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Virulence, Serotype, and Molecular Characteristics of Environmental Strains of *Cryptococcus neoformans* var. *gattii*

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Four strains of *Cryptococcus neoformans* var. *gattii* originating from *Eucalyptus camaldulensis*, three from Australia and one from San Francisco, were tested for their serotype, virulence for mice, and a number of genetic and molecular characteristics. All were found to be serotype B and showed significantly higher virulence for mice than did the type strains of *C. neoformans* var. *gattii* and *Filobasidiella neoformans* var. *bacillispora*, which were obtained from human cryptococcosis cases. Electrophoretic karyotypes of the strains from Australia were identical, although they were collected from sites at least 15 to 500 km apart. The electrophoretic karyotype of the strain from San Francisco was the same as that of the Australian isolates except for the mobility of one chromosome. On the contrary, no two isolates of serotype B (of a total of 11) from clinical sources were the same, regardless of their geographic origin. Furthermore, none of the clinical isolates showed a chromosomal banding pattern identical to that of *Eucalyptus*-originated strains. The *Eucalyptus*-originated strains failed to form dikaryons when crossed with the tester strains of the two varieties of *F. neoformans*. Hybridization analysis with a nucleic acid probe (AccuProbe *C. neoformans* Culture Confirmation Test; Gen-Probe Inc., San Diego, Calif.), however, showed signals of equal intensity for clinical strains and the *Eucalyptus*-originated strains. Various fungi phylogenetically related to *C. neoformans*, including a phenol oxidase-positive strain of *Cryptococcus laurentii* obtained from *E. camaldulensis*, were negative in the nucleic acid hybridization test. These observations confirm that, in spite of karyotypic differences and the lack of dikaryon formation with the tester strains of *F. neoformans*, *Eucalyptus*-originated *C. neoformans* var. *gattii* is the same organism as those isolated from cases of human infection. Furthermore, the *C. neoformans* culture confirmation test using a commercial nucleic acid probe is specific for *C. neoformans*.

The pathogenic yeast species *Cryptococcus neoformans* contains two varieties, *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii*. Each variety contains two serotypes, serotypes A and D for *C. neoformans* var. *neoformans* and serotypes B and C for *C. neoformans* var. *gattii* (18). The prevalence of the two varieties of *C. neoformans* involved in clinical disease is different in various geographic locations (3, 15), but there is a notably higher incidence of *C. neoformans* var. *gattii* isolations from non-AIDS patients in tropical and subtropical regions (5, 15, 16). *C. neoformans* var. *neoformans* is almost always the cause of cryptococcosis in AIDS patients regardless of geographic location (21).

Saprophytic sources of *C. neoformans* var. *neoformans* have repeatedly been reported (1, 2, 4, 7–9, 27), but until recently, *C. neoformans* var. *gattii* had not been isolated from nature. Ellis and Pfeiffer (6) reported in 1990 the isolation of *C. neoformans* var. *gattii* from *Eucalyptus camaldulensis* (red gum tree) in Australia. Since then, *C. neoformans* var. *gattii* has been isolated from *E. camaldulensis* growing near Fort Point, San Francisco (23). Identification of these *Eucalyptus*-originated strains was made on the bases of morphological, physiological, and biochemical characteristics.

In the present study, we tested *Eucalyptus*-originated strains of *C. neoformans* var. *gattii* from Australia and San Francisco for their serotype, virulence for mice, and a number of genetic and molecular characteristics. Eleven clinical strains of serotype B from various geographic areas were compared with *Eucalyptus*-originated strains for their karyotypes.

MATERIALS AND METHODS

Strains of *C. neoformans* var. *gattii* from *E. camaldulensis*. Three of the *Eucalyptus*-originated strains, B-4506, B-4507, and B-4508, were isolated by one of the investigators (D. Ellis) in Australia. B-4506 and B-4507 were isolated from the Barossa Valley, South Australia, at sites approximately 15 km apart. B-4508 was isolated from *E. camaldulensis* debris collected at Balranald, southwestern New South Wales. The site for B-4508 is approximately 500 km from the sites for B-4506 and B-4507. Strain B-4534 was isolated by T. Pfeifer from *E. camaldulensis* growing near Fort Point, San Francisco. These strains were cultured on yeast extract-peptone-glucose (YEPD) agar, as well as niger seed (birdseed) agar (26), to observe their colony characteristics.

Serotyping. Serotyping of *C. neoformans* was performed by previously described methods (15).

Mating test. The four strains of *C. neoformans* var. *gattii* isolated from *E. camaldulensis* were crossed in all possible combinations on V-8 juice agar (17), sucrose-yeast extract agar (12), and hay infusion agar (24). They were also crossed with tester strains of *Filobasidiella neoformans* var. *neoformans* and *Filobasidiella neoformans* var. *bacillispora*.

Contour-clamped homogeneous electric field (CHEF) separation of chromosomes. The electrophoretic karyotypes of the four *Eucalyptus*-originated strains of *C. neoformans* var.

