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Morphology, Physiology, and Virulence of Some Mutants of *Candida albicans*

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Five mutagens were used to induce mutants of *Candida albicans*. Ultraviolet light, *N*-nitroso-*N'*-methyl-*N*-nitrosoguanidine, and *N*-nitroso-*N*-methylurethane were effective mutagens which induced stable auxotrophs. *N*-nitroso-*N*-methylurethane produced the largest number and variety of mutants. Nitrous acid and hydroxylamine were ineffective as mutagens although they killed *C. albicans*. All mutagenic agents employed induced colonial variants, especially small colony forms. The morphology, physiology, and virulence of one methionine and two adenine auxotrophs was compared to that of the prototroph. The auxotrophs exhibited yeast-like morphology in complex media and had sugar fermentation patterns typical of *C. albicans*, and all were agglutinated by *C. albicans* antiserum. Chlamydospore production was absent in the nonpigmented adenine mutant, and the chlamydospores produced by the methionine auxotroph were distorted. Germ tubes were formed in human serum by the auxotrophs and prototroph. Virulence for mice was retained by all auxotrophs but generally at a reduced level. The methionine auxotroph, only slightly less virulent than the prototroph, was more virulent than a pigmented adenine mutant and a practically avirulent nonpigmented adenine auxotroph.

Candida albicans is generally considered to be an opportunistic yeast, showing pathogenicity toward hosts whose resistance is low. A characteristic of this organism that apparently contributes to its pathogenicity is the ability to convert to a filamentous form in vivo (8, 20). Numerous in vitro studies on the biochemistry of morphogenesis in this and other fungi indicate that sulfur metabolism is involved in the control of cellular form (11-15). This apparent biochemical relationship between morphogenesis and pathogenesis may be partially elucidated with the aid of biochemically deficient organisms. This study presents data on the induction of auxotrophs of *C. albicans* by various mutagenic agents, data on some of the biochemical and physiological characteristics of selected adenine or methionine auxotrophs, and data on their morphology and virulence for mice.

MATERIALS AND METHODS

Microorganisms and media. The prototrophic *C. albicans* used in these investigations was a clinical isolate from the Medical Division, Oak Ridge Associated Universities, designated here as W-3. The complete medium used in these studies was Sabouraud Dextrose Agar or Broth (BBL). The minimal medium was that of Balish and Svihla (1) without amino acid supplementation. When required, amino acids, purines or pyrimidines, or vitamin supplements were added to the minimal medium.

Stock cultures were maintained on the complete medium at 25 C and transferred weekly. Cells for the mutation experiments were grown on Sabouraud Dextrose Agar at 37 C for 8 hr, harvested, and washed (three times) in saline by centrifugation. Unless otherwise indicated, the cells, after treatment, were plated on Sabouraud Dextrose Agar plates and incubated at 37 C.

Induction of mutants. Ultraviolet light, nitrous acid, hydroxylamine (Fisher Scientific Company, Pittsburgh, Pa.), *N*-nitroso-*N'*-methyl-*N*-nitrosoguanidine (NG; Aldrich Chemical Company, Milwaukee) and *N*-nitroso-*N*-methylurethane (NMU; K & K Lab., Inc., Plainview, N.Y.) were used in these experiments. Ultraviolet (UV) irradiation was supplied by a model UVS-11 short wavelength lamp. The cells were spread on Sabouraud Dextrose Agar plates and exposed to the UV radiation at a distance of 12 inches from the UV light source for varying lengths of time. Survival was determined by plate counts on samples appropriately diluted and plated prior to UV exposure, so that a suitable survival was obtained with a given UV exposure. Treatment with nitrous acid was based

