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Auxotrophic Mutants of *Cryptococcus neoformans*†

ERIC S. JACOBSON* AND DEBORAH J. AYERS

Division of Infectious Diseases, Department of Medicine, Medical College of Virginia-Virginia Commonwealth University, Richmond, Virginia 23298, and Research Service, McGuire Veterans Administration Medical Center, Richmond, Virginia 23249

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Auxotrophic mutants of *Cryptococcus neoformans* have been obtained by using the methods of mutagenesis and replica-plating.

Cryptococcus neoformans is the etiological agent of a life-threatening meningitis. Treatment of this condition does not always lead to cure, and the drugs available require prolonged in-hospital administration and cause severe side effects. For these reasons we have been interested in developing tools for intensive study of this fungal pathogen by the method of biochemical genetics. Two fundamentals for establishment of a system of biochemical genetics already exist: (i) the demonstration that *C. neoformans* is capable of growth on a simple medium was made long ago (10); (ii) the sexual phase of *C. neoformans* has recently been described (8). Thus, there is reason to believe that formal genetic analysis will be applicable to a number of biological problems in this organism. We describe here the isolation of a variety of biochemical marker genes.

Strain 602 is a non-encapsulated variant of *C. neoformans* obtained from T. R. Kozel (7), and strains 401 and 404, from the National Institutes of Health collection, were a gift from H. J. Shadomy. Brain heart infusion was used as a complete medium, and Fries salts (1) supplemented with thiamine (0.4 μg/ml) was used as a minimal medium. The latter was composed of ammonium tartrate (5 g), potassium dihydrogen phosphate (1 g), ammonium nitrate (1 g), magnesium sulfate (heptahydrate, 0.5 g), trace element solution (1 ml), and water to 1 liter. The solution of trace elements contained, per liter, boric acid (57 mg), cupric sulfate (pentahydrate, 396 mg), manganous chloride (tetrahydrate, 72 mg), ammonium molybdate (37 mg), ferric chloride (hexahydrate, 0.98 g), zinc chloride (4.2 g), sodium chloride (100 g), and calcium chloride (dihydrate, 100 g). The carbon source was always glucose (2%). Growth in the liquid phase was followed turbidimetrically.

Mutagenesis was performed by a modification of the method using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine described by Delic et al. (4). Cells were grown in shake flasks at 37°C in brain heart infusion broth until the late logarithmic phase of growth. The density was adjusted to 150 Klett units (red filter), and the pH was adjusted to 9.0. Batches of cells were then exposed to various concentrations of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (0.3, 0.6, and 1.0 mg/ml) for 1 h at room temperature with agitation. The cells were then washed by centrifugation, resuspended in brain heart infusion broth, diluted 1:10 in fresh broth, and allowed to grow into stationary phase. They were then serially diluted and plated on brain heart infusion agar. Auxotrophic clones were identified by the technique of replica-plating (9). This was followed by inoculation into shake flasks containing minimal medium supplemented with Casamino Acids, a mixture of purines and pyrimidines, or a mixture of vitamins. The exact nutritional requirements were defined by the scheme of Holladay (6), in which several shake flasks containing minimal medium are supplemented with several compounds according to a pattern so that the exact growth requirements of a mutant can be inferred by the pattern of growth in the various media. We attempted to increase the final yield of auxotrophs by using 2-deoxyglucose, but this technique was not helpful.

The data in Table 1 summarize the logistics of three mutant hunts. The auxotrophic mutants we have obtained thus far are listed in Table 2. It can be seen that we have obtained auxotrophs

TABLE 1. *Induction of auxotrophic mutants*[a]

NTG concn (mg/ml)	Proportion of cells surviving mutagenesis (range)	No. of clones replicated	No. of auxotrophic clones	Yield of auxotrophic mutants (%)
0.3	10^{-2}-10^{-4}	2,451	6	0.24
0.6	10^{-3}-10^{-5}	2,810	9	0.32
1.0	10^{-5}-10^{-6}	2,525	2	0.08

[a] Data represent the combined results of three separate mutant hunts. NTG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

† This work is dedicated to David N. Gilbert, in admiration.

TABLE 2. *Auxotrophic mutants of C. neoformans*

Strain no.	Requirement	Mating type	Genetic background
C-9	Methionine	α	602 (acapsular)
C-13	Methionine	α	602 (acapsular)
C-15	Arginine	α	602 (acapsular)
C-46	Lysine	α	401
C-82	Cytosine	a	404
C-94	Lysine	α	401
C-107	Niacin	α	401
C-111	Niacin	α	401
C-145	Cytosine	α	401
C-154	Adenine	α	401
C-155	Cysteine	α	401
C-164	Cysteine	α	401
C-180	Arginine	α	401
C-183	Cytosine	α	401
C-196	Cytosine	α	401
C-202	Pantothenate	a	404
C-241	Cytosine	a	404

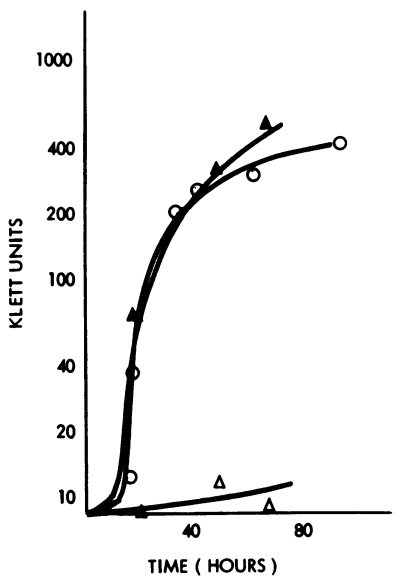

FIG. 1. *Growth of two strains of C. neoformans. Symbols:* (○) *wild type;* (△, ▲) *strain C-9 (a methionine-requiring auxotroph): open symbols represent growth on minimal medium; closed symbols represent growth on minimal medium supplemented with 5 mM* L*-methionine.*

in both the a and α mating types (8). We have also introduced auxotrophic mutations into the genetic background of strain 602, an acapsular mutant. In Fig. 1 are shown the growth characteristics of the first auxotroph we obtained. The growth of this mutant was restored to normal by exogenous methionine. Other mutants listed in Table 2 have been characterized in an analogous manner (data not shown).

Mutants of *C. neoformans* have been deliberately sought on two previous occasions, to our knowledge. Bulmer et al. (3) isolated seven nonencapsulated mutants from an encapsulated clinical isolate, six of which reverted to the encapsulated state over several months. Block et al. (2) isolated mutants resistant to 5-fluorocytosine; these are of interest because of the problem of early development of resistance to this clinically useful drug. Although the non-encapsulated strain 602 may not have been obtained originally by design, it has been a most useful tool for study of the interaction of the cryptococcal capsule with leukocytes (7). In this communication we have described the production of multiple genetic markers in an important human fungal pathogen. We intend that these will serve for exploitation of the sexual phase of this yeast (5, 8) in a formal system of biochemical genetics.

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