

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

Cletus A. D'Souza¹, Ferry Hagen⁵, Teun Boekhout⁵, Gary M. Cox^{1,2}, and
Joseph Heitman^{1-4*}

Departments of Molecular Genetics and Microbiology¹, Medicine², and
Pharmacology and Cancer Biology³, the Howard Hughes Medical Institute⁴,
Duke University Medical Center
Durham, North Carolina 27710

CBS⁵ - Centraalbureau voor Schimmelcultures, Uppsalaalaan 8, 3584 CT Utrecht,
The Netherlands

*Correspondence to: Dr. Joseph Heitman, Department of Molecular Genetics and Microbiology, 322 CARL Bldg., Duke University Medical Center, Research Drive, Durham, NC 27710
Phone: (919) 684-2824
Fax: (919) 684-5458
email: heitm001@duke.edu

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 *pkr1* (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 *pkr1* 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 (variety *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 *pkr1* 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 (reviewed in 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 *MATα* mating type locus in serotype D strains (Kwon-Chung et al. 1992).

Cryptococcal infection is initiated by the inhalation of spores or dessicated 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 intracellular survival of fungal cells within macrophages (Granger et al. 1985) (Feldmesser et al. 2000).

Based on antigenicity of the capsular polysaccharides, *C. neoformans* can be classified into four distinct serotypes (A, B, C, and D), all of which are human pathogens. The four serotypes are further classified into three varieties: serotype A corresponds to variety *grubii*, serotypes B and C to variety *gattii*, and serotype D to variety *neoformans* (Franzot et al. 1999; Kwon-Chung and Bennett 1984). While strains of the *neoformans* and *grubii* varieties typically infect immunocompromised individuals, variety *gattii* strains infect immunocompetent individuals (reviewed in 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* (Alspaugh et al. 1997) (Alspaugh et al. 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 *pkr1* mutant cells during infection. Pkr1 does not regulate virulence in serotype D strains and, unlike serotype A *pkr1* strains, serotype D *pkr1* 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 infecting 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 as well as moderately immunocompromised individuals from three continents. We determined if 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 *gattii* variety strains that normally infect immunocompetent individuals.

Materials and Methods

***C. neofformans* strains, plasmids, and media**

All strains used in this study are listed in Table 1. *C. neofformans* strains were grown on standard *S. 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 (FA) (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 earlier (Lengeler et al. 2001) using the Crypto Check serotyping kit from Iatron Laboratories Inc. (Tokyo, Japan). Serotyping was confirmed by PCR using serotype-specific primers as described (Lengeler et al. 2001).

AFLP analysis

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

Isolation of the *PKR1* gene from clinical isolates

PCR was conducted using genomic DNA from the clinical isolates as template and the following primers: JOHE3245 (TTCCTCCACAGCTCTACTC) and JOHE3180 (CTGTTGGAGAAAATCAGGG) 50 µl PCR reactions contained 1x Ex-Taq buffer, 1 mM dNTPs, 1.25 U 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 sec, 55°C for 30 sec, 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 *PKR1*, that was cloned in the TOPO-TA cloning vector pCR2.1 (Invitrogen).

Southern hybridization

Genomic DNA was isolated as described (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 32 P-dCTP (Amersham). The template for the *PKR1* gene was a 1.6 kb *Bam*HI cDNA fragment.

***PKR1* complementation test**

A 4 kb *Hind*III fragment bearing the entire *PKR1* gene was inserted into plasmid pCH233 that 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 YPD 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 µg/ml nourseothricin. Genomic DNA was isolated from nourseothricin-resistant transformants, and Southern analysis verified that the *PKR1* gene had been introduced into the clinical isolates.

Virulence assays

In the murine inhalational 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 µl 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 with twice-daily inspections. 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 ≤ 0.05. The protocol was approved by the Duke University Animal Use Committee.

PCR to determine *gattii* varietal status of clinical isolates

The *PLB1* gene (*gattii*)-specific PCR primers JOHE7819 (AAAACGCTTCATTATGAAG) and JOHE7824 (GCAAAAAAGAACAAAAAA) were used to determine if the clinical isolates were of the *gattii* variety. PCR was also carried out with genomic DNA templates from the clinical isolates and A/D specific primers JOHE7830 (TTTCTGCTTTCACCCATT) and JOHE7833 (TGAACAAATACATCATGC). 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 sec, 52°C for 30 sec, 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 an ~1.0 kb product.

The *SOD1* gene (*gattii*)-specific PCR primers JOHE7773 (GATCCTCACGCCATTACG) and JOHE7775 (GAATGATGCGCTTAGTTGGA) were also used to determine if the clinical isolates were of the *gattii* variety. Again, PCR was carried out with A/D specific primers JOHE7777 (TTCAACCACGAATATGTA) and JOHE7779 (AAGCCTCTCATCCATATCTT). PCR reaction conditions were: 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 52°C for 30 sec, 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 an ~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 (variety *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- or host-dependent.