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on the procedure of Kakar, Zimmerman, and Wagner (9). Washed cells were suspended in 0.1 M acetate buffer (*p*H 4.1) to a standard optical density, and sufficient sodium nitrate was added to give a 0.3 M concentration. Samples were diluted and plated after successive intervals of incubation at 25 C. The response of the organism to hydroxylamine was determined with varying concentrations of hydroxylamine hydrochloride in 0.3 M phosphate buffer (*p*H 6.0) with 10^{-3} M $MgCl_2$ and 1.0 M NaCl at 25 C. The latter reaction was stopped by dilution into cold 0.05 M tris(hydroxymethyl)aminomethane (Tris) buffer (*p*H 8.0) containing 2% acetone (19). For treatment with NG, washed cells were suspended in buffer (*p*H 7.7; 0.1 M citric acid and 0.2 M $Na_2HPO_42H_2O$) containing different concentrations of NG. Dilutions were made in the same buffer (without NG) after successive time intervals of exposure at 25 C (10). The effect of different concentrations of NMU on the organism was determined in a 0.1 M phosphate buffer (*p*H 7.0) solution. Incubation was at 25 C, and the reaction was stopped by dilution in 0.1 M phosphate buffer (7). Nutritionally deficient organisms were detected by replica plating colonies developing from those cells surviving the mutagenic treatment to minimal medium. The nutritional requirements of the mutants were determined by streaking the isolates on the minimal medium containing individual supplements.

Characteristics of adenine and methionine auxotrophs. Because of their potential use in biochemical and pathogenicity studies, the adenine and methionine auxotrophs were characterized for sugar fermentation, chlamydospore production, germ-tube formation in human serum and agglutination in *C. albicans* antiserum. For sugar fermentations the organisms were grown on glucose-free nutrient agar, inoculated into Phenol Red Broth Base (Difco) containing 3% carbohydrate, and incubated at 37 C. Chlamydospore production was determined on Corn Meal Agar (BBL) containing 1% Tween 80 with incubation at 37 C for 24 hr and 25 C for 96 hr. Germ-tube formation was verified by inoculating yeast cells (5×10^6) harvested from 18-hr Sabouraud Dextrose Agar slants into human serum and incubating for 3 hr at 37 C. Agglutination of all strains with *C. albicans* antiserum (Difco) was carried out at room temperature.

Growth and morphology of prototroph and auxotrophs. The cultures were grown on Sabouraud Dextrose Agar slants at 37 C, harvested at the end of a 48-hr growth period, washed, and suspended in physiological saline. Similar numbers (3×10^6) of washed cells were inoculated into 100 ml of Sabouraud Dextrose Broth and minimal medium plus supplements of adenine or methionine, either singly or in combination, and incubated at 37 C on a rotary shaker. Growth of the organisms was based on the increase in dry weight calculated by subtracting the dry weight of the inoculum from the dry weight of the culture after 20 hr. Wet mounts at 20 hr were used to ascertain the morphology of the cells.

S-adenosylmethionine determination. Cells were harvested from the various media, washed, and extracted with 1.5 M perchloric acid. These extracts were neutralized with 2 M $KHCO_3$, and *S*-adenosylmethionine was isolated and identified by the method of Schlenk and Ehninger (18).

Virulence of prototroph and adenine or methionine auxotrophs. Virulence of the parent and the mutant strains of *C. albicans* was determined by intravenous (iv) inoculation of 30-to 40-day-old white mice (male and female) with 0.1 ml of a standardized suspension of organisms. Cells for inoculation were harvested from Sabouraud Dextrose Agar slants after 48 hr at 37 C and washed (three times) by alternate suspension in saline and centrifugation. The number of organisms in a suspension was determined by direct microscopic count and verified by plate counts. Survivors were sacrificed approximately 5 weeks after injection and examined for gross kidney lesions.

RESULTS

Induction of mutants. All of the mutagenic agents selected were lethal for *C. albicans* under the conditions employed (Fig. 1A, B, C, D, E). The agents were generally applied to achieve 90% or more inactivation in a relatively short period of time. Hydroxylamine hydrochloride was the least lethal, and 12 hr was required for 90% inactivation with the highest concentration used. Inactivation of the organism by UV light was dependent on the conditions for survival of the treated organisms. A decrease in the survival incubation temperature resulted in a substantial increase in survival (ref. 5; see Fig. 1F). All of the mutagenic agents employed also induced small colony forms. Application of the tetrazolium overlay technique (16) to the small colonies indicated that they were not respiratory mutants. Rather, they appeared to be the result of a decreased growth rate and were similar to those reported by Bianchi (2, 3). This small colony size was an unstable character, and the transfer of these small colonies to fresh media resulted in the development of both normal-sized and small colonies. The possibility that these small colonies represented a UV-resistant part of the population was investigated by successive irradiations and selection of survivors, but no increased resistance to UV irradiation was observed.

