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Investigation of the basis of virulence in serotype A strains of *Cryptococcus neoformans* from apparently immunocompetent individuals

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Abstract *Cryptococcus neoformans* serotype A strains commonly infect immunocompromised patients to cause fungal meningitis. To understand the basis of serotype A cryptococcal infections in apparently immunocompetent patients, we tested two hypotheses: the strains were naturally occurring hypervirulent *pkrl* (PKA regulatory subunit) mutants, or the strains were hybrids with *C. neoformans* var. *gattii* strains that normally infect immunocompetent individuals. Analysis of clinical isolates obtained from apparently immunocompetent individuals from three continents revealed that none were *pkrl* mutants, but several exhibited phenotypes consistent with perturbations in cAMP signaling. Additionally, none of the strains were unusual hybrids with *gattii* strains. Except for one strain that was an AD hybrid, all others were serotype A (var. *grubii*) isolates. Taken together, our findings indicate that the ability of these clinical isolates to infect apparently normal individuals may be attributable to mutations other than *pkrl* and/or underlying immune system impairment in patients.

Keywords *Cryptococcus* · Virulence · Fungal pathogen · Protein kinase

Introduction

Cryptococcus neoformans is an encapsulated, basidiomycetous yeast that is one of the most common fungal pathogens in AIDS and other immunocompromised patients (for reviews, see Casadevall and Perfect 1998; Hull and Heitman 2002). The prevalence of cryptococcal meningitis has increased worldwide as a result of human immunodeficiency virus (HIV) infection, solid organ transplantation, cytotoxic chemotherapy, and systemic corticosteroid use. Known virulence factors include an antiphagocytic polysaccharide capsule (Chang et al. 1996; Granger et al. 1985; Kwon-Chung and Rhodes 1986), melanin produced in the cell wall as an antioxidant (Kwon-Chung et al. 1982c; Salas et al. 1996; Wang et al. 1995), production of the enzyme urease (Cox et al. 2000), the ability to grow at 37°C (Kwon-Chung and Rhodes 1986; Odom et al. 1997), and the *MAT α* mating type locus in serotype D strains (Kwon-Chung et al. 1992).

Cryptococcal infection is initiated by the inhalation of spores or desiccated yeast cells into the lung; and the organism then spreads via the bloodstream to the central nervous system, where it causes meningoencephalitis that is fatal if untreated. *C. neoformans* is a facultative intracellular pathogen and macrophages that have engulfed fungal cells contain multiple intracellular vesicles filled with capsular polysaccharide (Feldmesser et al. 2000). The capsule likely contributes to virulence by interfering with immune cell function as shed capsular antigen, inhibiting phagocytosis of fungal cells and enhancing the intracellular survival of fungal cells within macrophages (Granger et al. 1985; Feldmesser et al. 2000).

Based on the antigenicity of the capsular polysaccharides, *C. neoformans* can be classified into four distinct serotypes (A, B, C, D), all of which are human pathogens. The four serotypes are further classified into three varieties: serotype A corresponds to var. *grubii*,

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serotypes B and C to var. *gattii*, and serotype D to var. *neoformans* (Franzot et al. 1999; Kwon-Chung and Bennett 1984). While strains of the *neoformans* and *grubii* varieties typically infect immunocompromised individuals, var. *gattii* strains infect immunocompetent individuals (for a review, see Sorrell 2001). The molecular basis of these unique predilections of cryptococcal infection is largely unknown. Because serotype A strains typically infect immunocompromised patients, here we investigated the basis of virulence in serotype A strains isolated from apparently immunocompetent individuals.

A conserved G α protein-cAMP-PKA signal transduction cascade operates during virulence and differentiation of *C. neoformans* (Alsbaugh et al. 1997; 2002; D'Souza et al. 2001). In serotype A strains, the Pka1 catalytic subunit of protein kinase A regulates mating, melanin and capsule production, and virulence. The Pkr1 regulatory subunit of PKA is also a critical component; and serotype A mutants lacking Pkr1 overproduce capsule and are hypervirulent. Thus, both the larger capsule size and increased release of immunosuppressive capsular polysaccharides likely contribute to enhanced virulence in *pkrl* mutant cells during infection. Pkr1 does not regulate virulence in serotype D strains

and, unlike serotype A *pkrl* strains, serotype D *pkrl* mutant strains do not significantly differ in virulence from wild-type strains (Hicks et al. 2004). Because the virulence of *C. neoformans* serotype A strains can be increased by mutations, we hypothesized that this may be occurring in strains that infect individuals with no apparent defects in immune system function. Here, we analyzed serotype A clinical isolates obtained from individuals with no apparent immune system deficiency and from moderately immunocompromised individuals across three continents. We determined whether their virulence potential could be accounted for by alterations in the PKA pathway that controls virulence and also tested the possibility that the isolates may be unusual hybrids with var. *gattii* strains that normally infect immunocompetent individuals.

Materials and Methods

C. neoformans strains, plasmids, and media

All strains used in this study are listed in Table 1. *C. neoformans* strains were grown on standard *Saccha-*