The clinical isolates were verified as *C. neoformans* by a variety of tests, including melanin production on Niger seed agar (not shown), urease activity (not shown), carbohydrate assimilation tests using the API20C AUX yeast identification system (bioMerieux) (not shown), and serotyping (Table 1). In addition, PCR with *C. neoformans* serotype and/or mating-type specific primers (Table 2) and amplified fragment length polymorphism (AFLP) analysis (Figure

1) confirmed that most isolates were *C. neoformans* serotype A and MAT α , with the exception of strain TH1 that is an α AD α hybrid isolate. Further mating type testing with a different set of mating-type and/or serotype-specific primers confirmed this conclusion (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 low-iron medium (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 Figure 2 shows representative isolates. Isolates 38, 41, and 56 were hypocapsular (data not shown), as observed for the cAMP signaling mutants *gpa1* and *pka1* (Alspaugh et al. 1997; D'Souza et al. 2001). Like *pka1* 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 (not shown). Interestingly, cAMP also induced melanin production in strain 516 but not in strain STG1 (not shown). Strains ICPMR and 28 produced intermediate to large capsules, similar to the PKA hyperactivated *pkr1* 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 hypcapsular and the AD hybrid strain TH1 were included. In the murine model of infection, 10^5 yeast cells of 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 (Figure 3). The clinical isolates were all virulent, leading to 100% lethal infection, but all were less virulent than strain H99 in the murine inhalational model.

Molecular investigation of clinical isolates as candidate *pkr1* mutants

To investigate at the molecular level if the clinical isolates were *pkr1* mutants, we first used PCR to test if deletions or insertions were present in the *PKR1* gene. No such mutations were detected in any strain by this analysis (not shown). To investigate if strains 28 and ICPMR contained more subtle *pkr1* mutations, the *PKR1* gene was introduced by transformation with a plasmid bearing the *PKR1* gene and the nourseothricin selectable marker (*nat1*). Transformants were selected based on resistance to nourseothricin and confirmed to contain the linked *PKR1* gene by Southern blot. Capsule production was assessed in the transformed strains after growth in low-iron capsule-inducing medium. As shown in Figure 4, cAMP-responsive capsule production was restored in the *pkr1* + *PKR1* control strain. Interestingly, in two

independent ICPMR + *PKR1* transformants cAMP-responsive wild-type capsule production was restored (Figure 4), whereas the 28 + *PKR1* transformant continued to produce enlarged capsules in a cAMP-insensitive fashion (not shown). Extensive sequence analysis of the ICPMR *PKR1* gene revealed no evidence for any mutations that would result in amino acid substitutions in the *PKR1* gene product. These findings indicate that alterations other than in the *PKR1* gene likely underlie the enhanced capsule production, and that overexpression of the wild-type *PKR1* gene can repress capsule production in this isolate.

The clinical isolates are not intervarietal hybrids with *gattii*

Because serotype B or C *gattii* variety strains can infect immunocompetent individuals, we tested if serotype A isolates from apparently immunocompetent hosts represent hybrid strains formed by fusion of *grubii* strains with the *gattii* variety. 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 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 CGB and malate agar media. The Canavanine Glycine Bromthymol agar (CGB) test is based on the ability of *gattii* variety strains to utilize glycine as the sole source of carbon and

nitrogen (Kwon-Chung et al. 1982b). Ammonia released upon glycine degradation alkalinizes the medium, changing the color from yellow to blue (Min and Kwon-Chung 1986). Additionally, the canavanine in the medium inhibits the growth of variety *neoformans* and *grubii* strains by disrupting RNA and protein synthesis, whereas strains of variety *gattii* are resistant to canavanine (Polacheck and Kwon-Chung 1986). As shown in Figure 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 a serotype 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 *gattii* variety 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 (Figure 5B). However, none of the clinical isolates utilized malate as the sole carbon source, providing further evidence that these strains are not variety *gattii* or intervarietal hybrids.

In addition, we conducted PCR with variety-specific primers designed based on sequences of the genes *PLB1* and *SOD1* (Figure 6), as well as *URA5* (not shown). As shown in Figure 6, the *PLB1* and *SOD1-gattii* specific primers yielded *gattii* specific products with control strains but no product with the clinical isolates, indicating that none of these strains are of the *gattii* variety. 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 intervarietal 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 (Figure 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 melanin production in strain 516, it does not induce melanin production in strain STG1, like the cAMP-unresponsive *pka1* 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 *pkr1* mutation. Mating competency was also assessed and found to be varied among the clinical isolates.