The effectiveness of each agent as a mutagen under the conditions employed can be seen in Table 1. Nitrous acid and hydroxylamine hydrochloride at survival levels of less than 0.1% and 1.0%, respectively, failed to produce any biochemically deficient mutants. The other agents were effective mutagens and induced a variety of auxotrophs. Ultraviolet light and NG were comparable as far as the number of auxotrophs (four mutants in 10^3 survivors at survival levels below 1.0%), but NG produced a larger variety of mutants. The most effective was NMU in both the number (10 mutants in 10^3 survivors at survival

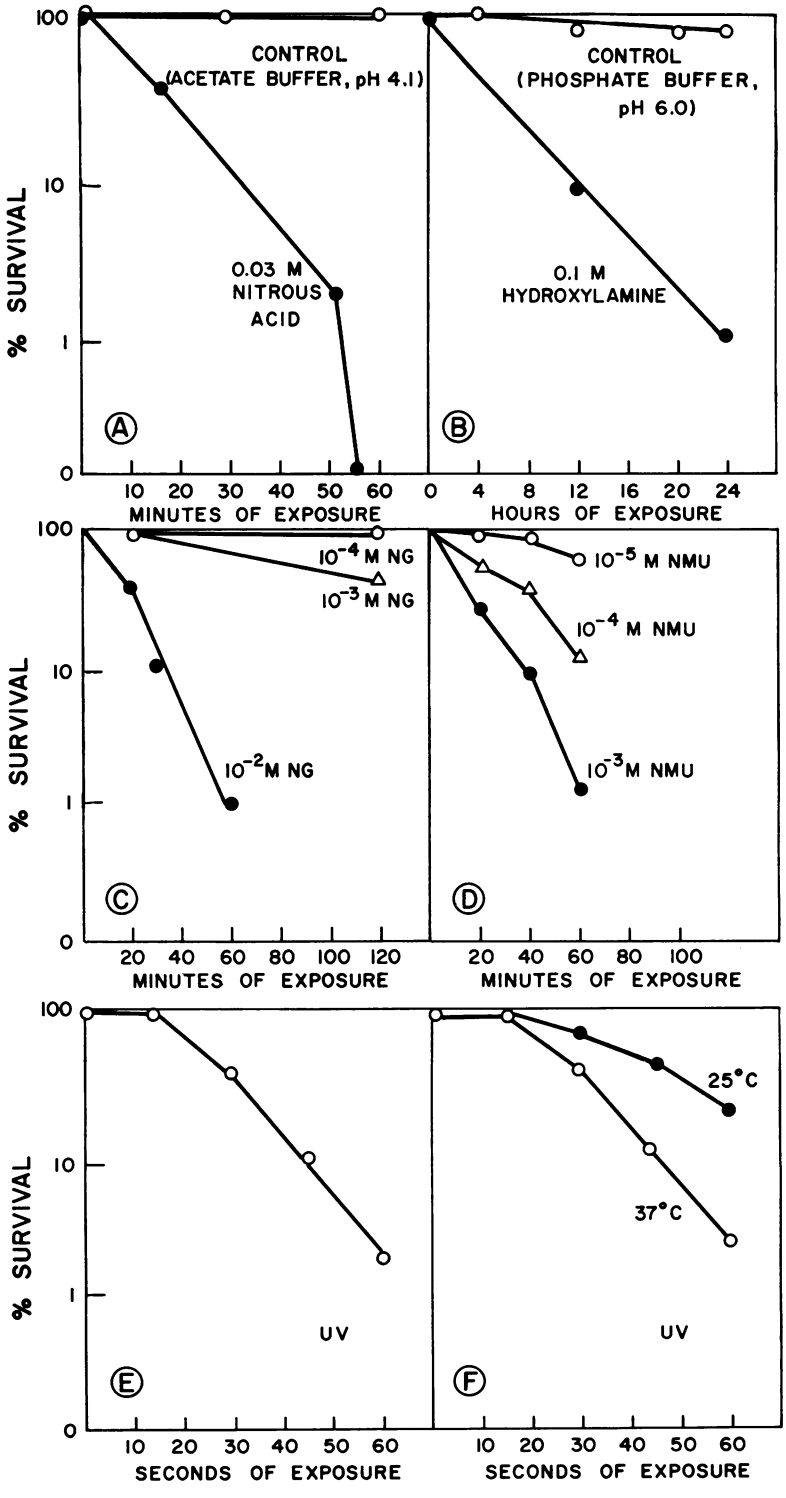

FIG. 1. *Effect of various mutagenic agents on the viability of Candida albicans.*

levels below 1.0%) and the variety of mutants. Multiple isolates of some mutants were obtained. The most common of these were histidine auxotrophs followed by leucine, tryptophane and arginine, and adenine. Rough colony forms were also induced by these agents, but no attempt was made to quantitate their induction.

TABLE 1. *Effectiveness of mutagenic agents for the induction of Candida albicans auxotrophs*

Mutagen	Per cent survival	Mutants/10^3 survivors	Auxotrophic types[a]
Nitrous acid (0.03 M)	<0.1	0	None
Hydoxylamine hydrochloride (0.1 M)	1.0	0	None
N-methyl-*N'*-nitro-nitroso-guanidine (0.01 M)	9.0 2.0 <1.0	2 1 4	Histidine, tryptophan, leucine, lysine, adenine, guanine
N-nitroso-*N*-methylurethane (0.001 M)	4.0 1.0 <1.0	7 11 10	Arginine, leucine, adenine, histidine, tryptophan, isoleucine, methionine
Ultraviolet light	5.0 <1.0	1 4	Histidine, guanine, tryptophan

[a] Arranged in descending order of frequency of isolation for all survival levels of each mutagen.