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TABLE 1. Hybridization expressed as RLU obtained from strains tested with the *C. neoformans* DNA AccuProbe

Isolate	Source	Serotype	RLU
C. neoformans var. *neoformans*			
ATCC 32045 (type strain)	Patient	D	508,879
NIH 68	Patient	A	748,473
NIH 371	Cuckoo dropping	A	579,528
Filobasidiella neoformans var. *neoformans*			
NIH 12 (type strain)	Patient	D	578,328
NIH 433	Patient	D	598,153
Filobasidiella neoformans var. *bacillispora*	Patient	C	585,547
NIH 191 (type strain)			
C. neoformans var. *gattii*			
B-3939 (type strain)	Patient	B	588,200
B-4506	*E. camaldulensis*	B	607,743
B-4507	*E. camaldulensis*	B	570,224
B-4508	*E. camaldulensis*	B	556,702
B-4534	*E. camaldulensis*	B	588,924
NIH 403	Patient	C	588,578
C. laurentii B-4535 (Mel^+ CGB^+)	*E. camaldulensis*		3,024
Filobasidium uniguttulatum (*C. uniguttulatus*)			
CBS 1727 (type strain)	Human nail		9,320
CBS 1730	Human nail		6,037
Filobasidium floriforme (*C. albidus*)			
CBS 6241 (type strain)	*Hibiscus siriaca*		3,730
CBS 6242	*Hibiscus siriaca*		8,585
Filobasidium capsuligenum (*C. capsuligena*)			
CBS 1906 (type strain)	Sake-moto		10,972
CBS 4736	Wine cellar		3,737

gattii were compared on the same gel with those of 11 clinical isolates of serotype B and with that of an unusual strain of *Cryptococcus laurentii* that produces brown colonies (Mel^+) on niger seed agar. The clinical isolates were obtained from Africa, Brazil, the United States, and Australia. The Mel^+ *C. laurentii* strain was isolated from debris (a mixture of leaves, bark, fruit, and flowers) collected from beneath an *E. camaldulensis* tree on the University of California campus in Los Angeles by one of the investigators (D. H. Howard) and produced a positive reaction on canavanine-glycine-bromthymol blue (CGB) agar, as *C. neoformans* var. *gattii* does (18). The electrophoretic karyotypes of 10 colonies each recovered from the brain, lung, and spleen of a mouse fatally infected with B-4507 and B-4534 were compared among themselves as well as with those of B-4507 and B-4534.

Cells were prepared for pulsed-field electrophoresis by using a modification of a previous method (28). All isolates were grown on MIN agar (6.7 g of yeast nitrogen base without amino acids [Difco, Detroit, Mich.], 20 g of glucose, and 20 g of agar [all per liter]) for 16 h. Approximately 6×10^8 cells were removed and suspended in 1.0 ml of washing buffer (50 mM EDTA, 10 mM Tris, pH 7.5), washed twice, and resuspended in TEME (100 mM Tris, pH 8.0, 5 mM EDTA, 0.5% 2-mercaptoethanol). Cells were then incubated for 30 min at 30°C. Suspensions were pelleted, washed once in MES-sorb [20 mM 2(*N*-morpholino)ethanesulfonic acid, pH 6.0, 1 M sorbitol], and resuspended in 160 μl of SCE (0.1 M Na citrate, pH 5.8, 0.01 M EDTA, 1 M sorbitol) made 6 mM in the nuclease inhibitor aurintricarboxylic acid (Sigma Chemical Co., St. Louis, Mo.). Forty microliters of spheroplasting solution (45 mg of mureinase [U.S. Biochemical, Cleveland, Ohio] per ml in SCE) was added to the suspensions. After brief mixing, 300 μl of low-melting-point plug agarose (FMC Bioproducts, Rockland, Maine) made up in 125 mM EDTA, pH 7.5, and held at 37°C was added to the suspensions. Cells were briefly mixed and carefully pipetted into a microtiter plate held on ice. Solidified plugs were removed to LET (45 mM EDTA, pH 9.0, 10 mM Tris, pH 8.0) made 6 mM in aurintricarboxylic acid. Tubes were incubated at 30°C for 20 h. LET was removed, and NDS (LET, 1% sarcosine, pH 9.0, 2 mg of proteinase K per ml) was added. The plugs were then incubated at 50°C for 24 h. The plugs were prepared for electrophoresis by two rinses in 10 volumes of running buffer and melting at 67°C. Samples were carefully loaded into the wells of a 0.6% gel (chromosomal grade agarose; Bio-Rad, Richmond, Calif.) with a 1.0-ml syringe and a 19-gauge needle. Electrophoresis was performed in a Bio-Rad CHEF DRII apparatus by using dual ramped switch times of 50 to 130 s for 16 h followed by 170 to 300 s for 32 h at 125 V. Running buffer was 0.5× TBE (1× TBE is 0.089 M Tris, 0.089 M boric acid, and 0.002 M EDTA [pH 8.0]) maintained at 12°C.