To avoid bias in our identification of potential *pkr1* 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 *pkr1* mutations by complementation tests with the cloned *PKR1* gene. The enhanced capsule phenotype of isolate ICPMR appeared to be either complemented or suppressed by 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 *pkr1* mutants, but the 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 *pkr1* mutants. Isolation of the cryptococcal isolates RNS13a-e from a variety of tissues further supports the idea that the 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 murine inhalational 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 to the mouse. The virulence experiment also indicated that there is no direct correlation between virulence and capsule size of these non-isogenic strains, as reported in earlier studies (Dykstra et al. 1977; Littman and Tsubura 1959).

The clinical isolates analyzed here may have either been acquired from nature and caused acute 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 in 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 if these strains were hybrid strains with one parent of the *gattii* variety. 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 *gattii* variety 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 genetic 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 supported the idea that the *gattii* variety may represent a distinct species from the *grubii* and *neoformans* varieties, and it has been proposed that the *gattii* variety 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 *pkr1* that confers the ability to infect healthy individuals. Alternately, their infectious potential may have been 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-, 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 (Reviewed in (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.

Acknowledgements

We thank Jim Kronstad and John Perfect for advice and comments on the manuscript, Cristl Arndt for technical assistance, and John Bennett, Wieland Meyer, Eric Jacobson, Barbara Alexander-Lodge, and Tom Harrison for strains. This work was supported by NIAID R01 grants AI39115 and AI42159 (to J.H). This work was also supported by P01 award AI44975 from the NIAID to the Duke University Mycology Research Unit. Joseph Heitman is a Burroughs Wellcome Scholar in Molecular Pathogenic Mycology and an associate investigator of the Howard Hughes Medical Institute.

References

- Alspaugh JA, Perfect JR, Heitman J (1997) *Cryptococcus neoformans* mating and virulence are regulated by the G-protein a subunit GPA1 and cAMP. *Genes & Dev.* 11:3206-3217
- Alspaugh JA, Pukkila-Worley R, Harashima T, Cavallo LM, Funnell D, Cox GM, Perfect JR, Kronstad JW, Heitman J (2002) Adenylyl cyclase functions downstream of the Ga protein Gpa1 and controls mating and pathogenicity of *Cryptococcus neoformans*. *Eukaryot Cell* 1:75-84
- Bennett JE, Kwon-Chung KJ, Theodore TS (1978) Biochemical differences between serotypes of *Cryptococcus neoformans*. *Sabouraudia* 16:167-74
- Boekhout T, Theelen B, Diaz M, Fell JW, Hop WC, Abeln EC, Dromer F, Meyer W (2001) Hybrid genotypes in the pathogenic yeast *Cryptococcus neoformans*. *Microbiology* 147:891-907
- Brasier C (2000) The rise of the hybrid fungi. *Nature* 405:134-5
- Brasier CM, Cooke DE, Duncan JM (1999) Origin of a new *Phytophthora* pathogen through interspecific hybridization. *Proc Natl Acad Sci U S A* 96:5878-5883
- Casadevall A, Perfect JR (1998) *Cryptococcus neoformans*. ASM Press, Washington
- Chang YC, Penoyer LA, Kwon-Chung KJ (1996) The second capsule gene of *Cryptococcus neoformans*, CAP64, is essential for virulence. *Infect. Immun.* 64:1977-1983
- Cox GM, Mukherjee J, Cole GT, Casadevall A, Perfect JR (2000) Urease as a virulence factor in experimental Cryptococciosis. *Infect. Immun.* 68:443-448
- D'Souza CA, Alspaugh JA, Yue C, Harashima T, Cox GM, Perfect JR, Heitman J (2001) Cyclic AMP-dependent protein kinase controls virulence of the fungal pathogen *Cryptococcus neoformans*. *Mol Cell Biol* 21:3179-91
- Dykstra MA, Friedman L, Murphy JW (1977) Capsule size of *Cryptococcus neoformans*: control and relationship to virulence. *Infect. Immun.* 16:129-35
- Feldmesser M, Kress Y, Novikoff P, Casadevall A (2000) *Cryptococcus neoformans* is a facultative intracellular pathogen in murine pulmonary infection. *Infect. Immun.* 68:4225-4237
- Franzot SP, Salkin IF, Casadevall A (1999) *Cryptococcus neoformans* var. *grubii*: Separate varietal status for *Cryptococcus neoformans* serotype A isolates. *J. Clin. Microbiol.* 37:838-840
- Fraser JA, Subaran RL, Nichols CB, Heitman J (2003) Recapitulation of the sexual cycle of the primary fungal pathogen *Cryptococcus neoformans* variety *gattii*: implications for an outbreak on Vancouver Island. *Eukaryot Cell* 2:1036-1045
- Granger DL, Perfect JR, Durack DT (1985) Virulence of *Cryptococcus neoformans*: regulation of capsule synthesis by carbon dioxide. *J. Clin. Invest.* 76:508-516
- Hicks JK, D'Souza CA, Cox GM, Heitman J (2004) Cyclic AMP-dependent protein kinase catalytic subunits have divergent roles in virulence factor production in two varieties of the fungal pathogen *Cryptococcus neoformans*. *Eukaryot Cell* in press