Characteristics of selected adenine and methionine auxotrophs. Adenine deficient auxotrophs were induced with both NG and NMU treatment and were selected for characterization and additional studies. The adenine mutant, designated V-NG-45, produced small, smooth, white colonies on Sabouraud Dextrose Agar at 37 and 25 C. Another adenine mutant, designated 1-NMU-5, produced a pink to red pigment when grown with a limiting quantity of adenine at 25 C. At 37 C the colonies were uniform in size, smooth, and white. The mutant resembled one that was isolated by Sarachek (17) and described by Bish and Sarachek (4). A single methionine auxotroph (VII-NMU-1) was isolated after treatment with NMU. This isolate produced smooth, uniform colonies on Sabouraud Dextrose Agar.

Typical fermentation patterns were obtained with the mutants except that a delay in sucrose fermentation was observed with all mutants, and a delay in gas production in glucose and maltose was observed with the nonpigmented adenine mutant (V-NG-45) and the methionine mutant (VII-NMU-1) (Table 2). Chlamydospores were produced after 5 days of incubation on Corn Meal Agar with 1% Tween 80 by the wild-type and pigmented adenine mutant. However, the chlamydospores produced by the methionine auxotroph were distorted, and no chlamydospores were produced by the nonpigmented adenine mutant. All strains produced germ tubes in human serum and were agglutinated by *C. albicans* antiserum.

TABLE 2. *Characteristics of prototrophic and auxotrophic types of Candida albicans*

Organism	Culture age (hr)	Sugar fermentation				Chlamydospore production	Agglutination
		Glucose	Sucrose	Lactose	Maltose		
W-3 (prototroph)	24	A	—	—	A		+
	48	AG	A	—	AG		
	120	AG	A	—	AG	+	
I-NMU-5 (pigmented adenine auxotroph)	24	A	—	—	A		+
	48	AG	—	—	AG		
	120	AG	—	—	AG	+	
	192	AG	A	—	AG		
V-NG-45 (nonpigmented adenine auxotroph)	24	A	—	—	A		+
	48	A	—	—	A		
	120	AG	A	—	AG	—	
VII-NMU-1 (methionine auxotroph)	24	A	—	—	A		+
	48	A	—	—	A		
	120	AG	A	—	AG	+	