Table 1 *C. neoformans* clinical isolates from apparently normal individuals

Strain	Serotype	Country of origin	Tissue isolated from	Brief patient history	Source
TH1	AD	UK	CSF	Intravenous drug abuse, meningitis, non-immunosuppressed, no travel history, CD4 cell count normal	T. Harrison
EF	A	USA	CSF	49-year-old, female, insulin dependent-diabetes, meningitis	B. Alexander-Lodge
NIH438	A	USA	CSF	Non-HIV with meningitis	E. Jacobson
8	A or D	USA	CSF	Untreated, previously normal	J. Bennett
13	A	USA	CSF	Untreated, previously normal	J. Bennett
14	A	USA	CSF	Untreated, previously normal	J. Bennett
25	A	USA	CSF	Untreated, previously normal	J. Bennett
28	A	USA	CSF	Untreated, previously normal	J. Bennett
30	A or AD	USA	CSF	Untreated, previously normal	J. Bennett
32	A	USA	CSF	Untreated, previously normal	J. Bennett
38	A	USA	CSF	Untreated, previously normal	J. Bennett
41	A	USA	CSF	Untreated, previously normal	J. Bennett
43	A	USA	CSF	Untreated, previously normal	J. Bennett
51	A	USA	CSF	Untreated, previously normal	J. Bennett
56	A	USA	CSF	Untreated, previously normal	J. Bennett
516	A	USA	Sinus above rib lesion	Previously normal, rib osteomyelitis, failing fluocytosine therapy	J. Bennett
Duyn	A	Australia	Unknown	Non-HIV	W. Meyer
ICPMR 98-181-1361	A	Australia	Bronchial wash	Non-HIV, CD4 lymphocytopenia patient, immunosuppressed	W. Meyer
PAH-21b	A	Australia	Skin	Non-HIV, asthma patient on steroids	W. Meyer
PAH-22	A	Australia	Lung	Non-HIV, systemic lupus erythematosus patient on steroids	W. Meyer
QEH-6	A	Australia	CSF	Non-HIV	W. Meyer
RNS-13a	A	Australia	Blood	Non-HIV, multiple myeloma patient	W. Meyer
RNS-13b	A	Australia	Urine	Non-HIV, multiple myeloma patient	W. Meyer
RNS-13c	A	Australia	CSF	Non-HIV, multiple myeloma patient	W. Meyer
RNS-13d	A	Australia	Bronchial wash	Non-HIV, multiple myeloma patient	W. Meyer
RNS-13e	A	Australia	Lingual wash	Non-HIV, multiple myeloma patient	W. Meyer
STG-1	A	Australia	Lung	Non-HIV	W. Meyer
W-20	A	Australia	CSF	Non-HIV	W. Meyer
W-24	A	Australia	CSF	Non-HIV	W. Meyer

romyces cerevisiae media (Sherman 1991). The selective medium for biolistic transformation (Toffaletti et al. 1993), Niger seed agar (Alspaugh et al. 1997), low-iron medium (LIM; Vartivarian et al. 1995), filament agar (Wickes et al. 1996), V8 agar (Alspaugh et al. 1997), CGB agar (Kwon-Chung et al. 1982b; Min and Kwon-Chung 1986), and YNB-1% malate agar (Bennett et al. 1978) were prepared as previously described.

Serotyping

Serotype analysis was performed as described by Lengeler et al. (2001), using the Crypto Check serotyping kit (Iatron Laboratories, Tokyo, Japan). Serotyping was confirmed by PCR, using serotype-specific primers as described by Lengeler et al. (2001).

AFLP analysis

DNA isolation and AFLP analysis of clinical isolates was performed as described by Boekhout et al. (2001).

Isolation of the *PKRI* gene from clinical isolates

PCR was conducted using genomic DNA from the clinical isolates as template and the following primers: JOHE3245 (TTCTCCACAGCTCTACTC) and JOHE3180 (CTGTTGGAGAAAATCAGGG). The 50- μ l PCR reactions contained 1 \times Ex-Taq buffer, 1 mM dNTPs, 1.25 units Ex-Taq (Takara), and primers JOHE3245 and JOHE3180. PCR reaction conditions were: 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min, with a final extension for 5 min at 72°C. Both primer pairs yielded a 2.5-kb PCR product, bearing the promoter and entire open reading frame of *PKRI*, that was cloned in the TOPO-TA cloning vector pCR2.1 (Invitrogen).

Southern hybridization

Genomic DNA was isolated as described by Perfect et al. (1993). DNA was digested with restriction enzymes, subjected to agarose gel electrophoresis, and analyzed by Southern blot (Sambrook et al. 1989). The radiolabeled probe was synthesized using the Prime-It II DNA labeling kit (Stratagene) and ³²P-dCTP (Amersham). The template for the *PKRI* gene was a 1.6-kb *Bam*HI cDNA fragment.

PKRI complementation test

A 4-kb *Hind*III fragment bearing the entire *PKRI* gene was inserted into plasmid pCH233, which bears the nourseothricin resistance selectable marker (*nat1*) for *C. neoformans*. The resulting plasmid pCD62 was then

introduced into the clinical strains by biolistic transformation (Toffaletti and Perfect 1994) and transformants were isolated on yeast extract/peptone/dextrose (YPD) medium with 1 M sorbitol. After 4 h incubation at 30°C, cells were collected, washed with water, diluted, and plated onto YPD medium containing 70 μ g/ml nourseothricin. Genomic DNA was isolated from nourseothricin-resistant transformants; and Southern analysis verified that the *PKRI* gene had been introduced into the clinical isolates.

Virulence assays

In the mouse inhalation model, 10⁵ yeast cells of the *grubii* type strain (H99) and strains 28, ICPMR, and STG1 were each used to infect ten 4- to 6-week-old A/Jcr mice by intranasal inoculation. Mice were first anesthetized by intraperitoneal administration of phenobarbital and then suspended by their incisors on a silk thread to fully extend the neck. Inocula were prepared in 50- μ l volumes and slowly pipetted directly into the nares; and the mice were kept suspended for 10 min. The mice were fed ad libitum and monitored twice daily. Mice that appeared to be moribund or in pain were sacrificed by CO₂ inhalation. Statistical analysis of murine cryptococcosis was performed by the Kruskal–Wallis test for significance. Significance in data was considered at *P* values \leq 0.05. The protocol was approved by the Duke University Animal Use Committee.

