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Genetic and Phenotypic Characterization of Capsule Mutants of *Cryptococcus neoformans*

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Stable mutants with reduced capacity to produce capsules were isolated from suspensions of *Cryptococcus neoformans* after treatment of the wild type with a mutagen. The mutants could be assigned one of two phenotypes, hypocapsular or acapsular. Hypocapsular mutants were immunochemically and physicochemically indistinguishable from the wild type, whereas acapsular mutants lacked a major capsular antigen and a negatively charged exterior. In genetic analysis, the mutant trait segregated as a Mendelian gene (1:1) when random basidiospores from an outcross were studied, and analysis of products of single meiotic events from outcrossed mutants was likewise consistent with meiotic segregation. Two-factor crosses yielded the expected four classes of progeny, with recombinants equal to parentals. We concluded that chromosomal genes are responsible for synthesis of the cryptococcal capsule and that random basidiospore analysis represents a useful technique for genetic analysis in this species.

Cryptococcus neoformans is the etiological agent of a life-threatening meningitis. The organism is surrounded by a characteristic polysaccharide capsule which has been shown to represent a virulence factor for the organism (1, 2, 6, 7). Thus, acapsular mutants were sought and obtained by Bulmer (2), and a single acapsular strain was identified and isolated by Kozel and Cazin (7). Acapsular strains are of reduced virulence and are more readily ingested by leukocytes than are encapsulated strains. Incubation of acapsular cells with capsular polysaccharide restores their resistance to ingestion (1, 6). Most of the mutants obtained by Bulmer were soon lost by reversion, but Kozel's acapsular strain 602 has been under continuing investigation. Because of the discovery of a sexual cycle in this species (5, 10–12) and the ease with which biochemical markers can be obtained (4, 17), we decided to examine the inheritance of the cryptococcal capsule. In this report we describe the production and phenotypic and genetic characterization of capsule mutants.

MATERIALS AND METHODS

Origin of strains and method of culture. All strains except one were derived from B3501 (mating type α) and B3502 (mating type **a**) (11), which were referred to previously as 401 and 404, respectively (4). Both are of serotype D. Strain 602 was a gift from T. R. Kozel, University of Nevada, Reno. Production of the auxotrophic mutants has been described (4). In control experiments, auxotrophic mutants were characterized genetically in a manner analogous to that described in detail below for capsule mutants. Thus, 1:1 segregation was observed in random basidiospores from outcrosses of auxotrophs to the wild type ($P > 0.6$ by the chi-square test), random recombination between auxotrophs was observed when one auxotroph was crossed to another and random basidiospores were studied ($P > 0.6$), and segregation of parental genes was observed in each four-chain postmeiotic complex dissected from outcrossed, auxotrophic mutants. Fries salts solution (4) with thiamine (0.4 μg/ml) was used as a minimal medium; when used to grow auxotrophs, this medium was supplemented with 0.05% of the appropriate amino acid or nucleic acid base or 1 μg of the appropriate vitamin per ml. Brain heart infusion (40 g/liter) was used as a complete medium. The energy source in both media was glucose (20 g/liter), incubations were performed at 37°C, and liquid cultures were agitated. Littman's capsular medium (13) was made up to study production of capsular polysaccharide. This medium was supplemented with appropriate growth factors when capsular production by auxotrophs was to be studied. Growth in liquid media was followed turbidimetrically, using the red filter (no. 66) in a Klett photometer (Klett Manufacturing Co., New York).

Production of mutants. We obtained capsule mutants by isolating nonglistening colonies. Encapsulated wild-type cells were treated with either *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Sigma Chemical Co., St. Louis, Mo.) (4) or UV irradiation. UV mutagenesis was accomplished by placing suspensions of logarithmic phase cells (Klett reading of 150) in a petri dish (20 by 100 mm) and irradiating by means of a G15T8 germicidal lamp (General Electric Corp., Bridgeport, Conn.) placed 35 cm above the surface of a magnetic stirring platform. The stirred suspension was sampled at regular intervals for 1 h. Samples were serially

diluted, plated on solid capsular medium, and incubated in the dark. Nonglistening colonies were picked after 4 days, and their capsular defects were confirmed with the India ink technique (14).

Electron microscopy. Yeast cells in the logarithmic phase of growth in capsular medium were fixed for electron microscopy in 2% glutaraldehyde buffered by 0.1 M sodium phosphate at a pH of 7.4, followed by 1% osmium tetroxide in the same buffer. After this, they were immobilized in 1.5% agar and dehydrated in ethanol. Epon 812 or Spurrs embedding medium was used for embedding, sections were cut and mounted on copper grids, and alcoholic uranyl acetate (6.5%), followed by Reynolds lead citrate, was used for staining. Electron microscopy was performed with an A.E.I. model 801 instrument (A.E.I. Ltd., Manchester, England).

