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Genetic Association of Mating Types and Virulence in *Cryptococcus neoformans*

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A pair of congenic *Cryptococcus neoformans* var. *neoformans* strains, B-4476 (*a* mating type) and B-4500 (α mating type), that presumably differ only in mating type was constructed. This pair and their progeny, five α type and five *a* type, were tested for virulence in mice. In the parent strains as well as the progeny, α type was clearly more virulent than *a* type. In addition, death tended to occur earlier among the α-strain-infected mice that died than among the mice that died by infection caused by *a* strains. These data strongly suggest the genetic association of virulence with mating type in this human fungal pathogen.

Cryptococcus neoformans occurs in two mating types, α and *a*, controlled by a single-locus, two-allele system (9). When crossed on an appropriate medium, opposite mating types fuse to form a basidiomycete species, *Filobasidiella neoformans* (4). A survey of mating types among environmental isolates (mostly from pigeon droppings) as well as clinical isolates revealed that the α type is 30 to 40 times more frequent than the *a* type (6). The skewed ratio of the two mating types in such samples could not be explained genetically, since crosses between the two tester strains in the laboratory produce α and *a* progeny in the expected 1:1 ratio (9).

To explain the predominance of the α type among clinical isolates, Kwon-Chung and Hill studied the virulence of the two types for mice (9). Progeny of two consecutive generations derived from the cross between the two type strains (α and *a*) of *F. neoformans* were used for the study (9). The virulence of α and *a* strains varied, but no clear association was found between mating type and virulence.

Pulsed-field gel electrophoresis has enabled us to separate the chromosomes of the type cultures of *F. neoformans* var. *neoformans* (NIH12 and NIH433) and the tester strains. It was found that the two mating types differed significantly in their karyotypes, which was not surprising given that the strains had come from disparate geographical locations. This indicated that the isolates used in the previous studies of the relationship between mating types of *C. neoformans* and virulence were nonisogenic. It became clear that isogenic strains had to be constructed before the relationships of any two phenotypes could be accurately compared; however, true isogeny cannot be achieved, because *C. neoformans* never switches mating type according to our experience. In this study, we constructed a set of congenic α and *a* strains and isolated the progeny to compare their virulence in mice, as well as their temperature tolerance at 42.5°C, the average rectal temperature of pigeons.

MATERIALS AND METHODS

Strains and media. The tester strains B-3501 (α type) and B-3502 (*a* type) (4) were used to construct a set of congenic strains (B-4476 [*a*] and B-4500 [α]) (see Results). Cultures were maintained on yeast extract-peptone-glucose (YEPD) agar slants. The degree of capsule formation in vitro and phenoloxidase activity were tested on YEPD agar and niger seed agar, respectively. The doubling time of each isolate was tested in YEPD broth at 37°C as previously described (10).

Isolation of single basidiospores. Strains of two opposite mating types were crossed on V-8 juice agar (7), and single basidiospores were randomly isolated by micromanipulation (4). The mating types of the single-basidiospore cultures were determined by backcrossing.

Construction of congenic strains. A congenic set of strains that differ only in mating type was constructed as follows. B-3501 (α) was crossed with B-3502 (*a*), and single-basidiospore cultures of an α strain and *a* strain were isolated and designated B-4476 (*a* type) and B-4478 (α type). B-4476 was mated with B-4478, and an α offspring from this union was backcrossed to B-4476 to yield second-generation progeny. The process of isolating α single-basidiospore cultures and backcrossing to B-4476 was repeated a total of 10 times to produce B-4500, an α strain that is congenic with B-4476.