Virulence for mice. To test virulence for mice, three *Eucalyptus*-originated strains (B-4506, B-4507, and B-4534) and the type strain of *C. neoformans* var. *gattii*, B-3939 (serotype B, ATCC 32269), were used. Strain B-3939 and a type strain of *F. neoformans* var. *bacillispora* (teleomorph of *C. neoformans* var. *gattii*), NIH 191 (serotype C, ATCC 32608), were previously shown not to cause fatal infection in mice within 30 days after intravenous injection of 10^6 or 10^7 cells. The yeast cells were grown on YEPD agar for 48 h and suspended in sterile saline. Serial dilutions were made to contain 5×10^6 viable cells per ml as determined by hemocytometer count and by a plate count. Three female BALB/c white mice, each weighing 19 to 20 g, were used for each strain and were injected with 0.2 ml of inoculum (10^6 cells) in the lateral tail vein. The survival of mice was observed during a 30-day period. The fungi were recovered from organs of mice by plating aseptically minced brains, lungs, and spleens on YEPD agar.

DNA-RNA hybridization. The DNA probe (AccuProbe)

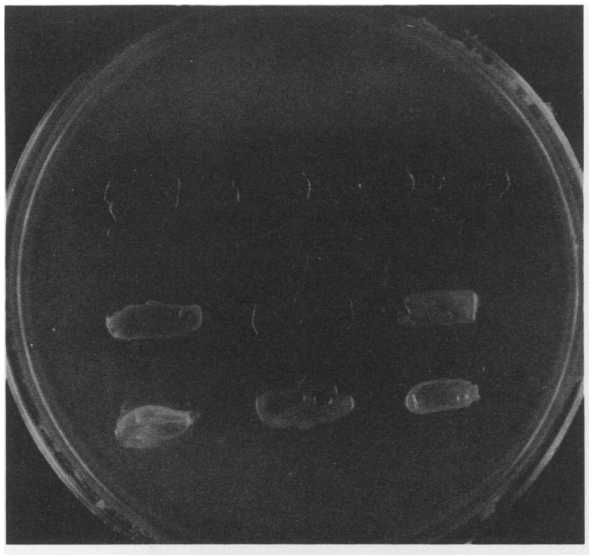

FIG. 1. Colonies of serotype B *C. neoformans* var. *gattii* strains grown on niger seed agar for 24 h at 30°C. Upper row: four *Eucalyptus*-originated strains (B-4506, B-4507, B-4508, and B-4534). Middle and bottom rows: six clinical strains (middle row, B-3939, B-444, and NIH 179; bottom row, NIH 198, NIH 184, and NIH 409).

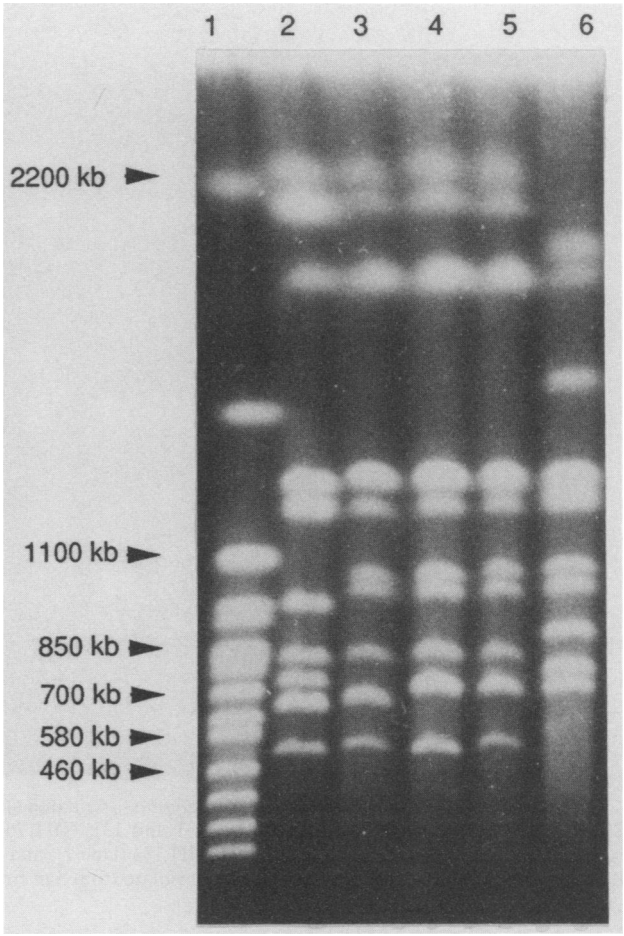

FIG. 2. Karyotypes of *C. neoformans* var. *gattii* strains from *E. camaldulensis* and the type strain of *C. neoformans* var. *gattii*. Lanes: 1, *Saccharomyces cerevisiae*; 2, B-4534, from *E. camaldulensis* in San Francisco; 3 to 5, B-4506, B-4507, and B-4508, respectively, from *E. camaldulensis* in Australia; 6, B-3939, the type strain of *C. neoformans* var. *gattii*.