PCR to determine *gattii* varietal status of clinical isolates

The *PLBI* gene (*gattii*)-specific PCR primers JOHE7819 (AAAACGCTTTTCATTTATGAAG) and JOHE7824 (GCAAAAAAGAACAACAAAAA) were used to determine whether the clinical isolates were var. *gattii*. PCR was also carried out with genomic DNA templates from the clinical isolates and A/D specific primers JOHE7830 (TTTCTGCTTTTCACCCATT) and JOHE7833 (TGAAACAATACATCATGC). Control strains used were H99 (serotype A), WM276 (serotype B), VPC1-87 (serotype C), and JEC21 (serotype D). PCR reaction parameters using Ex-Taq polymerase were: 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 1.5 min. PCR products were run on a 1% agarose gel. Both the *gattii*-specific and A/D-specific primers yielded a ca. 1.0-kb product.

The *SOD1* gene (*gattii*)-specific PCR primers JOHE7773 (GATCCTCACGCCATTACG) and JOHE7775 (GAATGATGCGCTTAGTTGGA) were also used to determine whether the clinical isolates were var. *gattii*. Again, PCR was carried out with A/D specific primers JOHE7777 (TTCAACCACGAATATGTA) and JOHE7779 (AAGCCTTCATCCATATCTT). PCR reaction conditions were: 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 1.5 min. PCR products were run on a 1% agarose gel.

Both the *gattii*-specific and A/D-specific primers yielded a ca. 1.0-kb product.

Results

Collection of *C. neoformans* clinical isolates for testing

The strains collected in this study were obtained from a variety of sources from three different continents: North America, Europe, and Australia (see Table 1). Strains met two criteria to be included. First, they were serotype A (var. *gattii* strains of serotypes B and C were excluded, due to their known propensity to infect immunocompetent individuals). Second, they were isolated from previously (prior to cryptococcal infection) healthy individuals with normal immune function. Although these criteria were strictly adhered to, some limitations became apparent later. One limitation was delay or difficulty in obtaining patient records due to maintenance of privacy. Isolates that were later found to be derived from patients of compromised health were retained in the study. For example, strain ICPMR was obtained from an HIV-negative patient with idiopathic T-cell lymphocytopenia. Our rationale was that inclusion of strains from patients with varying levels of immune impairment would assist in assessing whether virulence potential was strain-dependent or host-dependent.

The clinical isolates were verified as *C. neoformans* by a variety of tests, including melanin production on Niger seed agar (data not shown), urease activity (data not shown), carbohydrate assimilation tests using the API20C AUX yeast identification system (bioMérieux; data not shown), and serotyping (Table 1). In addition, PCR with *C. neoformans* serotype and/or mating type-specific primers (Table 2) and AFLP analysis (Fig. 1) confirmed that most isolates were *C. neoformans* serotype A and MAT α , with the exception of strain TH1, which was an α ADa hybrid isolate. Further mating type testing with a different set of mating type- and/or serotype-specific primers confirmed this conclusion (data not shown).

Capsule production in clinical isolates

To test the hypothesis that the serotype A clinical isolates chosen for this study had alterations in PKA signaling, capsule production was analyzed. Strains were grown in LIM with or without 10 mM cAMP for 5 days and India ink-stained capsules were then observed by microscopy. Strains varied in their ability to produce capsule and Fig. 2 shows representative isolates. Isolates 38, 41, and 56 were hypocapsular (data not shown), as observed for the cAMP signaling mutants *gal* and *pkal* (Alspaugh et al. 1997; D'Souza et al. 2001). Like *pkal* mutants, these strains were not responsive to cAMP. We also identified isolates that responded to cAMP by producing larger capsules, including isolates STG1 and 516 (data not shown).

Interestingly, cAMP also induced melanin production in strain 516 but not in strain STG1 (data not shown). Strains ICPMR and 28 produced intermediate to large capsules, similar to the PKA hyperactivated *pkrl* mutant (D'Souza et al. 2001).

Virulence of clinical isolates

The virulence of strains 28 and ICPMR relative to the serotype A reference strain H99 was tested. For comparison, the STG1 strain (which is hypocapsular) and the AD hybrid strain TH1 were included. In the murine model of infection, 10^5 yeast cells of the wild type (H99) and isolates 28, TH1, ICPMR, and STG1 were used to infect 4- to 6-week-old A/Jcr mice by intranasal inoculation; and survival was monitored (Fig. 3). The clinical isolates were all virulent, leading to 100% lethal infection, but all were less virulent than strain H99 in the mouse inhalation model.

Molecular investigation of clinical isolates as candidate *pkrl* mutants

To investigate at the molecular level whether the clinical isolates were *pkrl* mutants, we first used PCR to test whether deletions or insertions were present in the *PKRI* gene. No such mutations were detected in any strain by this analysis (data not shown). To investigate whether strains 28 and ICPMR contained more subtle *pkrl* mutations, the *PKRI* gene was introduced by transformation with a plasmid bearing the *PKRI* gene and the nourseothricin selectable marker (*nat1*). Transformants were selected based on resistance to nourseothricin and were confirmed to contain the linked *PKRI* gene by Southern blot. Capsule production was assessed in the transformed strains after growth in low-iron capsule-inducing medium. As shown in Fig. 4, cAMP-responsive capsule production was restored in the *pkrl* + *PKRI* control strain. Interestingly, in two independent ICPMR + *PKRI* transformants, cAMP-responsive wild-type capsule production was restored (Fig. 4), whereas the 28 + *PKRI* transformant continued to produce enlarged capsules in a cAMP-insensitive fashion (data not shown). Extensive sequence analysis of the ICPMR *PKRI* gene revealed no evidence for any mutations that would result in amino acid substitutions in the *PKRI* gene product. These findings indicate that alterations other than in the *PKRI* gene likely underlie the enhanced capsule production and that overexpression of the wild-type *PKRI* gene can repress capsule production in this isolate.

