methionine mutant:			Segregants	No. of segregants in arginine mutant A642 × B523
	A623 × A558	A623 × A641	A558 × A641		
his	35	1	0	*ade arg*	3
his/his met	2	0	0	*his arg*	1
?[a]	17	2	0	*his arg/ade arg*	2
his/trp	2	0	0		
his trp	2	0	0		
trp	2	0	0	*arg/his ade*	1
his/?	3	0	0		
his met	0	2	0	*arg/ade arg*	1
				arg/his arg	1
				ade/arg/his arg	1
his/met	0	1	0	*ade/arg*	1
met arg	0	3	0	*his ade/arg ade*	1
met/?	0	1	0		
met trp/met arg	0	0	1		

[a] ?, Requirement other than *his*, *met*, or *trp* which was not ascertained.

there were two instances of segregants recombinant for the outside markers, and the mixed colonies were +/*his trp*. The third cross, A623 × A641, segregated for combinations with some cases in which a single requirement for *his* or *met* segregated in addition to the parental requirement.

With regard to segregants among auxotrophs in the methionine cross A623 × A558, two of the three auxotrophs were *his* requiring, and the requirement of one could not be ascertained. In the cross A623 × A641, the predominant class was a mixture of the two parentals (17 of 35), whereas the next most frequent class was a mixture of His^- and Met^- Arg^- (7 of 35). The rest required one or the other parental markers either singly or in mixture with *his* or *met*. In the cross A558 × A641, 66 of the 89 auxotrophs tested were mixtures of two parentals, whereas the remaining were *met trp* requirers. Among the arginine cross auxotrophs, the requirement of the majority (16 of 33) could not be ascertained, as it was something other than the input markers, whereas the remaining were auxotrophic for one or the other of the parental markers or mixtures of the two.

Although the adenine auxotrophs were independent isolates, they failed to show complementation at a significant level, since there was no sector prototrophic for adenine of 44 colonies tested.

DISCUSSION

To establish a genetic system, at least the following three criteria must be met: (i) generation of novel phenotypes by mutagenesis, (ii) testing of the genetic basis of the novel phenotypes by complementation and mapping, and (iii) induction of new phenotypes by new combinations of parental characters (recombination). In the experiments described in this paper, we used spheroplast fusion in *C. albicans* to meet criteria (ii) and (iii). Our ultimate goal is to establish a parasexual genetic system in this medically important fungus.

Spheroplast fusion has been used for several years to induce artificial cell fusion among fungi (1, 9), and Sarachek et al. (7) and Poulter et al. (5) used it to hybridize *C. albicans*. Our goal in the present studies was to begin a systematic classification of the auxotrophs produced by a two-step mutagenic technique to be described elsewhere (Kakar et al., submitted for publication). We hope to begin to establish a set of reference auxotrophic mutations which will allow the biochemical and genetic manipulation of *C. albicans* to expand our knowledge of its biology and pathogenicity.

Our method of testing complementation was designed to minimize reversion as a way of obtaining prototrophs, since all fusion products were regenerated on medium which was supplemented for the requirement in question, thus avoiding any selection for revertants. Under these conditions, any prototrophs which arise are most likely the result of fusion, not of reversion.

In our studies, we isolated five independent isoleucine-valine auxotrophs; four of these were auxotrophic for another marker and could be tested in pairwise combinations for complementation. All produced prototrophs at frequencies which were much greater than could be expected by the reversion rates given above. The one pair which showed relatively weak complementation, *ilv2* × *ilv3*, nevertheless gave 4 prototrophs of 8 large colonies tested and 2 of 45 small colonies tested. Although not highly efficient complementation, this is still well above background and may indicate that these two mutations are in different locations. An alternative explanation is that these mutations are heteroallelic and the *ilv* prototrophs are the result of mitotic gene conversion, a process commonly observed in heteroalleles in yeasts (6). Because of the low complementation efficiency in this cross, we feel that we can be sure only of having demonstrated three complementation groups.