- Hull CM, Heitman J (2002) Genetics of *Cryptococcus neoformans*. Annu Rev Genet 36:557-615
- Kwon-Chung KJ, Bennett JE (1984) Epidemiologic differences between the two varieties of *Cryptococcus neoformans*. Am J Epidemiol 120:123-30
- Kwon-Chung KJ, Bennett JE, Rhodes JC (1982a) Taxonomic studies on *Filobasidiella* species and their anamorphs. Antonie van Leeuwenhoek 48:25-38
- Kwon-Chung KJ, Boekhout T, Fell JW, Diaz M (2002) Proposal to conserve the name *Cryptococcus gattii* against *C. hondurianus* and *C. bacilliporus* (*Basidiomycota, Hymenomycetes, Tremellomycetidae*). Taxon 51:804-806
- Kwon-Chung KJ, Edman JC, Wickes BL (1992) Genetic association of mating types and virulence in *Cryptococcus neoformans*. Infect. Immun. 60:602-605
- Kwon-Chung KJ, Polacheck I, Bennett JE (1982b) 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
- Kwon-Chung KJ, Polacheck I, Popkin TJ (1982c) Melanin-lacking mutants of *Cryptococcus neoformans* and their virulence for mice. J. Bacteriol. 150:1414-1421
- Kwon-Chung KJ, Rhodes JC (1986) Encapsulation and melanin formation as indicators of virulence in *Cryptococcus neoformans*. Infect. Immun. 51:218-223
- Lengeler KB, Cox GM, Heitman J (2001) Serotype AD strains of *Cryptococcus neoformans* are diploid or aneuploid and are heterozygous at the mating-type locus. Infect Immun 69:115-122
- Littman ML, Tsubura E (1959) Effect of degree of encapsulation upon virulence of *Cryptococcus neoformans*. Proc Soc Exp Biol Med 101:773-777
- Min KH, Kwon-Chung KJ (1986) The biochemical basis for the distinction between the two *Cryptococcus neoformans* varieties with CGB medium. Zentralbl Bakteriol Mikrobiol Hyg [A] 261:471-480
- Newcombe G (2003) Native *Venturia inopina* sp. nov., specific to *Populus trichocarpa* and its hybrids. Mycol Res 107:108-116
- Odom A, Muir S, Lim E, Toffaletti DL, Perfect J, Heitman J (1997) Calcineurin is required for virulence of *Cryptococcus neoformans*. EMBO J. 16:2576-2589
- Perfect JR, Casadevall A (2002) Cryptococcosis. Infect Dis Clin North Am 16:837-74, v-vi
- Perfect JR, Katabchi N, Cox GM, Ingram CW, Beiser CL (1993) Karyotyping of *Cryptococcus neoformans* as an epidemiological tool. J. Clin. Microbiol. 31:3305-3309
- Polacheck I, Kwon-Chung KJ (1986) Canavanine resistance in *Cryptococcus neoformans*. Antimicrob Agents Chemother 29:468-473
- Salas SD, Bennett JE, Kwon-Chung KJ, Perfect JR, Williamson PR (1996) Effect of the laccase gene, CNLAC1, on virulence of *Cryptococcus neoformans*. J. Exp. Med. 184:377-386

- Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Sherman F (1991) Getting started with yeast. In: Guthrie C, Fink GR (eds) Methods in Enzymology, vol 194. Academic Press, Inc., San Diego, CA, pp 3-21
- Sorrell TC (2001) *Cryptococcus neoformans* variety *gattii*. Med Mycol 39:155-168
- Stephen C, Lester S, Black W, Fyfe M, Raverty S (2002) Multispecies outbreak of Cryptococcosis on southern Vancouver Island, British Columbia. Can Vet J 43:792-794
- Toffaletti DL, Perfect JR (1994) Biolistic DNA delivery for *Cryptococcus neoformans* transformation. In: Maresca B, Kobayashi GS (eds) Molecular Biology of Pathogenic Fungal: A Laboratory Manual. Telos Press, New York, pp 303-308
- Toffaletti DL, Rude TH, Johnston SA, Durack DT, Perfect JR (1993) Gene transfer in *Cryptococcus neoformans* by use of biolistic delivery of DNA. J. Bacteriol. 175:1405-1411
- Vartivarian SE, Cowart RE, Anaissie EJ, Tashiro T, Sprigg HA (1995) Iron acquisition by *Cryptococcus neoformans*. J Med Vet Mycol 33:151-156
- Wang Y, Aisen P, Cadadevall A (1995) *Cryptococcus neoformans* melanin and virulence: mechanism of action. Infect. Immun. 63:3131-3136
- Wickes BL, Mayorga ME, Edman U, Edman JC (1996) Dimorphism and haploid fruiting in *Cryptococcus neoformans*: association with the alpha-mating type. Proc Natl Acad Sci U S A 93:7327-7331

Figure Legends

Figure 1. AFLP analysis of clinical isolates. Clustering of AFLP banding patterns of isolates by UPGMA (unweighted pair group method using arithmetic means). Control strains were H99 (**aA**), 125.91 (**aA**), JEC20 (**aD**), and JEC21 (**aD**). 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, and 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 to the other clusters.