TABLE 3. *Growth and morphology of prototrophic and auxotrophic Candida albicans on various media*

Organism	Medium	Dry weight[a] (mg/100 ml)	Morphology
W-3 (prototroph)	SAB	130	Yeast
	Minimal[b]	113	Yeast
	Minimal + adenine[c]	103	5% Filaments[d]
	Minimal + methionine[e]	143	Yeast
	Minimal + adenine and methionine[f]	135	10% Filaments
V-NG-45 (nonpigmented adenine auxotroph)	SAB	19	Yeast
	Minimal + adenine	25	Yeast
	Minimal + adenine and methionine	32	Yeast
I-NMU-5 (pigmented adenine auxotroph)	SAB	82	Yeast
	Minimal + adenine	68	5% Filaments
	Minimal + adenine and methionine	96	10% Filaments
VII-NMU-1 (methionine auxotroph)	SAB	95	Yeast
	Minimal + methionine	130	10% Filaments
	Minimal + adenine and methionine	76	10% Filaments

[a] Increase in dry weight obtained by subtracting dry weight of inoculum from total dry weight after 20 hr incubation at 37 C. Values represent typical results from three experiments.
[b] Glucose-salts-biotin with 1.0 mg/ml $(NH_4)_2SO_4$. Auxotrophs were unable to grow on this medium.
[c] 0.4 mg/ml.
[d] Percentage of yeast cells possessing germ tubes.
[e] 2.0 mg/ml.
[f] 2.0 mg/ml of methionine; 0.4 mg/ml of adenine.

Growth and morphology of prototroph and auxotrophs. The growth of the prototroph, the two adenine auxotrophs, and the methionine auxotroph was determined in Sabouraud Dextrose Broth and the glucose-salts-biotin medium with necessary supplementation (Table 3). The growth of the prototroph in the minimal medium was stimulated by the addition of methionine as was the growth of both of the adenine auxotrophs. Supplementation of the minimal medium with adenine had a slightly inhibitory effect on the prototroph and the methionine auxotroph but was essential for the growth of the adenine auxotrophs, V-NG-45 and I-NMU-5. Neither the prototroph nor the auxotrophs were able to grow on adenine or methionine as the sole nitrogen source.

The morphology of the prototroph and all auxotrophs was predominantly yeastlike after 20 hr of growth. Supplementation of the minimal medium with either adenine or methionine stimulated filament formation in all organisms except the nonpigmented adenine auxotroph. The total percentage of filaments in the population of cells was not large, however, with no more than 10% filaments in any case.

Subsequent studies on the methionine auxotroph indicated that cysteine was unable to support growth alone or in a combination with $(NH_4)_2SO_4$. Cysteine was actually inhibitory to the growth of this auxotroph as well as the prototroph.

Accumulation of S-adenosylmethionine by prototrophic and auxotrophic types. Data in Table 4 show the capacity of prototrophic and auxotrophic strains to accumulate *S*-adenosylmethionine in a supplemented minimal medium. The zero-hour sample values were obtained from 48-hr Sabouraud Dextrose Agar slant cultures. The 12- and 20-hr samples were obtained after incubation in the indicated media on a rotary shaker at 37 C. A decrease of *S*-adenosylmethionine levels was observed with the prototroph and adenine auxotrophs grown in minimal medium supplemented with adenine. The addition of methionine to this medium resulted in an increase in the *S*-adenosylmethionine levels in the prototroph and one adenine auxotroph (V-NG-45) The latter two strains maintained high levels of *S*-adenosylmethionine through the 20-hr incubation with a slight decrease indicated at 20 hr. The other adenine auxotroph (I-NMU-5) showed consistently high levels of *S*-adenosylmethionine for 20 hr with adenine and methionine in the minimal medium.