Production of antiserum against cryptococcal polysaccharide. We prepared rabbit antiserum against cryptococcal polysaccharide purified by ethanol precipitation and cetyltrimethylammonium bromide precipitation (3) (a gift from E. Reiss, CDC, Atlanta, Ga.). Polysaccharide (10 μg) was complexed with 10 μg of methylated bovine serum albumin (8), and the complex was emulsified in 0.5 ml of Freund complete adjuvant (Difco Laboratories, Detroit, Mich.), to which we added 5 mg of *Mycobacterium butyricum* (Difco). This amount was injected intramuscularly into each of six rabbits: two New Zealand, two Dutch white, and two Flemish giants. The rabbits were boosted at 4- to 6-week intervals with an identical preparation after test bleeding and were bled 10 to 14 days after each boost.

Indirect fluorescent-antibody staining. Cultures for indirect fluorescent-antibody staining were grown to mid-log phase in capsular medium and inactivated by addition of an equal volume of 2% formaldehyde on the day before harvesting (15). The cells were removed by centrifugation, washed three times with 0.85% saline, and suspended in saline at an optical density of 0.6 at 700 nm as measured in a Coleman, Jr. spectrophotometer (Coleman Instruments, Maywood, Ill.). This suspension was stored at 4°C. Fluorescein-conjugated goat anti-rabbit globulin was obtained from Arnel Products, Inc., Brooklyn, N.Y. We slightly modified the procedure for immunofluorescent-antibody staining described by Palmer et al. (15) because the acapsular mutants adhere poorly to glass slides. Absorbed serum was prepared by treating immune serum with 10 successive batches of inactivated cells of optical density 0.6. The first absorption was performed by centrifuging a volume of cell suspension, discarding the saline, adding serum to achieve the original volume of the suspension, mixing, incubating for 30 min at room temperature, centrifuging, decanting the serum, and discarding the pellet. Ten successive absorptions were performed with 10 equal volumes of inactivated cells to produce each absorbed serum.

Partition of cells. The technique used for the partitioning of cells was described by Walter (18) and was applied to *C. neoformans* by Kozel et al. (9). Cells were grown to mid-logarithmic phase in 10 ml of capsular medium and were washed three times with sodium phosphate buffer (pH 6.8, 0.44 M). The top and bottom phases of the partition system were produced by mixing 5% (wt/vol) dextran T-500 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) and 4% polyethylene glycol 6000 (Sigma) in sodium phosphate buffer (pH 6.8, 0.1 M) and allowing the phases to separate for 30 min into a positively charged top phase and a negatively charged bottom phase. Approximately 10^8 cells were suspended in 3 ml of the top phase of the partition medium. A portion of this suspension was mixed with an equal volume of the bottom phase and incubated at room temperature for 30 min. The top phase was removed, and dilutions of the original suspension, top phase, and combined interface plus bottom phases were plated on solid capsular medium. Colonies were counted after 5 days of incubation.

Genetic analysis. Crosses were made as described by Kwon-Chung (11), using hay infusion agar (16) as the crossing medium. Outcrosses were made by inoculating together a mutant and the wild-type, tester strain of opposite mating type. Basidiospores were scraped with a wire loop from an area of sporogenous hyphae several millimeters removed from the margin of the yeastlike colony. Such a sample typically contained more than 85% single basidiospores, 10% basidiospore pairs or clusters, and less than 5% yeast cells, as estimated by light microscopy. The basidiospores were suspended by vigorous agitation in sterile water, and the suspension was plated on brain heart infusion agar. Cells were scored for viability by comparing numbers of single cells and small colonies at 20 h with a phase-contrast microscope. Approximately 60% of the spores were viable. When colonies were macroscopically visible, they were transferred to small flasks of the appropriate liquid media, and the capsular phenotypes or nutritional requirements were determined. Capsule production was scored by use of the India ink technique after subculture in capsular medium. The complete products of single meiotic events (four intertwined chains of basidiospores) were isolated as single complex units with a micromanipulator (Lawrence Precision Machine Co., Hayward, Calif.) under a compound microscope. The units were then inoculated onto brain heart infusion agar. When each complex, postmeiotic colony had grown to visible size, it was streaked on agar, and 20 individual clones were studied for nutritional dependence or capsular morphology.