Figure 2. Capsule production by the clinical isolates. Strains were grown in inducing low iron media (LIM) with or without 10 mM cAMP for 5 days. Cells from the cultures were stained with a standard India ink preparation and photographed (400X). The WT 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.

Figure 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 WT strain.

Figure 4. Introduction of the *PKR1* gene and effects on capsule synthesis.

Capsule production of strains ICPMR, ICPMR + *PKR1*, and *pkr1* + *PKR1* grown in inducing low-iron media (LIM) with or without 10 mM cAMP for 5 days. Cells from the cultures were stained with a standard India ink preparation and photographed (400X). The WT 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.

Figure 5. 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 to the serotype B strain WM276 and serotype C strain VPC1-87 that grew on CGB to produce a blue color change, compared to the serotype A strain H99 and serotype D strain JEC21 that 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 to the serotype B strain WM276 and serotype C strain VPC1-87 which could grow on

malate as a sole source of carbon, compared to the serotype A strain H99 and serotype D strain JEC21 which could not grow on malate as sole carbon source.

Figure 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).

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	IV drug abuse, meningitis, non-immunosuppressed, no travel history, CD4 cell count normal	T. Harrison
EF	A	USA	CSF	49 yr old, female, insulin dependent-diabetes, meningitis	Barbara 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		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

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

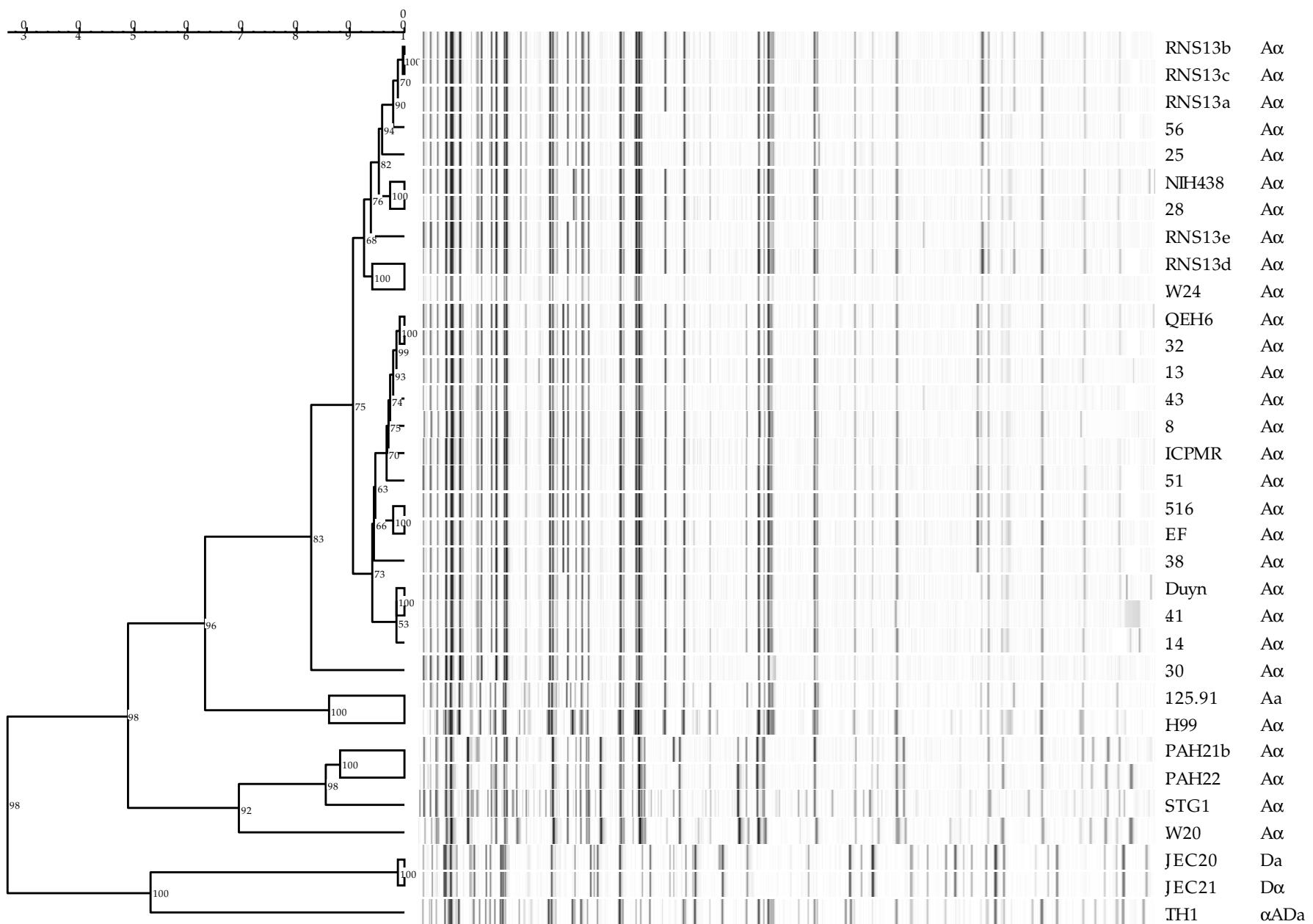
Strain	GPA1 -A	PAK1 -A	GPA1 -D	PAK1 -D	STE12α -A or D	STE20a -A	STE20α -A	STE20a -D	STE20α -D	Strain type
H99	+	+	-	-	+	-	+	-*	-	A α
125.91	+	+	-	-	-	+	-	-*	-	Aa
JEC20	-	-	+	+	-	-	-	+	-	Da
JEC21	-	-	+	+	+	-	-	-*	+	D α
CDC94-383	+	+	+	+	+	-	-	+, -*	-	α ADA
ATCC 48184	+	+	+	+	+	+	-	-*	+	aAD α
8	+	+	-	-	+	-	+	-*	-	A α
13	+	+	-	-	+	-	+	-*	-	Aa
14	+	+	-	-	+	-	+	-*	-	A α
25	+	+	-	-	+	-	+	-*	-	A α
28	+	+	-	-	+	-	+	-*	-	A α
30	+	+	-	-	+	-	+	-*	-	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 98-181-1361	+	+	-	-	+	-	+	-	-	A α
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