used for the confirmation of *C. neoformans* cultures was supplied by Gen-Probe Inc. (San Diego, Calif.). The probe is a single-stranded, rDNA sequence specific for *C. neoformans* linked to an acridinium ester (10). Approximately 1 mm^2 of a yeast colony grown on a YEPD agar slant for 72 h at 30°C was inoculated into a tube containing glass beads and 200 μl of lysing agent. The cells were sonicated for 15 min in a water bath sonicator at room temperature to release the RNA. The tube was then heated to 95°C for 15 min to inactivate viable cells. One hundred microliters of yeast lysate was added to a *C. neoformans* AccuProbe tube. The probe was allowed to hybridize with the rRNA present in the lysate at 60°C for 15 min. A selection reagent was added to inactivate unbound probe, and the tube was incubated at 60°C for 5 min. Chemiluminescence was measured in a luminometer (LEADER I; Gen-Probe), and relative light units (RLU) were recorded. The final chemiluminescent signal detected is proportional to the amount of hybridized probe. Any reaction greater than 50,000 RLU was considered positive. The positive control strain used was the type strain of *C. neoformans*, ATCC 32045 (CBS 132).

The strains used for the test (Table 1) were the four *Eucalyptus*-originated *C. neoformans* var. *gattii* strains, clinical isolates of the two varieties of *C. neoformans*, and various species of yeasts taxonomically related to *C. neoformans*, including a strain of Mel^+ *C. laurentii* isolated from *E. camaldulensis*.

RESULTS

Serotype, virulence, and molecular characteristics of *Eucalyptus*-originated strains of *C. neoformans* var. *gattii*. All four strains of *Eucalyptus*-originated *C. neoformans* var. *gattii* were serotype B. All four strains produced highly mucoid colonies on YEPD agar similar to colonies of the clinical isolates of *C. neoformans* var. *gattii*. They were very virulent for mice, killing all nine (100%) between 11 and 26 days postinjection. The brains, lungs, and spleens of the dead mice yielded confluent growth of *C. neoformans* var. *gattii*. The numbers of yeast CFU were highest in the brain (too numerous to count), with fewer CFU in the spleen (150 to 200 CFU) and the lung (72 to 100 CFU). The three mice injected with cells of the type strain, B-3939, appeared healthy and gained weight during the 30-day period. They were sacrificed on day 30, and the organs were cultured. CFU were not recovered from the spleens or lungs of any of the mice. Two of the three mice contained 198 and 238 CFU in the brain, while the remaining mouse showed no CFU in the brain.

On niger seed agar, all four strains produced chocolate brown colonies within 24 h. The expression of phenol oxidase activity was markedly higher than it was for the majority of the clinical isolates of serotype B *C. neoformans* var. *gattii*. Figure 1 shows the colony characteristics of the four *Eucalyptus*-originated cultures and six strains from clinical specimens which have been used as reference strains for serotype B in our laboratory. Clinical isolates of serotype B *C. neoformans* var. *gattii* have been generally known to take longer to produce brown colonies on niger seed agar than isolates of *C. neoformans* var. *neoformans* (22).