RESULTS

Phenotypic characterization. (i) Appearance of mutants. Capsule mutants were readily obtained: such mutants represented 0.1 to 0.5% of the surviving population with nitrosoguanidine and 0.5 to 0.8% with UV. As viewed in the light microscope with India ink, some strains showed no capsule at all (acapsular mutants), whereas others (hypocapsular mutants) showed small capsules that were much thinner than those of wild-type strains (Table 1). The appearance of the capsule was constant in any given strain and did not vary over the 15 months during which it was observed. Some cultures of hypocapsular mutants exhibited a small proportion of cells with capsules and a majority without. When these were streaked on solid capsular medium, all colonies appeared identical and each colony

TABLE 1. Morphological, immunochemical, and physicochemical properties of wild-type strains and capsule mutants

Strain	Thickness (mean ± standard error) of capsule as fraction of diameter of cell wall[a] by following technique:		Immunofluorescent staining reaction[b]	% Cells in top phase[c]
	Light microscopic	Electron microscopic		
B-3501	0.43 ± 0.13	0.30 ± 0.16	+	86
B-3502	0.18 ± 0.09	0.27 ± 0.17	+	88
602	<0.05[d]	0	−	1
Cap43	~0.05	0.01 ± 0.03	+	89
Cap48	0.08 ± 0.04	0.06 ± 0.05	+	
Cap53	0.06 ± 0.02	0.06 ± 0.02	+	85
Cap55	<0.05	0	−	1
Cap59	<0.05	0	−	1
Cap64	<0.05		−	1

[a] Diameter of cell wall was 5.1 ± 1.0 μm for B-3501 and 6.0 ± 1.2 μm for B-3502.

[b] Rabbit antiserum against cryptococcal polysaccharide was absorbed 10 times with cells of the acapsular strain 602 and used for indirect immunofluorescent staining of cells of the strains indicated.

[c] Cells were allowed to partition in the electrochemical field generated by the charged two-phase system described in the text. The top phase retains negatively charged particles. The top phase of the partitions performed with strains B-3501, B-3502, Cap43, and Cap53 was turbid from suspended cells, whereas the top phase of those performed with strains 602, Cap55, Cap59, and Cap64 was not turbid.

[d] This value represents the lower limit of detection by light microscopy.

gave rise to the same mixed population. Electron microscopy confirmed the light microscopic observations (Table 1).

(ii) Immunochemical characterization of mutants. Antibody was detected after a single boost with an indirect fluorescent-antibody technique. The highest titer of antibody (1:50) was obtained in Dutch rabbits. Preincubation of immune serum with cryptococcal polysaccharide abolished the immunofluorescent reaction, but absorption with 0.5 M D-mannose, sodium D-glucuronate, or D-xylose (constituents of the capsular polysaccharide) alone or in combination did not diminish the reaction. All strains exhibited some degree of immunofluorescence when nonabsorbed immune serum was used for staining.

Immune absorption studies. Antiserum absorbed with cells of strain 602 did not react at all with acapsular cells but did react with wild-type cells and hypocapsular cells (Table 1). In fact, any one of several strains could be used to absorb antiserum; any strain which did not react against antiserum absorbed with mutant 602 could be used to absorb fresh antiserum, with an equivalent reagent resulting from absorption.

(iii) Partitioning behavior of mutants. In an electrochemical field, both wild-type strains and all hypocapsular strains went predominantly to the top, positively charged phase, whereas acapsular mutants 602, Cap55, Cap59, Cap64, Cap66, Cap67, and Cap70 remained in the interface or lower phase (Table 1). The partition behavior appeared to depend upon charge because the affinity of encapsulated cells for the top phase was abolished by removal of the interphase potential difference by making up the partition system with sodium chloride instead of sodium phosphate (data not shown). We attempted to use this partition as a basis for selecting uncharged mutants from a population of mutagenized cells. We found no acapsular colonies by this method and no strains which were different from the wild type in their partition behavior.

Genetic characterization. (i) Outcrosses. Random basidiospore analysis was performed on outcrosses of capsule mutants (Table 2). Mendelian (1:1) segregation was observed. Attempts to study mutant 602 were not successful, as the mutant did not cross with any of the wild-type strains.