The clinical isolates are not intervarietal hybrids with *gattii*

Because serotype B or C var. *gattii* strains can infect immunocompetent individuals, we tested whether sero-

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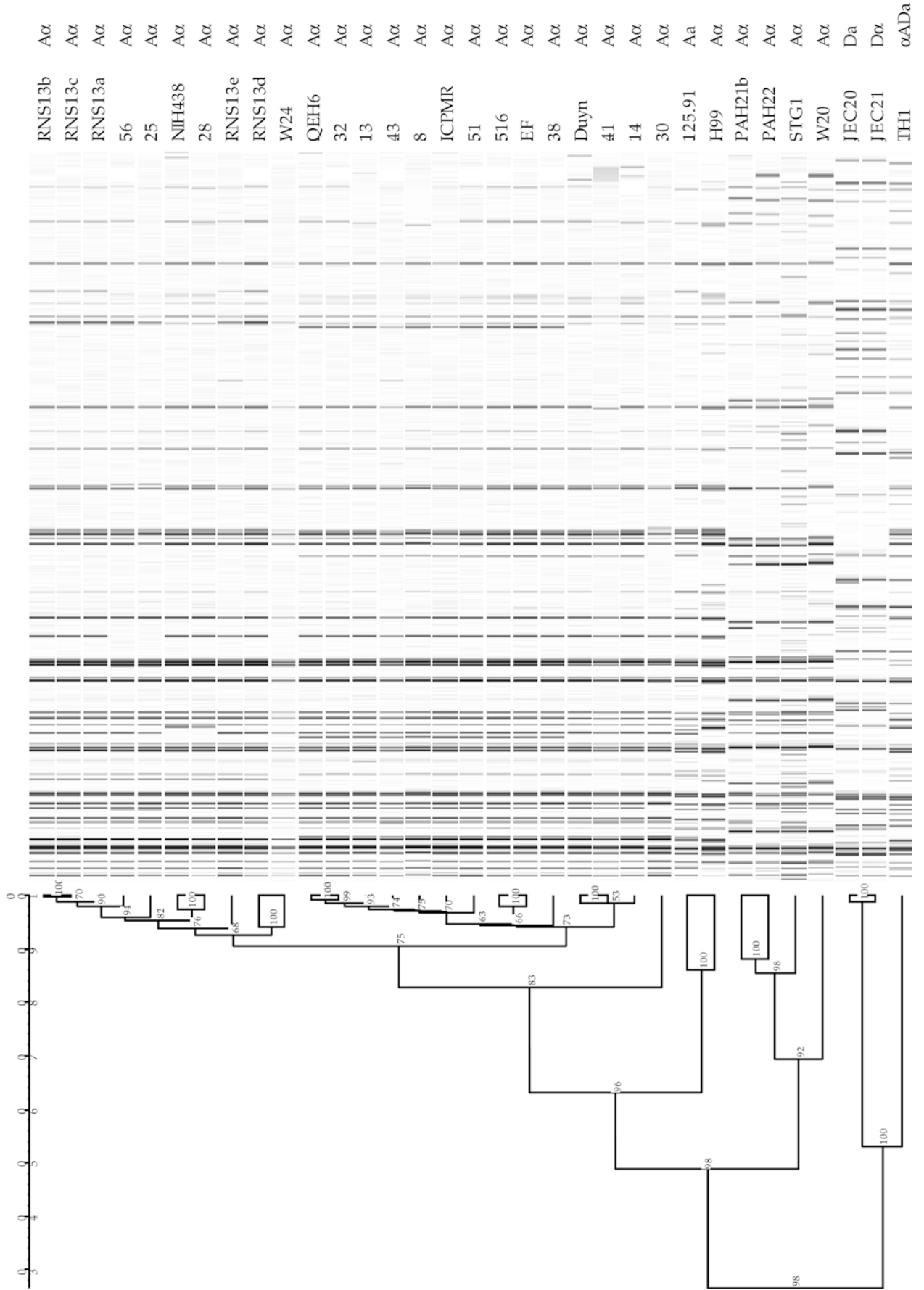




Fig. 1 AFLP analysis of clinical isolates. Clustering of AFLP banding patterns of isolates by the unweighted pair group method, using arithmetic means. Control strains were H99 (α A), 125.91 (α A), JEC20 (α D), and JEC21 (α D). Isolates from RNS13b to W24 form a distinct cluster, as do QEH6 to 14. Isolate 30 forms an individual cluster, as do reference isolates 125.91 and H99. Four isolates (PAH21b, PAH22, STG1, W20) form a distinct cluster sharing approximately 46% similarity only with the other clusters. This cluster is characterized by the presence and absence of at least ten unique bands when compared with the other clusters

type A isolates from apparently immunocompetent hosts represented hybrid strains formed by fusion of *grubii* strains with var. *gattii*. Although the clinical isolates were all serotyped as A, serotyping is a relatively insensitive test and some strains that are AD hybrids by molecular analysis can type as only serotype A or serotype D (Lengeler et al. 2001). We therefore applied additional stringent and sensitive tests of this hypothesis. A number of biochemical tests have been formulated to distinguish *gattii* from *neoformans* and *grubii* varieties. These include growth on canavanine glycine bromthymol (CGB) and malate agar media. The CGB agar test is based on the ability of var. *gattii* strains to utilize glycine

as the sole source of carbon and nitrogen (Kwon-Chung et al. 1982b). Ammonia released upon glycine degradation alkalizes the medium, changing the color from yellow to blue (Min and Kwon-Chung 1986). Additionally, the canavanine in the medium inhibits the growth of var. *neoformans* and *grubii* strains by disrupting RNA and protein synthesis, whereas strains of var. *gattii* are resistant to canavanine (Polacheck and Kwon-Chung 1986). As shown in Fig. 5a, the serotype B (WM276) and serotype C (VPC1-87) positive control strains grew on CGB agar and produced a blue color, whereas none of the clinical isolates grew on CGB agar. One caveat to this analysis is that it has been reported that hybrid progeny of a cross between a serotype B and D strain failed to grow on CGB agar (Kwon-Chung et al. 1982a).