Pulsed-field gel electrophoresis. Agarose plugs containing yeast cells of *C. neoformans* were prepared by a modification of a previous method (20). Isolates were grown on minimal agar (yeast nitrogen base with 2% glucose) plates for 18 h. Cells were washed and treated as described; however, incubation in TEME was at 30°C instead of 37°C. Mureinase (U.S. Biochemical, Cleveland, Ohio) at a final concentration of 6.4 mg/ml was used to spheroplast the cells. Spheroplasting was at 30°C, while lysis was at 50°C. Plugs were rinsed twice for 1 h in 0.5× Tris-borate-EDTA, melted at 67°C, and carefully loaded into a 0.7% agarose gel (chromosomal-grade agarose; Bio-Rad, Richmond, Calif.) by using a syringe (1 ml) and 19-gauge needle. Electrophoresis was performed in a contour-clamped homogeneous electric-field DRII unit (Bio-Rad) by using a dual ramped switch time of 70 to 150 s for 15 h followed by 170 to 300 s for 33 h at 125 V. Buffer temperature was held constant at 12°C.

Temperature tolerance. Yeast cells grown on YEPD agar for 48 h were plated (10^3 cells per plate) on YEPD agar plates (preheated to 42.5°C) and incubated for 5, 8, and 24 h before removal of the plates and incubation for an additional 48 h at 30°C.

Experimental infection. The yeast cells were grown on YEPD agar for 48 h and injected into female BALB/c white mice (10^6 cells per mouse) intravenously (10 mice each) as described previously (10). The survival of mice was observed during a 30- or 100-day period.

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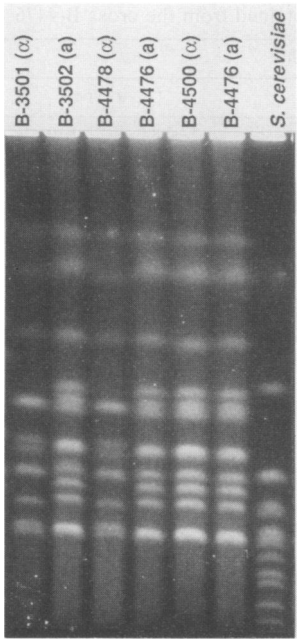

FIG. 1. Chromosomal banding patterns of the ancestral strains (B-3501 and B-3502), parental strains (B-4478 and B-4476), and congenic sets (B-4500 and B-4476).

Statistical analysis. The cumulative mortality among 2 groups of mice infected with B-4476 (*a*) and B-4500 (α) and 10 groups of mice infected with five α progeny and five *a* progeny of B-4476 × B-4500 were analyzed by the chi-square test.

RESULTS

Construction of congenic strains. The tester strains B-3501 (α) and B-3502 (*a*) showed phenotypic differences, including colony morphology on YEPD agar and niger seed agar. Colonies of B-3501 were shiny on YEPD, while those of B-3502 were dull, and the brown pigment of B-3501 colonies on niger seed agar was darker than that of B-3502. Karyotypes were also distinct between B-3501 and B-3502. Both strains appeared to have 13 chromosomes: B-3501 showed 10 separated bands, with 1 band being doubly intense and another band being more than doubly intense on the contour-clamped homogeneous electric-field gel (Fig. 1). B-3502 showed 11 separated bands, with 2 bands being doubly intense. Although the total number of chromosomes appeared the same in the two strains, the banding patterns of at least four chromosomes were different. The two isolates, however, had the same doubling time: 3 h at 37°C in YEPD broth.

The two F_1 progeny obtained from the cross B-3501 (α) × B-3502 (*a*), B-4476 (*a*) and B-4478 (α), also manifested phenotypic differences. B-4478 produced colonies that were slightly more shiny on niger seed agar than were B-4476 colonies. The karyotype of B-4476 was identical with that of B-3502, and that of B-4478 was identical with that of B-3501 (Fig. 1). B-4476 was crossed with B-4478, and an α progeny was randomly selected to backcross with B-4476. This procedure was repeated for 10 generations. By the sixth generation of backcrossing, the α strain showed a karyotype identical to that of B-4476. The 10th-generation α type was designated B-4500. B-4476 and B-4500 not only had identical karyotypes (Fig. 1) but also showed identical phenotypes as far as could be determined. B-4476 was crossed with B-4500, and 10 progeny (five *a* and five α) were selected to use for animal study. The 10 isolates showed identical phenotypes with regard to colony morphology, growth rate, and the expression of phenoloxidase activity.