Virulence of prototroph and auxotrophs for mice. Inoculation of suspensions of the prototroph and auxotrophs iv into mice indicated a difference in the virulence of the strains (Table 5). The prototroph was most virulent, and death of mice oc-

TABLE 4. *Accumulation of S-Adenosylmethionine by adenine and methionine auxotrophs of Candida albicans*

Supplement to minimal medium[a]	Organism[b]	S-adenosylmethionine[c] [μMoles/g (dry weight)]		
		Zero hour[d]	12 hr	20 hr
Adenine[e]	W-3	4.5	3.2	2.1
	V-NG-45	4.1	2.1	1.1
	I-NMU-5	18.7	6.5	2.5
Methionine[f]	VII-NMU-1	3.5	11.6	4.9
Adenine and methionine[g]	W-3	4.5	14.9	14.7
	V-NG-45	4.1	7.5	7.1
	I-NMU-5	18.7	18.3	16.8

[a] Glucose-salts-biotin with $(NH_4)_2SO_4$ as nitrogen source.

[b] W-3 (prototroph); V-NG-45 (nonpigmented adenine mutant); I-NMU-5 (pigmented adenine mutant); VII-NMU-1 (methionine auxotroph).

[c] Values are typical results based on three experiments.

[d] Organisms harvested from Sabouraud Dextrose Agar slant after incubation at 37 C for 48 hr.

[e] Adenine added in 0.4 mg/ml quantity.

[f] Methionine added in 2.0 mg/ml quantity.

[g] Adenine added in 0.4 mg/ml quantity; methionine in 2.0 mg/ml quantity.

curred in 10 days or less with doses at, or above, 10^6 organisms. A dose of 10^5 resulted in kidney infections in 40% of the animals inoculated. The methionine auxotroph was slightly less virulent. Death occurred more slowly with this strain, and a higher dose was necessary to kill mice; approximately five times as many organisms were required for a response equivalent to the prototroph. Kidney infections were prevalent in mice surviving the lowest dose used (10^5). The pigmented adenine auxotroph (I-NMU-5) was also considerably less virulent, at comparable doses, than the prototroph and slightly less virulent than the methionine auxotroph. With I-NMU-5, even at the highest dose, no deaths occurred prior to 10 days after challenge. The incubation period prior to death appeared to be the greatest difference between I-NMU-5 and the methionine mutant since the percentage of deaths and sublethal infections were essentially the same. The other adenine auxotroph (V-NG-45) exhibited the lowest virulence. No deaths occurred after inoculation with a dose of 10^7 organisms, but 40% of this group of animals had kidney lesions apparent at necropsy.

Gross lesions in the animals inoculated with any of these strains were present in kidneys. Massive accumulation of yeasts and filaments was observed microscopically in the kidneys of animals dying from the infection as well as those sacrificed prior to death. The animals that died soon after challenge generally showed involvement of both kidneys. Those animals surviving 35 days often showed involvement of only one kidney. Preliminary studies with the prototroph indicated that there was an accumulation of organisms in the kidney of massively infected animals by the time of death 20 hr after inoculation. Other organs examined that yielded cultures included the liver, spleen, and lung. The latter organs, with the exception of the lung, were positive 1 hr after injection and at death 20 hr after injection.

Intraperitoneal inoculation of equivalent amounts of each of the strains of organism did not result in any deaths or gross kidney infection.

DISCUSSION

The data presented in this paper indicate that adenine and methionine auxotrophs of *C. albicans* retain their pathogenicity although at a reduced level of virulence. A number of possible explanations for this effect may be derived from an examination of the results pertaining to the growth and morphology of the individual strains.

The most significant characteristic of the strains that can be correlated with their level of virulence is their in vitro rate of growth on all of the media employed. The prototroph had the fastest growth rate and the highest degree of virulence. The auxotrophs exhibited slower rates of growth and correspondingly lower levels of virulence. This slower rate of growth, if maintained in vivo, would allow the host more opportunity to eliminate the invading organisms and to restrict the development of the disease once it has been established. The most significant difference in virulence of two of the three auxotrophs when compared to the prototroph was the interval between the time of inoculation until death of the animal. This prolonged course of the disease might well be the consequence of a slower rate of growth of the organism in the lesion sites.