The electrophoretic karyotypes of the three *Eucalyptus*-

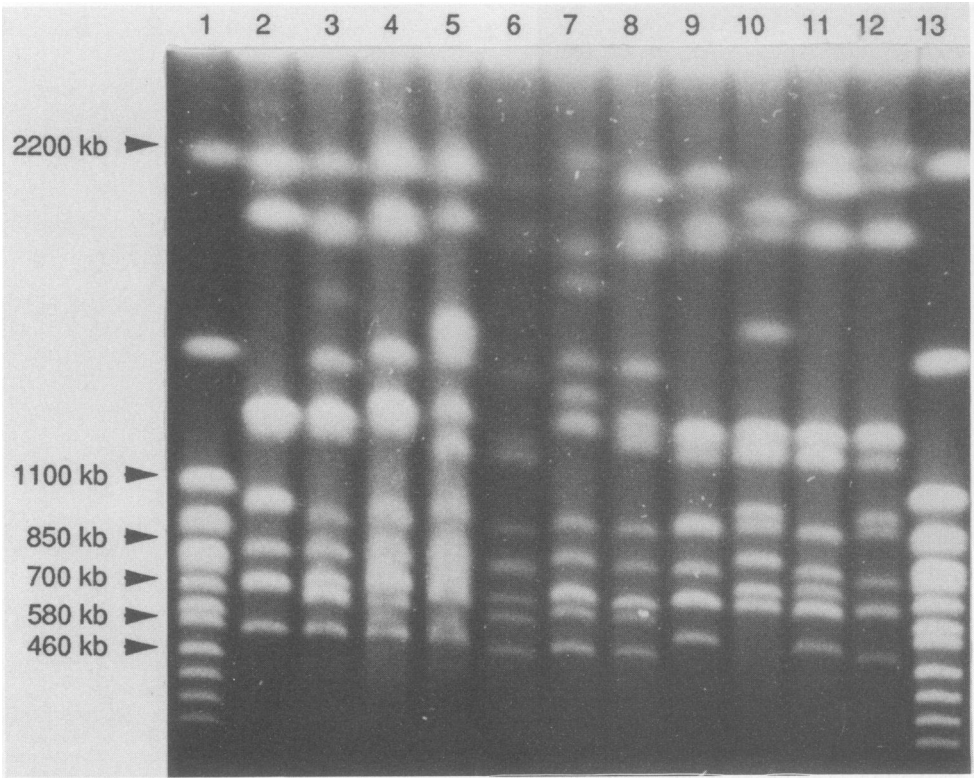

FIG. 3. Comparison of karyotypes of *Eucalyptus*-originated strains and nine serotype B clinical isolates from various geographic regions. Shown are *Saccharomyces cerevisiae* (lanes 1 and 13); NIH 76 from New Jersey (lane 2); NIH 744 (lane 3) and NIH 771 (lane 4) from Alabama; NIH 179 (lane 5), R-438 (lane 6), NIH 184 (lane 7), and NIH 189 (lane 8) from California; B-4536 (lane 9) and B-3939 (lane 10) from Zaire; B-4534 (lane 11), *Eucalyptus*-originated isolate from San Francisco; and B-4506 (lane 12), *Eucalyptus*-originated isolate from Australia.

originated strains from Australia were identical, in spite of their geographic differences (Fig. 2). A total of 10 clearly separated bands, with a size range of about 2.5 Mb to 520 kb, were found in these strains. Three of the 10 bands appeared to have been doublets, which makes the total number of chromosomes 13. The karyotype of the *Eucalyptus*-originated strain from San Francisco was the same as that of the Australian strains except in the mobility of one band (Fig. 2). On the contrary, no two clinical isolates had identical karyotypes (9 or 10 separated bands), and none of them had a karyotype identical to that of the *Eucalyptus*-originated strains either from Australia or from San Francisco (Fig. 3). The variation among the clinical isolates was mostly in the mobility of the largest three chromosomes. For example, the karyotype of the patient isolate from Australia (Fig. 4, lane 4) was the same as that of a *Eucalyptus*-originated isolate from Australia (Fig. 4, lane 3) except that the third and the fourth chromosomes were resolved in the patient isolate, resulting in 11 instead of 10 separated bands. Another example was found in one of the Brazilian patient isolates (Fig. 4, lane 1), which showed an identical pattern to that of the *Eucalyptus*-originated strain from San Francisco (Fig. 4, lane 2) except that the first two chromosomes migrated together, resulting in 9 instead of 10 separated bands.

The type strain of *C. neoformans* var. *gattii*, B-3939 (Fig. 3, lane 10), from an African patient showed a karyotype distinct from that of another African clinical isolate (Fig. 3, lane 9) as well as from those of the remaining nine clinical isolates from various geographical regions: the largest chromosome of about 2.2 Mb and the smallest chromosome of about 520 kb were missing, but a chromosome of about 1,800 kb which was not found in other strains was present (Fig. 3, lane 10). The Mel^+ strain of *C. laurentii* (Fig. 5, lane 5) isolated from *E. camaldulensis*, which mimicked *C. neoformans* var. *gattii* on CGB agar, had a karyotype distinct from those of *C. neoformans* var. *gattii* of both clinical and *Eucalyptus* origin. The electrophoretic karyotypes of the isolates recovered from the mice fatally infected with two *Eucalyptus*-originated strains, B-4507 and B-4534, were identical to those of the parent strains.

None of the four *Eucalyptus*-originated strains mated or produced dikaryotic hyphae when crossed with each other or with tester strains of the two varieties of *F. neoformans*. The α and *a* tester strains of *F. neoformans* var. *neoformans* mated and produced abundant basidia with viable basidiospores, while those of *F. neoformans* var. *bacillispora* failed to mate. The α and *a* tester strains of *F. neoformans* var. *neoformans*, however, previously produced dikaryotic hyphae when crossed with the opposite mating type of *F. neoformans* var. *bacillispora* (13).

The AccuProbe assay resulted in all isolates having RLU readings greater than 500,000, as was the case for the patient isolates of the two varieties of *C. neoformans* (Table 1).

AccuProbe assay results for other yeasts taxonomically related to *C. neoformans*. The perfect state of *C. neoformans*, *F. neoformans*, belongs to the family Filobasidiaceae. The family contains two other genera, *Filobasidium* and *Cystofilobasidium*, in addition to *Filobasidiella* (14). *Filobasidium* is phylogenetically closer to *Filobasidiella*, as determined by 18S and 25S rRNA sequence homology (11). To test the