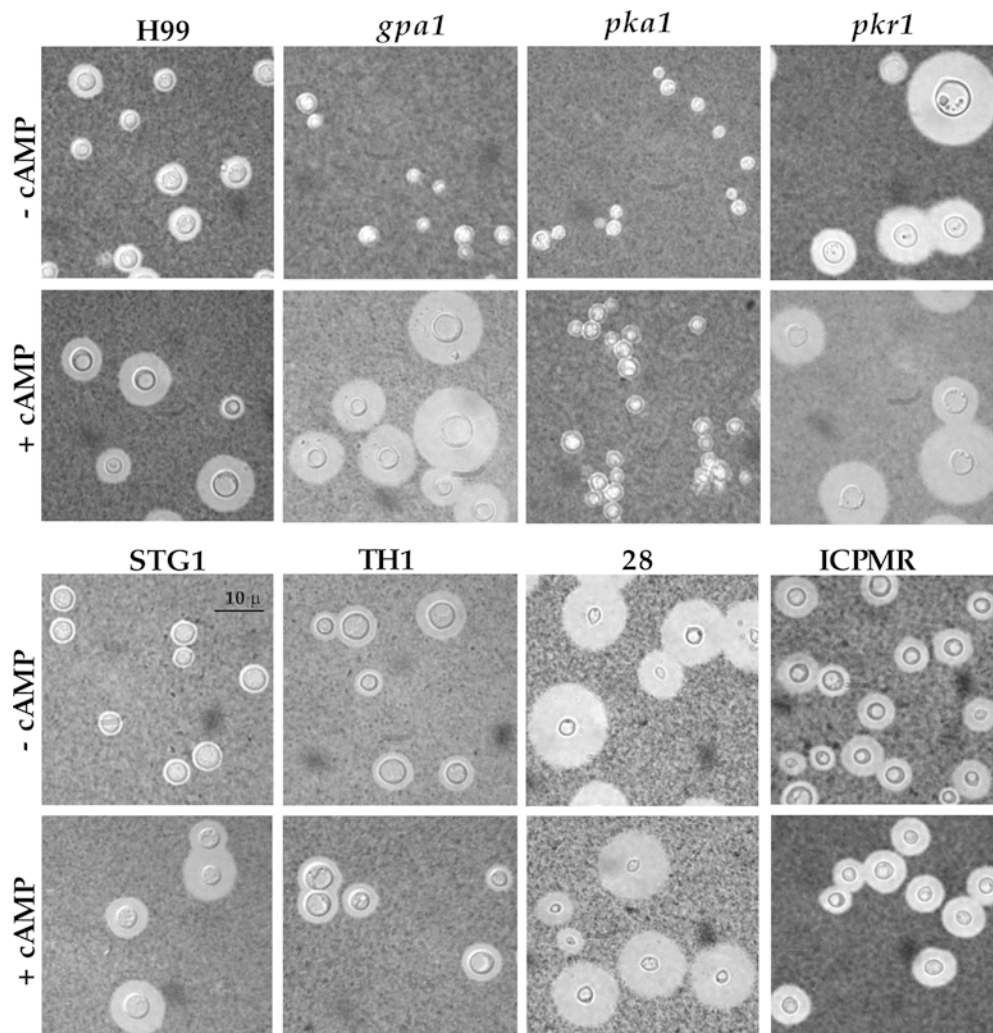
We next determined whether the clinical isolates could grow on 1% malate as the sole source of carbon, as do var. *gattii* strains (Bennett et al. 1978). The serotype B (WM276) and serotype C (VPC1-87) positive control strains grew on malate as a sole source of carbon (Fig. 5b). However, none of the clinical isolates utilized malate as the sole carbon source, providing further

Table 2 Results of PCR with *C. neoformans* serotype and/or mating type-specific primers

Strain	<i>GPAL</i> -A	<i>PAK1</i> -A	<i>GPAL</i> -D	<i>PAK1</i> -D	<i>STE12α</i> -A or D	<i>STE20a</i> -A	<i>STE20α</i> -A	<i>STE20a</i> -D	<i>STE20α</i> -D	Strain type
H99	+	+	-	-	+	-	+	- ^a	-	A α
125.91	+	+	-	-	-	+	-	- ^a	-	A α
JEC20	-	-	+	+	-	-	-	+	-	Da
JEC21	-	-	+	+	+	-	-	- ^a	+	D α
CDC94-383	+	+	+	+	+	-	-	+, - ^a	-	α ADa
ATCC 48184	+	+	+	+	+	+	-	- ^a	+	α AD α
8	+	+	-	-	+	-	+	- ^a	-	A α
13	+	+	-	-	+	-	+	- ^a	-	A α
14	+	+	-	-	+	-	+	- ^a	-	A α
25	+	+	-	-	+	-	+	- ^a	-	A α
28	+	+	-	-	+	-	+	- ^a	-	A α
30	+	+	-	-	+	-	+	- ^a	-	A α
32	+	+	-	-	+	-	+	-	-	A α
38	+	+	-	-	+	-	+	-	-	A α
41	-	+	-	-	+	-	+	-	-	A α
43	+	+	-	-	+/-	-	+	-	-	A α
51	+	+	-	-	+	-	+	-	-	A α
56	+	+	-	-	+	-	+	-	-	A α
516	+	+	-	-	+	-	+	-	-	A α
NIH438	+	+	-	-	+	-	+	-	-	A α
Duyn	+	+	-	-	+	-	+	-	-	A α
ICPMR	+	+	-	-	+	-	+	-	-	A α
98-181-1361										
PAH21b	+	+	-	-	+	-	+	-	-	A α
PAH22	+	+	-	-	+	-	+	-	-	A α
QEH6	+	+	-	-	+	-	+	-	-	A α
RNS13a	+	+	-	-	+	-	+	-	-	A α
RNS13b	+	+	-	-	+	-	+	-	-	A α
RNS13c	+	+	-	-	+	-	+	-	-	A α
RNS13d	+	+	-	-	+	-	+	-	-	A α
RNS13e	+	+	-	-	+	-	+	-	-	A α
STG1	+	+	-	-	+	-	+	-	-	A α
W20	+	+	-	-	+	-	+	-	-	A α
W24	+	+	-	-	+	-	+	-	-	A α
TH1	+	+	+	-	+	-	+	+	-	α ADa
EF	+	+	-	-	+	-	+	-	-	A α