In addition to the possible role of the growth rate, the morphology of the strains during growth must be examined, especially in view of the concept that the pathogenicity of *C. albicans* is the consequence of a rapid in vivo pseudohyphal formation (8, 20). Examination of the morphology of the prototroph and auxotrophs in vitro indicated that all strains formed germ tubes in human serum and, with the exception of one adenine auxotroph (V-NG-45), had the capacity

TABLE 5. *Virulence of prototroph and auxotrophs of Candida albicans for mice*[a]

Organism	Dose	Mortality	Days of survival[b]		No. of lesions[c]/ no. survivors
		No. dead/no. inoculated	Average	Range	
W-3 (prototroph)	1×10^5	0/5	—	—	2/5
	1×10^6	5/5	8	7–9	—
	5×10^6	5/5	4	2–5	—
	1×10^7	5/5	1	0–1	—
VII-NMU-1 (methionine auxotroph)	1×10^5	0/9	—	—	5/9
	1×10^6	7/10	17	7–28	2/3
	5×10^6	4/5	9	7–11	NE[d]
	1×10^7	9/10	6	1–19	0/1
I-NMU-5 (pigmented adenine auxotroph)	1×10^5	0/5	—	—	2/5
	1×10^6	3/5	24	18–28	2/2
	5×10^6	4/5	14	12–15	0/1
	1×10^7	4/5	12	11–13	0/1
V-NG-45 (nonpigmented adenine auxotroph)	1×10^6	0/5	—	—	0/5
	1×10^7	0/5	—	—	2/5

[a] Intravenous inoculation of cells harvested from Sabouraud Dextrose Agar after 48 hr at 37 C and washed (three times) in saline.
[b] Based only on those animals that died from the infection prior to sacrifice.
[c] Gross kidney lesions.
[d] Not examined.

to develop filaments as evidenced by the small percentage of filaments after 20 hr of incubation in minimal media. The production of germ tubes in vivo is of likely importance in resisting phagocytosis soon after inoculation, and each strain should be examined for this characteristic at periods less than 20 hr under in vitro and in vivo conditions.

An additional consideration in relation to the morphology and possible pathogenicity of these organisms is the role of sulfur amino acids and *S*-adenosylmethionine in these processes. Recent studies indicate a difference in methionine concentration in cell walls of yeast and pseudohyphal forms (6, 11) and a variation in *S*-adenosylmethionine levels in the vacuoles of yeast and pseudohyphal forms (1, 11). Generally, there seems to be a lower concentration of methionine in the walls of pseudohyphae with large amounts of *S*-adenosylmethionine in the vacuoles. Certain amino acids, including methionine, induce pseudohyphal growth in some strains of *C. albicans* (11). The prototrophic and auxotrophic strains studied here accumulated higher levels of *S*-adenosylmethionine when grown with methionine. The largest percentage of filaments for all strains except V-NG-45 was found under conditions giving maximal *S*-adenosylmethionine levels. This increased *S*-adenosylmethionine level may be the result of this increased percentage of filaments, or the increased filamentation may be the consequence of the increased *S*-adenosylmethionine levels.

In addition to the mechanical protection from phagocytosis afforded by the pseudomycelial form of *C. albicans*, it has been suggested that the virulence may result from absorption of methionine from the host resulting in the vacuolar accumulation of high levels of *S*-adenosylmethionine (1). The different levels of virulence in these prototrophic and auxotrophic strains may also be an indication that virulence is correlated with the capacity to accumulate *S*-adenosylmethionine. By this proposal, the I-NMU-5 adenine mutant should be the most virulent since it had the greatest capacity for *S*-adenosylmethionine accumulation. However, this organism was not as virulent as the prototroph or the methionine auxotroph. This might be explained by the variation in *S*-adenosylmethionine levels at zero and 12 hr of incubation. The two most virulent strains exhibited approximately a fourfold increase in adenosylmethionine, whereas the adenine mutant (I-NMU-5) exhibited a high but constant level. The second adenine mutant (V-NG-45) showed only a twofold increase. Thus, the rate of absorption of methionine is likely less or slower in these latter two strains.

In conclusion, it may be postulated that mutation of *C. albicans* to adenine or methionine auxotrophy does not eliminate the pathogenicity of the organism. However, variations in growth

rate, accumulation and metabolism of *S*-adenosylmethionine, and filamentation may be responsible for the alteration of virulence of these mutant forms.

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