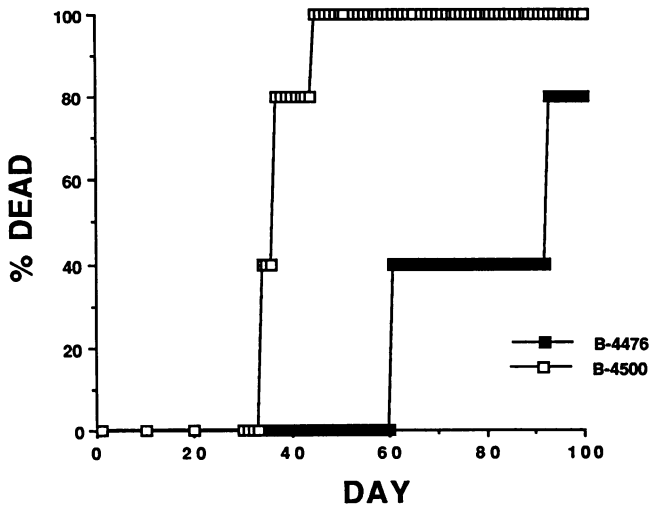

FIG. 2. Percent death of all mice infected with B-4476 and B-4500.

Virulence for mice. The congenic strains B-4476 (*a*) and B-4500 (α) and 10 randomly selected single-basidiospore progeny from the cross B-4476 × B-4500 were tested for virulence in mice. Isolate B-4500 was considerably more virulent than B-4476: 80% of mice infected with B-4500 (α) died within 36 days, and the rest of the mice died by day 43. Isolate B-4476 (*a*) required 93 days to cause fatal infection in 80% of the mice, and the remaining 20% appeared healthy until the study was terminated at day 100 (Fig. 2). No attempt was made to culture brains of these mice, since it has been shown that the death is directly related to the number of CFU in the brain (9). The increased virulence in α-type isolates was also clearly seen among the progeny of B-4476 × B-4500. As shown in Table 1 and Fig. 3, 80 to 90% of mice infected with α progeny died within 30 days, while only 20 to 50% of mice infected with *a* progeny died within the same period. Statistical analysis showed that the excess in mortality among the mice infected with α type is highly significant ($P < 0.001$). In addition, deaths tended to occur earlier among the mice infected with α than among the mice that died by infection caused by *a* isolates (Wilcoxon test, $u = 238.5$, $P = 0.012$). The cumulative percentages of total deaths of mice by α and *a* progeny have been compared in Fig. 4.

Survival of the two mating type strains at 42.5°C. The pigeon has been suspected as a carrier of *C. neoformans*, since the fungus was isolated from the crop, beak, and feet of wild pigeons (3). The strains of *C. neoformans* found in pigeon droppings must, then, have survived passage through the digestive tracts of these birds. It is possible that α strains survive better than *a* strains under this condition. Since the average rectal temperature of the pigeon is 42.5°C, we have tested temperature tolerance of the congenic pair and their 10 progeny (5 α and 5 *a*) by exposing them at 42.5°C for 5, 8, and 24 h. No cells (out of 10^3) of any strain survived exposure at this temperature for 8 h or longer. After 5 h of exposure, one or two cells of each strain survived and showed no difference between the mating types.