^aNon-specific banding

Fig. 2 Capsule production by the clinical isolates. Strains were grown in inducing LIM with or without 10 mM cAMP for 5 days. Cells from the cultures were stained with a standard India ink preparation and photographed (400×). The wild-type serotype A strain H99 served as a positive control for capsule production, *pkr1* as a control for increased capsule production, and strains *gpa1* and *pka1* as controls for hypoencapsulation



evidence that these strains are not var. *gattii* or inter-variety hybrids.

In addition, we conducted PCR with variety-specific primers designed based on sequences of the genes *PLB1* and *SOD1* (Fig. 6) and *URA5* (data not shown). As shown in Fig. 6, the *PLB1*- and *SOD1*-*gattii*-specific primers yielded *gattii*-specific products with the control strains but no product with the clinical isolates, indicating that none of these strains are var. *gattii*. The A/D specific primers, however, did produce PCR products, further confirming our serotyping and AFLP analysis that these strains are serotype A. These findings reveal that the clinical isolates from apparently normal hosts are not intervariety hybrids between *gattii* and either *grubii* or *neoformans*.

Discussion

To investigate the basis of virulence in *C. neoformans* serotype A clinical isolates from apparently immunocompetent individuals, we obtained isolates from sources on three different continents. The criteria for

inclusion of these strains in our study were that the strains were serotype A and were isolated from previously normal, non-HIV infected patients. Isolates from moderately immunocompromised patients were also included. We did not include *C. neoformans* strains of the *gattii* variety in this study because these naturally infect immunocompetent individuals.

We first established that the strains were indeed *C. neoformans* by a battery of tests, including melanin production, urease activity, carbohydrate assimilation using the API20C AUX yeast identification system, agglutination-based serotyping, and PCR-based serotyping. Also, by AFLP analysis these isolates clustered with serotype A control strains and were distinguished from serotype D isolates (Fig. 1). Next, we tested whether the strains exhibited phenotypes of *pkr1* mutants. Assessment of capsule production by India ink staining revealed that strain 28 produced enlarged capsules, like a *pkr1* mutant. Strain ICPMR made an intermediate-sized capsule. It is noteworthy that strains 516 and STG1 were both hypocapsular and responded to cAMP by producing larger capsules, like the cAMP-responsive *gpa1* mutant. Interestingly, while cAMP induced mel-

Fig. 3 Assessment of virulence in the clinical isolates. Groups of ten animals were each infected with 10^5 yeast cells of wild-type, 28, TH1, ICPMR, and STG1 strains via nasal inhalation. The numbers of mice surviving from each group were plotted over the course of 100 days. The average survival of mice is indicated in the figure along with corresponding *P* values for comparison with the wild-type strain

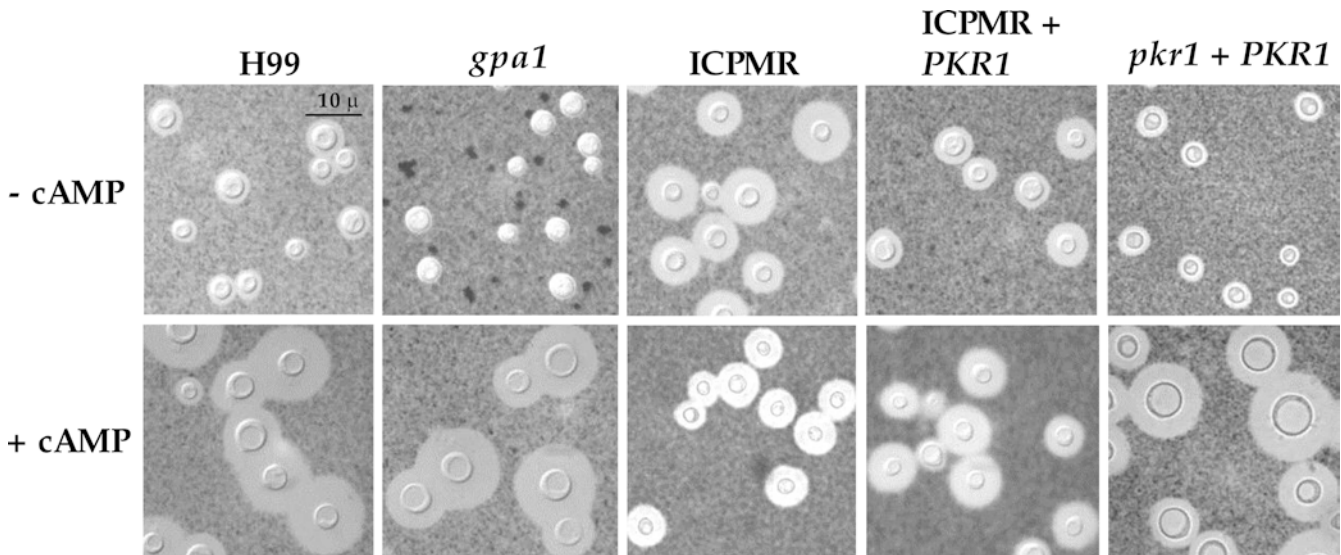
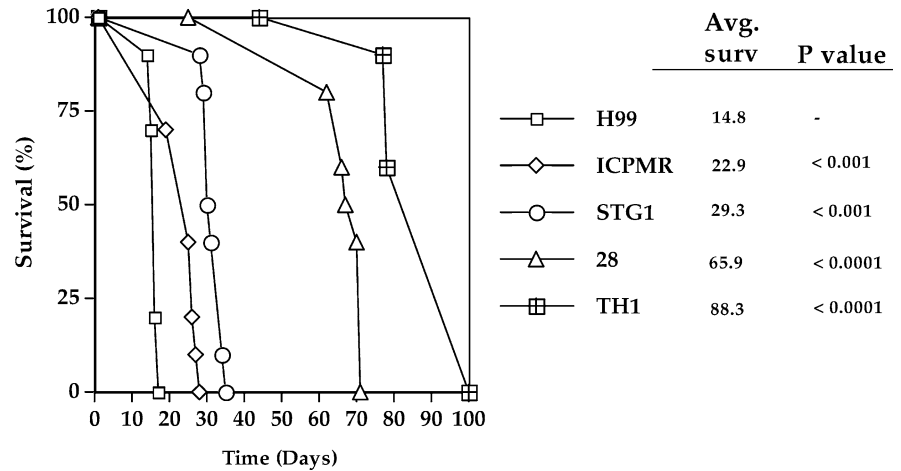