Similarly, of the four independent methionine mutants available to us, three for which double mutants were available were tested for complementation. A significantly large number of prototrophs for methionine were obtained among progeny of two of the three possible combinations. Thus, mutant *met2* seems to be nonallelic to mutants *met3-1* and *met3-2*, which in turn did not show any complementation and are therefore apparently allelic to one another. The single prototroph in the latter cross could most likely result from gene conversion from the heteroallelic situation.

We have in our stock collection three arginine-requiring mutants, only two of which could be analyzed. A large number of arginine prototrophs were obtained, suggesting that *arg1* and *arg2* are nonallelic to one another. Complementation between arginine mutants was also obtained when selection pressure was put on arginine as the outside marker in testing allelism for isoleucine-valine mutants *ilv3* and *ilv4*. The results for complementation between arginine mutants were thus doubly confirmed. One of the Arg^- mutants was derived from strain FC-18, and the second was derived from strain MG-30.

The two adenine mutants, both derived from strain MG-30 but of independent origin, did not produce adenine prototrophs among the regenerating fusion products and are thus allelic and represent mutational events in the same locus.

We do not know what process is occurring at the genetic level in our fusion products. The present results do not allow us to distinguish between plasmogamy and karyogamy, and both could lead either to stable prototrophs or to auxotrophs of one or the other parental type or, indeed, of recombinant phenotypes. However, the apparent aneuploids which segregated from several of the fusion products support the idea that karyogamy has occurred. It is important to point out, as observed by Sarachek et al. (7), that spheroplast fusion leads to multinucleate syncytia, not to exclusively pairwise crosses, and the excess of nuclear material may lead to drastic nuclear changes, such as significant chromosome loss. Evidence for the latter kind of event can be found in the fusion segregants with recombinant phenotypes (e.g., the Trp^- Ade^- Arg^- segregants listed in Table 3. These cells must have been derived from fusion products which lost at least one chromosome from each parent. A second example of such drastic chromosome loss is found in the cross A623 × A558, where an unknown auxotrophic requirement appeared as a result of chromosome loss after fusion. Evidence presented elsewhere (Kakar et al., submitted for publication) suggests that this unknown requirement may be lysine, since A50, the parent strain, appears to have been rendered heterozygous for a requirement for lysine by the mutagenic treatment used to induce the *trp1* mutation and render it homozygous.

In the case of the methionine-requiring fusions, one of the crosses (A623 × A558) segregated histidine-requiring cells at a high rate. This was not the case in the second cross, in which the same histidine-requiring parent, A623, was again involved. Unless there is some kind of selective advantage not apparent at this time, it is not possible to provide any satisfactory explanation without further tests. However, this does not in any way deter our conclusions about complementation.

In conclusion, the results show that spheroplast fusion can be successfully used as a means for establishing allelism through complementation. Most of the fusion products appear to complement through heterokaryon formation. The evidence suggests that, in some cases, heterozygote formation may also be involved. This is based on the fact of recovery of segregants, apparently resulting from chromosome loss, which had a single auxotrophic requirement, whereas both parents were doubly auxotrophic or had recombined requirements of both parents. The results are consistent with our similar observation of complementation for histidine markers reported elsewhere (Kakar et al., submitted for publication). In this way, we have identified at least three different loci in the isoleucine-valine, two in the methionine, and two in the arginine biosynthetic pathways.

In experiments reported elsewhere, we describe a two-step mutagenic technique which yields a large variety of auxotrophs from all strains for which it has been tested. We therefore feel that we have met the three criteria needed for establishment of a parasexual genetic system in *C. albicans*.

Our experiments set the stage for extending such an analysis to a variety of other loci known in this organism and for further characterization of the genetically determined loci through biochemical and other means.

ACKNOWLEDGMENTS

We thank R. M. Partridge for technical assistance.

This work was supported by Public Health Service grant AI 16567 from the National Institutes of Health to P. T. Magee.

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