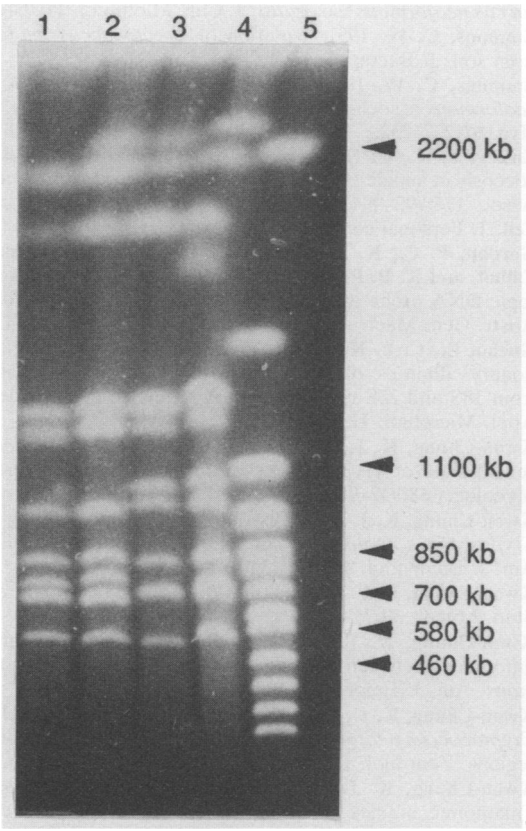

FIG. 4. Karyotypes of NIH 997 (lane 1) from a Brazilian patient, B-4534 (lane 2) from *E. camaldulensis* in San Francisco, B-4506 (lane 3) from *E. camaldulensis* in Australia, B-4537 (lane 4) from an Australian patient, and *Saccharomyces cerevisiae* (lane 5).

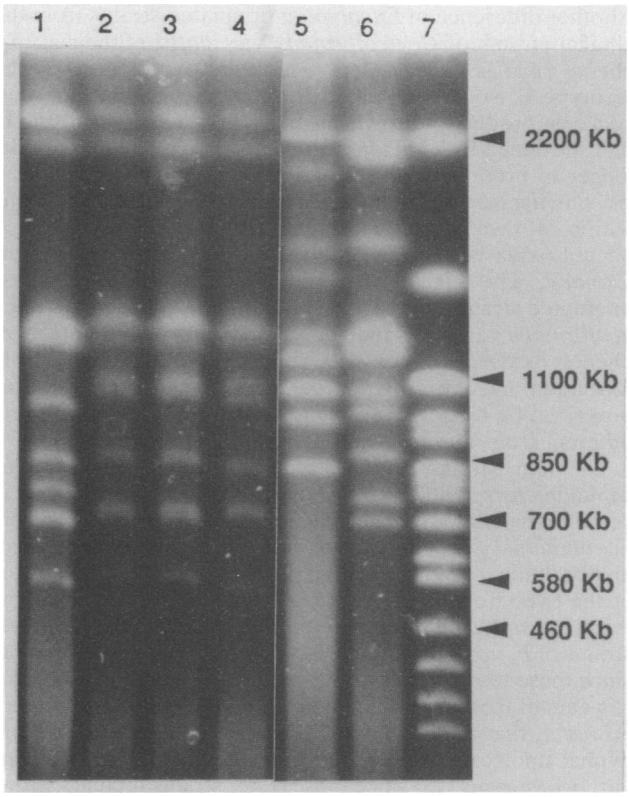

FIG. 5. Karyotypes of *Eucalyptus*-originated *C. neoformans* var. *gattii* and Mel$^+$ *C. laurentii*. Shown are *C. neoformans* var. *gattii* B-4534 (lane 1), B-4506 (lane 2), B-4507 (lane 3), and B-4508 (lane 4); Mel$^+$ *C. laurentii* (lane 5); B-3939 (lane 6), the type strain of *C. neoformans* var. *gattii*; and *Saccharomyces cerevisiae* (lane 7).

specificity of the AccuProbe, two mating strains of *Filobasidium floriforme*, *Filobasidium uniguttulatum*, *Filobasidium capsuligenum*, and a birdseed agar-positive isolate of *C. laurentii* from *E. camaldulensis* were compared with the two varieties of *C. neoformans* in the AccuProbe assay. Although *C. laurentii* does not produce a teleomorph belonging to the Filobasidiaceae, it is closely related to the members of the Filobasidiaceae on the basis of the nucleotide sequence homology of the large-subunit rRNA (9a). *C. laurentii* is also more closely related to *C. neoformans* var. *gattii* than to other species of the genus *Cryptococcus* in that it fails to assimilate nitrate and produces a positive reaction on CGB agar. As shown in Table 1, the hybridization results showed that only *C. neoformans*, including the type strains of *F. neoformans*, produced values exceeding 50,000 RLU. The *Filobasidium* species and *C. laurentii* all produced RLU of less than 11,000.

DISCUSSION

It was surprising to find that the karyotypes of three strains of *C. neoformans* var. *gattii* isolated from *E. camaldulensis* in Australia were identical and very similar to that of the San Francisco isolate because the karyotypes of *C. neoformans* var. *gattii* from clinical sources are so heterogeneous that we have yet to see two isolates with identical banding patterns. *E. camaldulensis* has been exported extensively from Australia to other geographic regions, including California (6). Pfeiffer and Ellis hypothesized that *C. neoformans* var. *gattii* was exported to California by contaminated seedlings or seed of Australian *E. camaldulensis* (23). The similar karyotypes of *Eucalyptus*-originated strains from San Francisco and Australia support their hypothesis of a common origin. Since the karyotypes of clinical strains were heterogeneous and different from those of *Eucalyptus*-originated strains, it is possible that chromosomal rearrangement occurs during infection in human tissue. Karyotypes of the isolates recovered from the mouse brain, however, showed no chromosomal rearrangement, but yeast cells from human infections may be subjected to a different type of stress conducive to rearrangement. Karyotypes of *C. neoformans* appear to be extremely stable in vitro since no change has been observed in the cultures that have been maintained on agar media for the past 15 years.