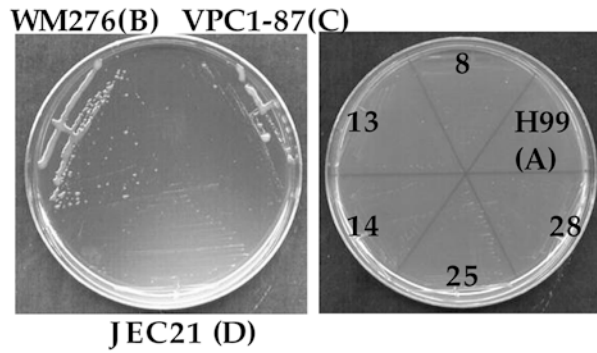
Fig. 4 Introduction of the *PKR1* gene and effects on capsule synthesis. Capsule production of strains ICPMR, ICPMR + *PKR1*, and *pkrl* + *PKR1* grown in inducing LIM with or without 10 mM cAMP for 5 days. Cells from the cultures were stained with a standard India ink preparation and photographed (400 \times). The wild-type serotype A strain H99 served as a positive control for normal capsule production and the strain *gpa1* served as a negative control. Both strains served as positive controls for cAMP induction of capsule production

nin production in strain 516, it did not induce melanin production in strain STG1, like the cAMP-unresponsive *pkal* mutant. These isolates may contain either previously characterized (*gpa1*, *pka1*) or novel cAMP signaling pathway mutations. Melanin production was found to be varied among the clinical isolates, with no clear correlation with capsule formation to allow inferences regarding the presence of a *pkrl* mutation. Mating competency was also assessed and found to be varied among the clinical isolates.

To avoid bias in our identification of potential *pkrl* mutant strains among the clinical isolates, we tested all strains for larger-scale mutations, such as deletions and insertions by PCR with *PKR1*-specific primers spanning

the entire gene. No such mutations were identified in any of the clinical isolates. Because point mutations could not be detected by this analysis, we further tested the presence of *pkrl* mutations by complementation tests with the cloned *PKR1* gene. The enhanced capsule phenotype of isolate ICPMR appeared to be either complemented or suppressed by the introduction of the *PKR1* gene. However, sequence analysis of two independent *PKR1* clones from the ICPMR strain and comparison with isolated *PKR1* genes from other serotype A isolates did not reveal any mutations. This suggests that overexpression of *PKR1* in ICPMR suppresses rather than complements an unknown mutation in this strain. This mutation results in cAMP-independent, constitutive capsule formation, as observed for *pkrl* mutants, but suppression rather than complementation by wild-type *PKR1* in the ICPMR strain points to alternative and potentially interesting targets in the cAMP signaling pathway. Based on these results, we conclude that the clinical isolates are not *pkrl* mutants. Isolation of the cryptococcal isolates RNS13a–e from a variety of tissues further supports the idea that the

A Growth on CGB agar



B Growth on YNB-malate

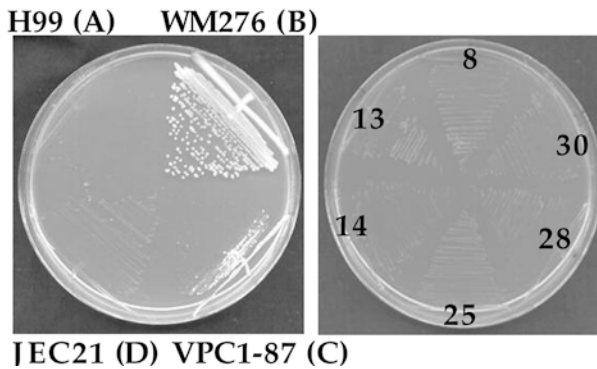


Fig. 5 a,b Clinical isolates are not intervarietal hybrids. **a** The clinical strains were grown on CGB agar for 4 days at 30°C (only one representative plate shown here). These were compared with the serotype B strain WM276 and serotype C strain VPC1-87, which grew on CGB to produce a blue color, compared with the serotype A strain H99 and serotype D strain JEC21, which failed to grow on CGB. **b** The clinical strains were grown on YNB-1% malate agar for 4 days at 30°C (only one representative plate shown here). These were compared with the serotype B strain WM276 and serotype C strain VPC1-87, which could grow on malate as a sole source of carbon, compared with the serotype A strain H99 and serotype D strain JEC21, which could not grow on malate as sole carbon source

strain(s) had no mutation that would confer tissue-specificity.