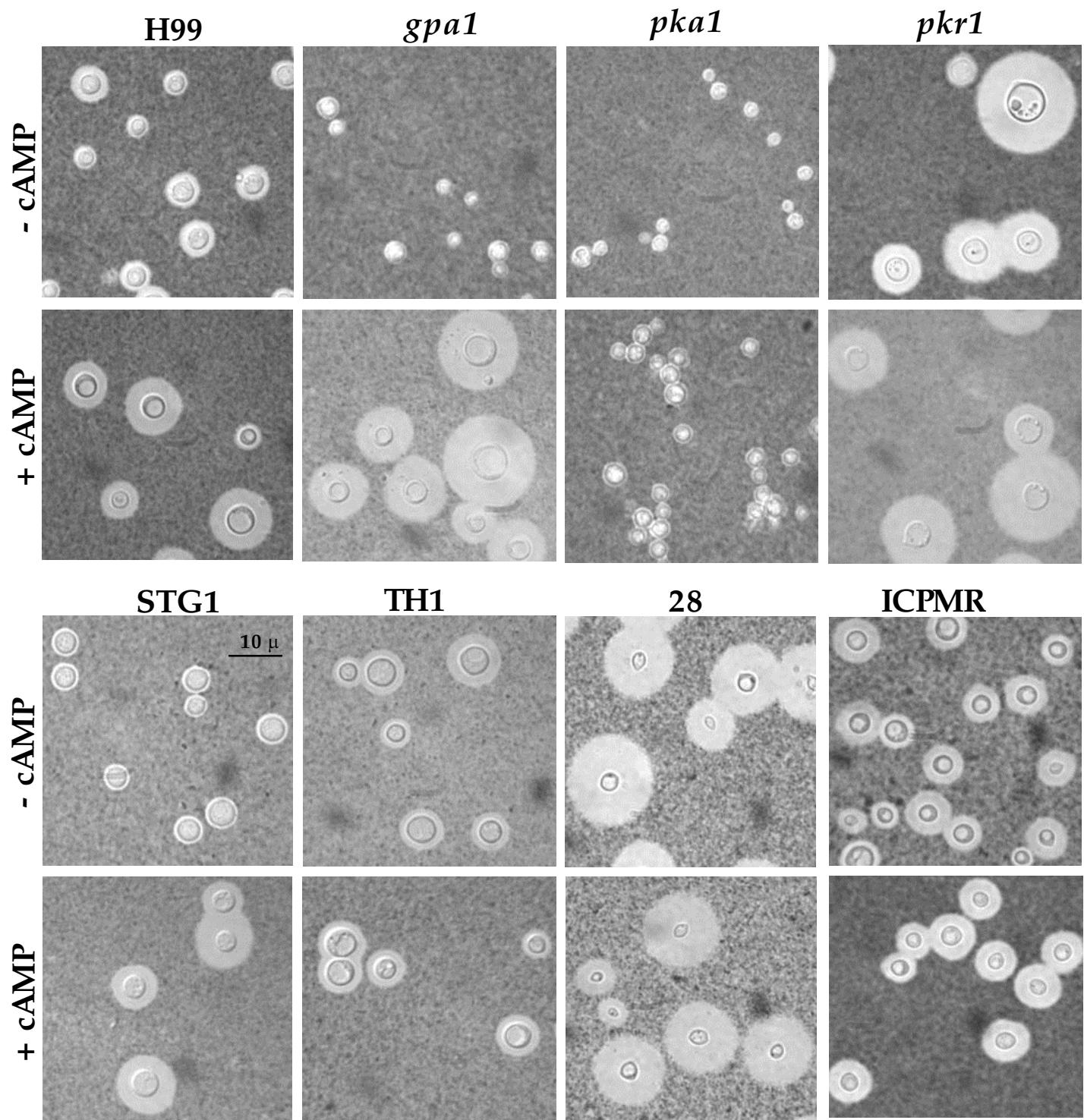
W24	+	+	-	-	+	-	+	-	-	A α
TH1	+	+	+	-	+	-	+	+	-	α ADA
EF	+	+	-	-	+	-	+	-	-	A α