It was also surprising to find the *Eucalyptus*-originated cultures to be so virulent for mice. The mouse has never been proven to be a good animal model for clinical isolates of *C. neoformans* var. *gattii*; several clinical strains of either serotype B or C tested in a previous study failed to cause fatal infection with 10^6 to 10^7 cells inoculated intravenously (17). As expected, the type strain, B-3939, failed to cause fatal infection, and the lungs and spleens of the mice receiving 10^6 cells were found to be free from CFU by day 30. The fungus was recovered only from the brains of two of three mice, and the CFU recovered were much fewer than from the mice injected with *Eucalyptus*-originated strains.

The mouse virulence of *Eucalyptus*-originated strains is about equal to that of the tester strains of *C. neoformans* var. *neoformans*. With the injection of 10^6 cells intravenously, usually 100% of the mice are dead within 4 weeks (17).

Another difference of *Eucalyptus*-originated strains from the clinical strains of *C. neoformans* var. *gattii* is their strong phenol oxidase activity on niger seed agar. Most of the serotype B isolates obtained from cases of human cryptococcosis produce a brown pigment with less intensity than that produced by *C. neoformans* var. *neoformans* and take longer to produce it (22). The brown pigment produced by the *Eucalyptus*-originated *C. neoformans* var. *gattii* strains within 24 h was as intense as that produced by the strongly phenol oxidase-positive strains of *C. neoformans* var. *neoformans*. The higher virulence for mice of *Eucalyptus*-originated strains compared with that of the type strain of *C. neoformans* var. *gattii* may be associated with their stronger phenol oxidase activity (19, 20). The degree of capsule formation and the growth rate at 37°C, two other features proven to be related to virulence for mice (19, 20), were no different from those of the type strain.

The four strains of *C. neoformans* var. *gattii* from *E. camaldulensis* failed to mate among themselves, and it is possible that all four strains of *C. neoformans* var. *gattii* from *E. camaldulensis* belong to the same mating type. A predominance of the α type has been reported among clinical isolates of the two varieties of *C. neoformans* (13). The four strains from *E. camaldulensis* failed to mate with the α and *a* tester strains of *F. neoformans* var. *gattii*, which was not surprising, since these tester strains have lost their mating ability during 15 years of laboratory maintenance on agar. It was expected, however, that the *Eucaplytus*-originated isolates would form hyphae upon crossing with the tester strains of *F. neoformans* var. *neoformans* (13) since these tester strains retained strong mating ability and the *Eucalyptus*-originated strains were maintained on agar medium for less than 2 years.

It is interesting that a Mel^+ strain of *C. laurentii* can be isolated from *E. camaldulensis*. The colonies of this strain grown on niger seed agar produced a brown color but of less intensity than that produced by *Eucalyptus*-originated *C. neoformans* var. *gattii*. The Mel^+ strain of *C. laurentii* produced a positive reaction on CGB agar and grew at 37°C, mimicking *C. neoformans* var. *gattii*; however, the *C. laurentii* isolate assimilated lactose. Although it is extremely rare, we have previously encountered an isolate of *C. laurentii* that was Mel^+. The strain was isolated from tar on a street in Long Beach, Calif., and was sent to us by the late Milton Huppert. It is possible that the strain may have originated from *E. camaldulensis* since this tree is indigenous to that area. Others have also observed Mel^+ *C. laurentii* strains (25).

The AccuProbe test for *C. neoformans* culture confirmation was very specific and confirmed the identity of *C. neoformans*. None of the yeasts known to be phylogenetically related to *C. neoformans* yielded positive results by this method.

REFERENCES

1. **Ajello, L.** 1958. Occurrence of *Cryptococcus neoformans* of saprophytic origin. Am. J. Hyg. **67:**72–77.
2. **Bauwens, L., D. Swinne, C. De Vroey, and M. De Meurichy.** 1986. Isolation of *Cryptococcus neoformans* var. *neoformans* in the aviaries of the Antwerp zoological gardens. Mykosen **29:** 291–294.
3. **Bennett, J. E., K. J. Kwon-Chung, and D. H. Howard.** 1977. Epidemiologic differences among serotypes of *Cryptococcus neoformans*. Am. J. Epidemiol. **105:**582–586.
4. **Cobcraft, R., H. Kronenberg, and T. Wilson.** 1978. *Cryptococcus* in bagpipes. Lancet **i:**1358–1360.
5. **Ellis, D. H.** 1987. *Cryptococcus neoformans* var. *gattii* in Australia. J. Clin. Microbiol. **25:**430–431.
6. **Ellis, D. H., and T. F. Pfeiffer.** 1990. Natural habitat of *Cryptococcus neoformans* var. *gattii*. J. Clin. Microbiol. **28:**1642–1644.
7. **Emmons, C. W.** 1951. Isolation of *Cryptococcus neoformans* from soil. J. Bacteriol. **62:**685–690.
8. **Emmons, C. W.** 1955. Saprophytic sources of *Cryptococcus neoformans* associated with the pigeon (*Columbia livia*). Am. J. Hyg. **62:**227–232.
9. **Ensley, P. K., C. E. Davis, and K. C. Fletcher.** 1979. Cryptococcosis in a male Beccari's crowned pigeon. J. Am. Vet. Med. Assoc. **175:**992–994.