DISCUSSION

Unequal prevalence of two opposite mating types among clinical isolates has been known in several pathogenic fungi

TABLE 1. Survival of mice infected with *a* and α single-basidiospore isolates obtained from the cross B-4476 × B-4500

Day	No. of mice[a] infected with:											
	a type						α type					
	sb1	sb3	sb6	sb9	sb12	Dead	sb2	sb5	sb8	sb10	sb13	Dead
17	10	10	10	10	10	0	10	10	10	10	10	0
18	10	10	9	10	10	1	8	6	9	10	10	7
19	10	10	9	10	10	1	6	6	9	8	8	13
20	10	10	9	9	10	2	6	3	9	7	7	18
21	10	9	9	9	10	3	5	3	9	5	6	22
22	10	9	9	9	10	3	5	3	8	5	5	24
23	10	9	9	8	10	4	5	3	7	5	5	25
24	10	7	9	8	10	6	4	3	5	5	5	28
25	8	7	8	8	9	10	4	2	5	5	4	30
26	8	7	7	7	8	13	2	2	5	4	4	33
27	8	7	7	6	8	14	2	2	5	4	3	34
28	8	7	7	6	8	14	2	2	4	3	3	36
29	6	7	7	5	8	17	2	2	2	1	2	41
30	5	7	7	5	8	18	1	2	2	1	2	42
Total no. dead	5	3	3	5	2	18	9	8	8	9	8	42

[a] For each isolate is indicated the number of surviving mice. The numbers of dead mice are cumulative.

with bipolar heterothallism. *Histoplasma capsulatum* is one such fungus causing systemic disease in humans and animals. The overall frequency of the (−) type is known to be much higher than that of the (+) type among clinical isolates of *H. capsulatum* (12). The two mating types, however, have been found with equal frequency among isolates obtained only from cases of disseminated histoplasmosis in severely immunocompromised patients (5). The prevalence of the two mating types is also equal among environmental isolates (12). An adequately designed genetic study to assess the differences in the virulence of the two mating types in *H. capsulatum* has not been made.

Various species of zoophilic and anthropophilic dermatophytes have shown extremely biased distribution of (+) and (−) types (11). In various anthropophilic dermatophytes, only one mating type is found (19, 21). The geophilic dermatophytes such as *Microsporum gypseum* or *Arthroderma simii*, however, have shown the two mating types in equal frequency among clinical isolates (17, 18). These results suggest that dermatophyte species successfully established as obligate human or animal parasites may be evolving into asexual fungi.

The α and *a* strains used as the ancestors for the construction of the congenic pair in this study were from a case of osteomyelitis (NIH12 strain) in the United States and from pigeon droppings (NIH433) collected in Denmark, respectively (4). Although the number of chromosomes separated by contour-clamped homogeneous electric-field electrophoresis was identical between the two strains, at least 4 of the 13 chromosome bands showed different mobilities (1). Such differences were expected, since a wide variation has been seen in the karyotypes among clinical and environmental isolates of *C. neoformans* var. *neoformans* (8). Since the occurrence of the sexual cycle must be a rare event in pigeon droppings or soil because of the lack of compatible mating types, chromosomal variation among isolates within the species can be as wide as that of the asexual fungi (20). In addition, genetic rearrangement may occur while the fungus is growing in the host tissue, as has been seen in some protozoan species (3). The second-generation pair, B-3501

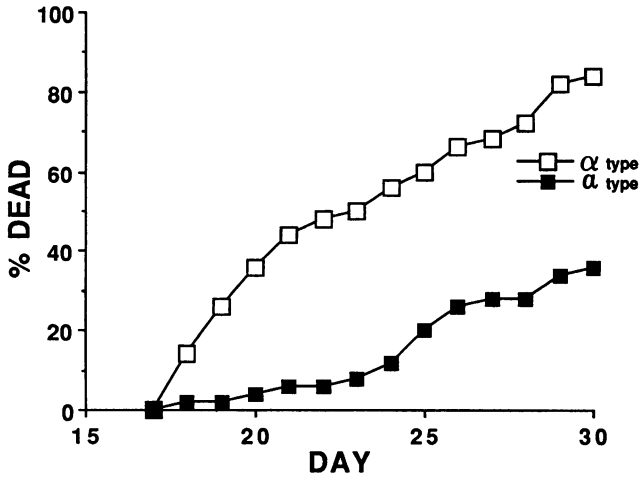

FIG. 3. Cumulative percentage of dead mice infected with five α strains and five *a* strains obtained from the cross B-4476 × B-4500. The total number of dead is calculated as 100%.