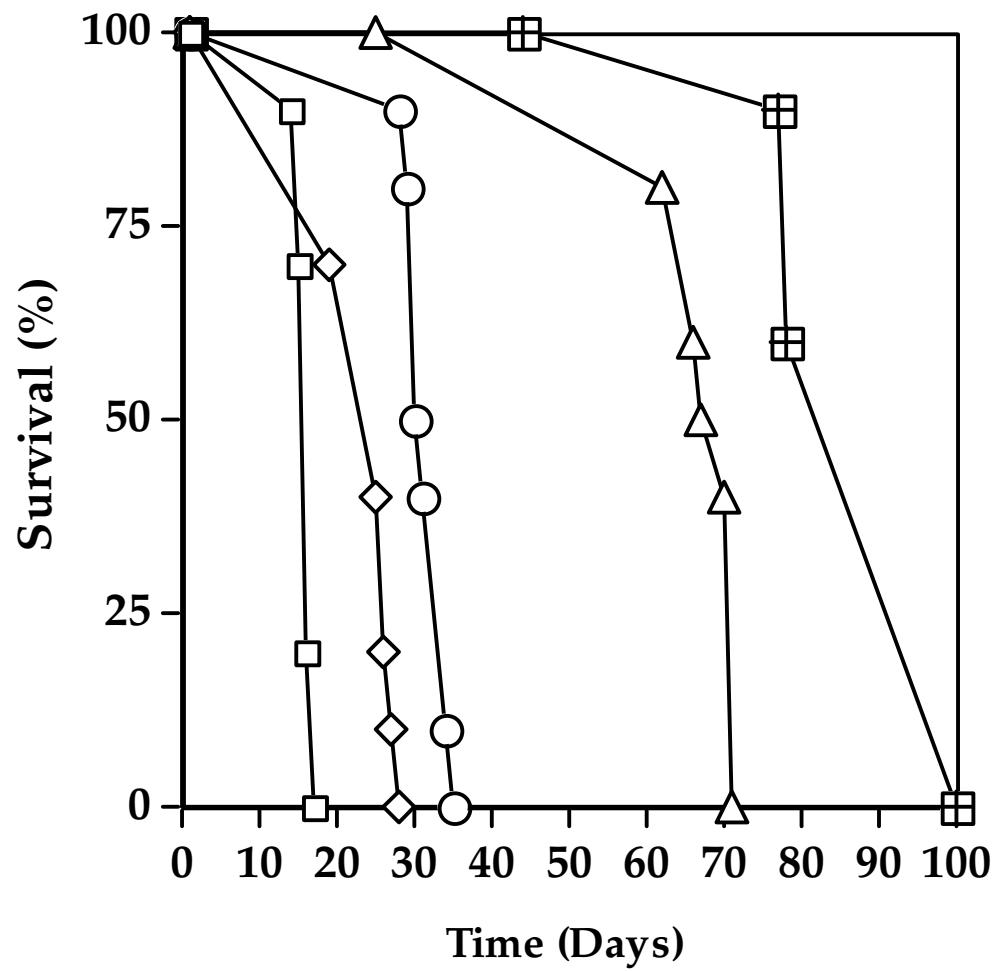
*NS= Non-specific banding

Pearson correlation (Opt:0.04%) [0.0%-100.0%]
AFLPabi310

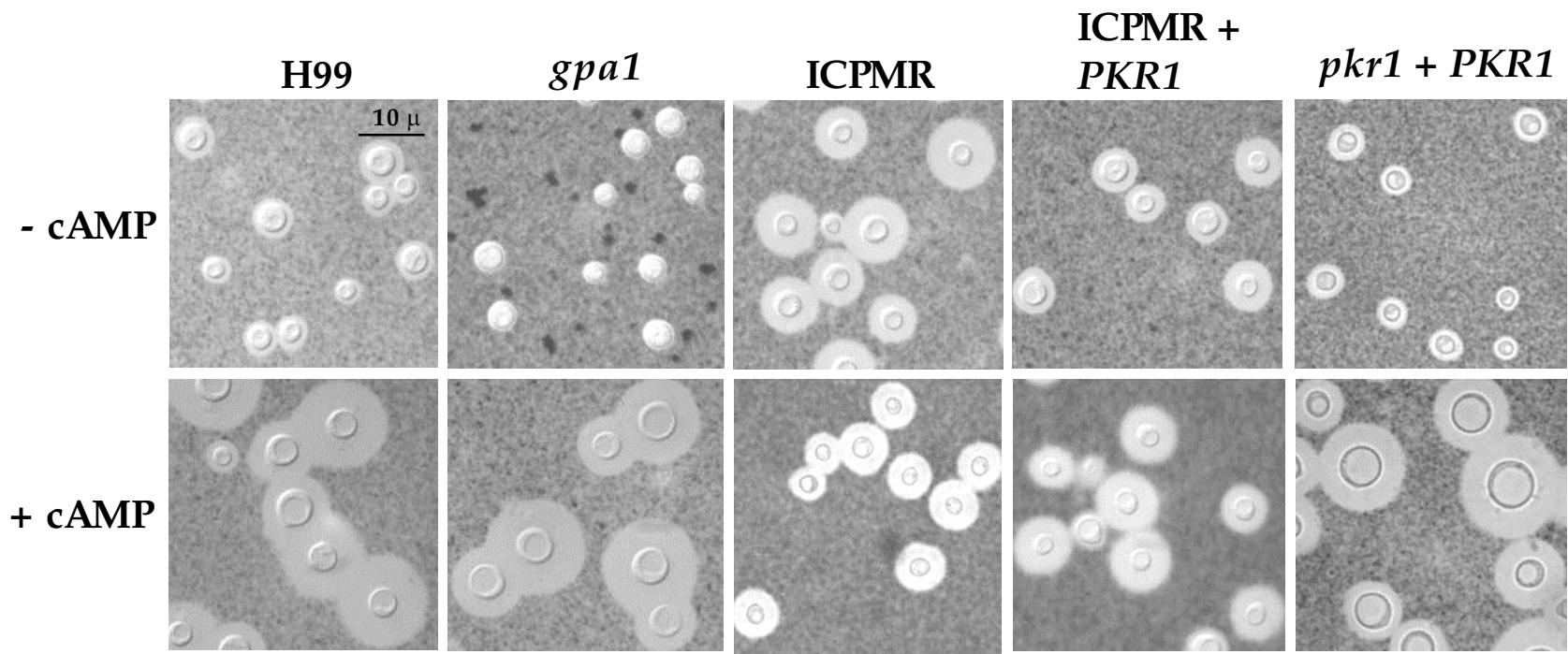
AFLPabi310





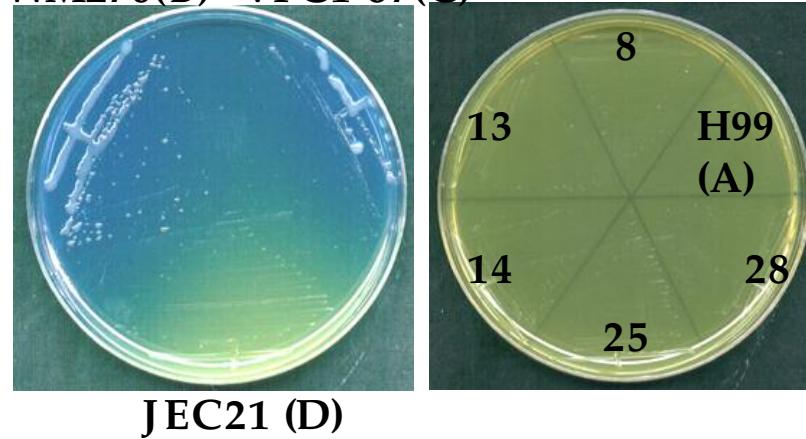


	Avg. surv	P value
H99	14.8	-
ICPMR	22.9	< 0.001
STG1	29.3	< 0.001
28	65.9	< 0.0001
TH1	88.3	< 0.0001



A Growth on CGB agar

WM276(B) VPC1-87(C)



B Growth on YNB-malate

H99 (A) WM276 (B)