By micromanipulation, all four spore chains (intermingled together) were removed from individual basidia resulting from outcrosses of capsule mutant strains Cap43, Cap51, Cap53, Cap55, and Cap57. Although individual postmeiotic cells were isolated after an unknown number of mitotic divisions, each meiotic event

TABLE 2. Outcrosses of capsule mutants

Outcross	No. of colonies with following capsular phenotype of progeny	
	Cap^+	Cap^-
Cap43 × B-3501	55	45
Cap44 × B-3501	46	54
Cap48 × B-3501	57	43
Cap53 × B-3501	47	53
Cap54 × B-3501	52	48
Cap55 × B-3502	41	59
Cap59 × B-3502	42	58

TABLE 3. Results of crosses of auxotrophic to capsule-negative strains

Strains crossed	Recombinants		Parentals	
	$Cap^+ Aux^+$	$Cap^- Aux^-$	$Cap^+ Aux^-$	$Cap^- Aux^+$
Nia30 ($Nia^- Cap^+$)[a] × Cap43 ($Nia^+ Cap^-$)	26	19	7	48
Nia30 ($Nia^- Cap^+$) × Cap48 ($Nia^+ Cap^-$)	44	5	15	36
Nia30 ($Nia^- Cap^+$) × Cap53 ($Nia^+ Cap^-$)	20	24	11	45
Nia30 ($Nia^- Cap^+$) × Cap54 ($Nia^+ Cap^-$)	20	20	23	35
Cyt27 ($Cyt^- Cap^+$)[b] × Cap55 ($Cyt^+ Cap^-$)	39	20	23	18

[a] Nia30 is a niacin auxotroph.
[b] Cyt27 is a cytosine auxotroph.

produced at least one normally encapsulated member and at least one mutant member.

(ii) Two-factor crosses. Capsule mutants were crossed to an auxotrophic strain. In each cross, the ratio of parental to recombinant progeny was within the range expected for random segregation of unlinked genes (Table 3). The segregation ratios for the capsular traits from strains Cap48 and Cap54 are consistent with 1:1 segregation. In the cases of strains Cap43 and Cap55, the segregation ratios for the capsular traits are consistent with Mendelian segregation if the data from Tables 2 and 3 are combined.

DISCUSSION

Because acapsular mutants can be identified grossly as "dry" colonies, they were much easier to obtain than were auxotrophs, even though the proportion of capsule mutants in irradiated suspensions was comparable to the proportion of auxotrophs obtained earlier. Our acapsular mutants showed no propensity to revert to the encapsulated state, as had those of Bulmer (1). It was convenient to assess the presence of a capsule by means of the India ink technique. Although this optical method has its limitations, there was good correlation between light microscopy and other methods. The similar immunochemical reaction of all of the acapsular mutants suggests that a particular antigen may be responsible for the morphological appearance of the capsule. That glucuronoxylomannan (3) is this antigen is suggested by the following points: (i) it is the major extracellular component of growing cultures of *C. neoformans* (3); (ii) a purified preparation of glucuronoxylomannan was used to produce our antiserum; (iii) glucuronoxylomannan was able to neutralize our antiserum; and (iv) cultures of acapsular mutants lack the glucuronoxylomannan (R. Cherniak, E. Reiss, and E. Jacobson, manuscript in preparation). Acapsular mutants behave physicochemically as though they lack the negative charge which is present on the surface of normally encapsulated and hypocapsular strains. We have demonstrated that all of the acapsular mutants described here are of greatly reduced virulence for mice (R. A. Fromtling, H. J. Shadomy, and E. S. Jacobson, Mycopathologia, in press).

The small size (2 μm) and multiplicity of the basidiospores of *C. neoformans* make it desirable to find a method of genetic analysis which does not involve micromanipulation. To a large extent, the present study represents a test of genetic analysis based upon collection of random basidiospores. Thus, random basidiospores from outcrosses of capsule mutants exhibited Mendelian (1:1) segregation of mutant and wild-type traits. In every case, each four-chain postmeiotic complex resulting from an outcross contained basidiospores of both parental types, confirming that parental traits were segregating in the products of meiosis. Parental markers were also followed through two-factor crosses with random basidiospore analysis. All of the four classes of progeny predicted were obtained, and equal proportions of parental and recombinant progeny indicated that randomly collected basidiospores represented accurately the products of meiosis. We have no ready explanation for the unequal size of the classes, although we believe that the mutant traits (especially the auxotrophic traits) tend to diminish the viability of basidiospores.

The mutants that we have described in the present study demonstrate that it is worthwhile to study the genetics of virulence in *Cryptococcus neoformans*. It follows from the genetic analysis that the capsule mutants represent chromosomal genes for the production of the cryptococcal capsule. A second conclusion is that the genes for capsule production described here are not linked to the auxotrophic markers to which they were crossed.

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