9a. **Fell, J.** Personal communication.

10. **Gordon, P. C., K. A. Clark, C. Milliman, M. Majlessi, L. A. Talbot, and K. D. Pusateri.** 1991. Development of rapid nonisotopic DNA probe assays for fungi: *Cryptococcus neoformans*. Abstr. Gen. Meet. Am. Soc. Microbiol. 1991 F-81, p. 422.
11. **Gueho, E., C. P. Kurtzman, and S. W. Peterson.** 1989. Evolutionary affinities of heterobasidiomycetous yeasts estimated from 18S and 25S ribosomal RNA sequence divergence. Syst. Appl. Microbiol. **12:**230–236.
12. **Kwon-Chung, K. J.** 1976. A new species of *Filobasidiella*, the sexual state of *Cryptococcus neoformans* B and C serotypes. Mycologia **68:**942–946.
13. **Kwon-Chung, K. J.** 1978. Distribution of α and *a* mating type of *Cryptococcus neoformans* among natural and clinical isolates. Am. J. Epidemiol. **108:**337–340.
14. **Kwon-Chung, K. J.** 1987. Filobasidiaceae—a taxonomic survey. Stud. Mycol. **30:**75–85.
15. **Kwon-Chung, K. J., and J. E. Bennett.** 1984. Epidemiologic differences between the two varieties of *Cryptococcus neoformans*. Am. J. Epidemiol. **120:**123–130.
16. **Kwon-Chung, K. J., and J. E. Bennett.** 1984. High prevalence of *Cryptococcus neoformans* var. *gattii* in tropical and subtropical regions. Zentralbl. Bakteriol. Hyg. **257:**213–218.
17. **Kwon-Chung, K. J., J. E. Bennett, and J. C. Rhodes.** 1982. Taxonomic studies on *Filobasidiella* species and their anamorphs. Antonie van Leeuwenhoek J. Microbiol. Serol. **48:**25–38.
18. **Kwon-Chung, K. J., I. Polacheck, and J. E. Bennett.** 1982. Improved diagnostic medium for separation of *Cryptococcus neoformans* var. *neoformans* (serotypes A and D) and *Cryptococcus neoformans* var. *gattii* (serotypes B and C). J. Clin. Microbiol. **15:**535–537.
19. **Kwon-Chung, K. J., I. Polacheck, and T. J. Popkin.** 1982. Melanin-lacking mutants of *Cryptococcus neoformans* and their virulence for mice. J. Bacteriol. **150:**1212–1220.
20. **Kwon-Chung, K. J., and J. C. Rhodes.** 1986. Encapsulation and melanin formation as indicators of virulence in *Cryptococcus neoformans*. Infect. Immun. **51:**218–223.
21. **Kwon-Chung, K. J., A. K. Varma, and D. H. Howard.** 1990. Ecology of *Cryptococcus neoformans* and prevalence of its two varieties in AIDS and non-AIDS associated cryptococcosis, p. 103–113. *In* H. Vanden Bossche and D. W. R. Mackenzie (ed.), Mycoses in AIDS patients. Plenum Press, New York.
22. **Nurudeen, T. A., and C. G. Ahearn.** 1989. Regulation of melanin production by *Cryptococcus neoformans*. J. Clin. Microbiol. **10:**724–729.
23. **Pfeiffer, T., and D. Ellis.** 1991. Environmental isolation of *Cryptococcus neoformans gattii* from California. J. Infect. Dis. **163:**929–930.
24. **Raper, K. B., and D. J. Fennell.** 1965. The genus *Aspergillus*, p. 39. The Williams & Wilkins Co., Baltimore.
25. **Salkin, I. F., M. R. McGinnis, and G. A. Land.** 1989. Evaluation of selective rapid procedures for the identification of *Cryptococcus neoformans* in the clinical laboratory, S-18. *In* Proceedings of the International Conference of *Cryptococcus* Cryptococcosis, Jerusalem.
26. **Staib, F.** 1962. *Cryptococcus neoformans* und *Guizotia abyssinica*—(Syn. *G. oleifera* D.C.). Z. Hyg. **148:**466–475.
27. **Swinne-Desgain, D.** 1975. *Cryptococcus neoformans* of saprophytic origin. Sabouraudia **13:**303–308.
28. **Wickes, B. L., J. E. Golin, and K. J. Kwon-Chung.** 1991. Chromosomal rearrangement in *Candida stellatoidea* results in a positive effect on phenotype. Infect. Immun. **59:**1762–1771.