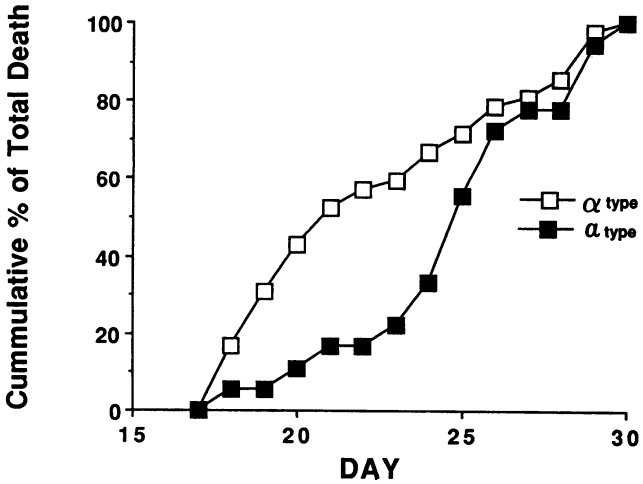

FIG. 4. Cumulative percentage of the total death of the mice infected with five α strains and five *a* strains obtained from the cross B-4476 × B-4500.

(α) and B-3502 (*a*), and the third-generation pair, B-4476 (*a*) and B-4478 (α), showed the same differences as NIH12 and NIH433. Previous classical genetics studies dealing with virulence factors where isogenic sets were needed were all done with the mutants derived from B-3501 and B-3502 (11, 16). Although some sets of parental isolates used for genetic analysis were the products of backcrossings either with B-3501 or B-3502 (11), it is highly unlikely that they were isogenic, since it took at least six generations of backcrossings to achieve congenicity in the strains described here.

It is generally assumed that cryptococcosis is acquired through inhalation of dehydrated airborne yeast cells from pigeon droppings or other pigeon-associated sources (3). If this is the case, the predominance of α strains among clinical isolates is understandable, since α strains are far more common than *a* strains in pigeon droppings or in soil contaminated with avian excreta (6). The question as to whether avian excreta are the primary environmental source has not been answered. Pigeon and other avian droppings are high in creatinine and other low-molecular-weight nitrogen sources, and some authors suggested that the droppings serve as a selective growth medium for *C. neoformans* in nature (15). This hypothesis does not appear sound, since *C. neoformans* var. *gattii* utilizes creatinine better than *C. neoformans* var. *neoformans* and yet has never been isolated from avian guanos (9).

Assuming that the pigeon is a carrier of *C. neoformans*, the skewed ratio of α and *a* types among environmental isolates was not due to different susceptibilities to 42.5°C, the average rectal temperature of pigeons (13). Both mating types were equally susceptible to high temperature, and over 99% died within 5 h of exposure. This observation does not negate the possibility that there is any difference in survival between α and *a* during the passage through the pigeon gut, since temperature must be only one of many factors *C. neoformans* cells are exposed to in the avian digestive tract. It could also be that the growth rates of the two mating types in pigeon droppings are different.

The data presented here demonstrate the linkage of mating type with virulence in *C. neoformans*. The mating type locus in *C. neoformans* is over 60 kb in length (unpublished data) and probably contains multiple genes, as is the case in other basidiomycetes (2, 14). The molecular basis for the enhanced virulence is unknown; however, it is possible that a virulence-enhancing gene is so closely linked to, but not a part of, the mating type determining system that they rarely segregate. The linkage between mating type and virulence could also arise by several other mechanisms: (i) a gene or genes that promote virulence could be embedded within the α mating type locus and, hence, do not segregate; (ii) a gene involved in α mating type behavior and a gene promoting virulence may both be positively regulated by a gene within the α mating type locus; or (iii) a gene directly involved in α behavior may enhance virulence. The detailed molecular and genetic analysis of the mating type loci will allow the dissection of genes involved in mating type determination and their effect on virulence.

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