Of four clinical isolates that were tested for virulence potential, all were infectious and led to 100% lethality in a mouse inhalation model. This said, these four isolates were all less virulent than the pathogenic serotype A control strain H99. However, these strains are not isogenic and H99 exhibits robust virulence. H99 is a clinical strain derived from a patient with Hodgkin's lymphoma and represents the type strain for *C. neoformans* var. *grubii*. In addition, these clinical isolates may differ in virulence potential in humans compared with the mouse. The virulence experiment also indicated that there is no direct correlation between virulence and capsule size for these non-isogenic strains, as reported in earlier studies (Dykstra et al. 1977; Littman and Tsubura 1959).

The clinical isolates analyzed here may have been either acquired from nature and caused an acute

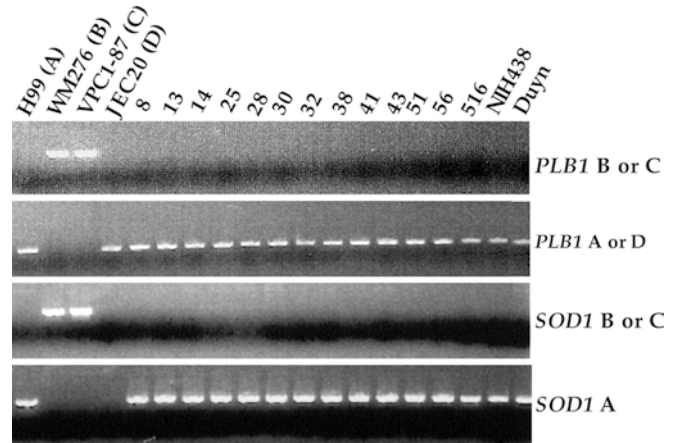


Fig. 6 PCR based on *PLB1* and *SOD1* genes and varietal specific primers. To confirm the varietal status of the clinical isolates, PCR was carried out with either *PLB1* (B/C)-specific primers and *PLB1* (A/D)-specific primers, or *SOD1* (B/C)-specific primers and *SOD1*(A)-specific primers. The PCR products were then electrophoresed in a 1% agarose gel and stained with ethidium bromide (representative reactions shown here)

infection, or reactivated from latent infections in the host. One argument against acute infections as the source is that these might be expected to result then in outbreaks of infection, similar to those occurring on Vancouver Island, Canada, associated with var. *gattii* infections (Fraser et al. 2003; Stephen et al. 2002). Most infections in both immunocompetent and immunocompromised hosts likely result from reactivation of latent infections, but some acute infections may also occur (such as in the injection drug-user in this series, or the patient with a topical skin infection). Likely all clinical infections result from some level of selection for virulence in the host; and this likely accounts for the long dormant periods between initial infection and re-emergence, during which the fungus must evade the host immune system. Further study will be required to define the molecular nature of changes that occur during latency in the host and their contribution to virulence potential and progression.

To further investigate the ability of the clinical isolates to infect apparently healthy individuals, we tested whether these strains were hybrid strains with one var. *gattii* parent. Interspecies hybridization has been found to be responsible for altered virulence in a variety of fungal pathogens (Brasier 2000; Brasier et al. 1999; Newcombe 2003). Two diagnostic biochemical tests and PCR with serotype-specific primers revealed that all except one isolate were serotype A. This ruled out var. *gattii* traits in these clinical strains as the reason for their ability to infect apparently healthy individuals. While AD hybrid strains are readily detected by serotyping, no AB, AC, DB, or DC hybrids have been described. However, because upon passage aneuploid AD strains that initially typed as AD can become only A or only D and still contain genetic material from both parental strains (Lengeler et al. 2001), we considered that unusual hybrid strains might serotype as A yet still contain ge-

netic material from serotype B or C. Our PCR and AFLP molecular analyses provide evidence against such a model and suggest that alternative molecular explanations other than intervarietal hybridization are likely operative here.

Recent AFLP analysis supports the idea that var. *gattii* may represent a distinct species from var. *grubii* and *neoformans*; and it has been proposed that var. *gattii* be reclassified as *Cryptococcus gattii* (Boekhout et al. 2001; Kwon-Chung et al. 2002). Our findings contribute to this distinction by revealing that, even amongst a broad collection of clinical isolates that are serotype A, only one intervarietal serotype AD hybrid strain was detected and no AB or AC hybrid strains were found.

Taken together, these clinical isolates may have a mutation(s) other than *pkrl* that confers the ability to infect healthy individuals. Alternately, their infectious potential may be an indirect result of an underlying disease of idiopathic nature in the host. Where patient history could be obtained, we found that all individuals were HIV-negative, but several did have an underlying disease that may have contributed (Table 1). For example, in the case of the patient from whom strain ICPMR was isolated, the patient suffered from CD4 syndrome. CD4 syndrome is an idiopathic lymphocytopenia in which, for unknown reasons, CD4 counts are low, making such individuals susceptible to cryptococcosis (for a review, see Perfect and Casadevall 2002). We conclude that the isolation of *C. neoformans* serotype A isolates from previously healthy individuals may be due to mutations conferring increased virulence potential, underlying disease that compromises host immunity, or both factors.

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