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A survey of heterobasidiomycetous yeasts for the presence of the genes homologous to virulence factors of *Filobasidiella neoformans*, *CNLAC1* and *CAP59*

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Among species of the heterobasidiomycetous yeasts, *Filobasidiella neoformans* is the only serious pathogen that causes fatal infections in both immunocompromised as well as immunocompetent patients. Three phenotypic characteristics, including growth at 37 °C, extracellular polysaccharide capsule and laccase activity, of *F. neoformans* are known to play major roles in the pathogenicity of the fungus. Several *CAP* genes involved in polysaccharide capsule formation, as well as the *CNLAC1* gene encoding a laccase, have previously been cloned and characterized. To analyse the presence of these *Cryptococcus neoformans* virulence factors in other heterobasidiomycetous yeasts, numerous species of heterobasidiomycetous yeasts were screened for the presence of laccase activity and a polysaccharide capsule. Species exhibiting laccase activity and possessing a glucuronoxylomannan (GXM) capsule were screened for homologues of both the *CAP59* gene and the *CNLAC1* gene of *F. neoformans*. Southern blots of genomic DNA from GXM capsule-producing species exhibited no discernible hybridization to the *CAP59* DNA sequence except for the two varieties of *F. neoformans* and *Cryptococcus podzolicus*. Although discernible, the hybridization band observed with the DNA of *C. podzolicus* was faint. Oligonucleotide primers constructed using the *CAP59* gene sequence also failed to yield PCR products from DNAs of these yeasts except for the two varieties of *F. neoformans*. These results, coupled with the absence of a *CAP59* homologue in the database, suggested the *CAP59* gene to be unique to *F. neoformans*. *C. podzolicus* was the only species besides *F. neoformans* that possessed a capsule and expressed strong laccase activity on various media containing phenolic compounds. A *CNLAC1* homologue was isolated from *C. podzolicus* while it was not detected in the species producing beige to faint tan colonies on media with phenolic compounds. Compared to the *CNLAC1* sequence of four serotypes of *F. neoformans*, the *CNLAC1* homologue of *C. podzolicus* showed the highest homology to that of serotype B/C strains and the lowest homology to that of serotype A strains.

Keywords: *Cryptococcus neoformans*, virulence gene homologues, basidiomycetous yeasts

INTRODUCTION

Filobasidiella neoformans Kwon-Chung [anamorph *Cryptococcus neoformans* (Sanfelice)Vuillemin] is a heterobasidiomycetous yeast which causes fatal meningoencephalitis mostly in immunocompromised patients. The two varieties of *F. neoformans* can be identified based on their serotypes: *F. neoformans* var. *neoformans* (serotype A/D) and *F. neoformans* var. *bacillispora* (serotype B/C). As evident from phylogenetic trees constructed on the basis of small- and

Abbreviations: DOPA, 3,4-dihydroxyphenylalanine; GXM, glucuronoxylomannan; ITS, internal spacer region.

The GenBank accession numbers for the sequences determined in this work are AF337642, L22866, AF337643, AF337644 and AF337645.

large-subunit rRNA sequences, the family Filobasidiaceae is polyphyletic and *F. neoformans* is more closely related to members of the Tremellaceae than to other members of the Filobasidiaceae (Swann & Taylor, 1993; Kwon-Chung *et al.*, 1995). These observations suggest a proximal evolution of *Filobasidiella* and members of the Tremellaceae and, consequently, *F. neoformans* should be classified under Tremellales rather than Filobasidiales (Kwon-Chung *et al.*, 1995; Fell *et al.*, 2000). As in the case of *F. neoformans*, anamorphs of most members of Tremellaceae are capsulated budding yeasts (Oberwinkler, 1987). The polysaccharide capsule of *C. neoformans* is mainly composed of glucuronoxylomannan (GXM), a linear α-1,3-linked mannan bearing 6-*O*-acetyl groups and monosaccharide side chains of xylose and glucuronic acid (Battacharjee *et al.*, 1978; Cherniak *et al.*, 1988). The GXM of polysaccharides isolated from *Filobasidium* species (*C. albidus* var. *albidus*) is reported to have a structural resemblance to that of *F. neoformans* (Ikeda *et al.*, 1991). The major capsular polysaccharide from *Tremella mesenterica* is also GXM with some variation: the xylose side chain is composed of multimers instead of monomer (Slodki, 1966). The polysaccharide capsule is known as a primary virulence factor of *F. neoformans*. Four genes, *CAP59*, *CAP64*, *CAP60* and *CAP10*, involved in capsule formation in *F. neoformans* have been identified and the loss of any of the four genes was found to result in a loss of capsule and virulence (Chang & Kwon-Chung, 1994, 1998, 1999; Chang *et al.*, 1996). The *CAP59* genes have been isolated from several strains of each serotype and the sequence comparison indicated $>90\%$ similarity between different serotypes (Nakamura *et al.*, 2000). Unlike other pathogenic yeasts, *F. neoformans* produces melanin when grown on media containing diphenolic compounds (Staib, 1962; Polacheck *et al.*, 1982; Williamson, 1994). Melanin-forming ability in this species was shown to play an important role in virulence (Kwon-Chung *et al.*, 1982; Rhodes *et al.*, 1982) and the *CNLAC1* gene product responsible for the formation of melanin was identified as a laccase (Williamson, 1994). Disruption of *CNLAC1* in a serotype D strain significantly prolonged the survival of infected mice (Salas *et al.*, 1996), confirming the role of melanin formation in the virulence of *F. neoformans*. Though haploid cells (yeast cells) of *Tremella* species lack the ability to produce melanin, basidiocarps of several *Tremella* species, such as *T. foliacea* and *T. subanomala*, are pigmented. However, *T. mesenterica* showed weak laccase and aryl-alcohol oxidase activity (Peláez *et al.*, 1995).

To determine the phylogenetics of virulence factors in *F. neoformans*, we screened various heterobasidiomycetous species for the ability to produce melanin and polysaccharide capsules. The genomic DNAs of species positive for both of these phenotypes were screened for sequences homologous to the *CAP59* and the *CNLC1* genes. Sequences homologous to *CAP59* were not detected in any heterobasidiomycetous genera that are known to produce GXM capsules. *Cryptococcus podzolicus* was found to be the only species containing a sequence homologous to *CNLAC1*. We obtained sequences from conserved regions of the *CNLAC1* homologue from *C. podzolicus* and compared them with sequences from strains of four *F. neoformans* serotypes.

METHODS

Strains. Table 1 lists the number of strains in each genus screened for the production of melanin. Table 2 summarizes the species (excluding members of the genera *Filobasidiella* and *Cryptococcus*) that reacted with either 3,4-dihydroxyphenylalanine (DOPA) or norepinephrine. Melanin-forming strains were tested for capsule production as described below.

Media and growth conditions. Yeast cultures were maintained on YEPD (1% yeast extract, 2% peptone, 2% glucose) agar slants and grown in the same medium, depending on strains, at either 30 °C or 25 °C. *Escherichia coli* was grown in Luria–Bertani broth or on agar supplemented with 150 μg ampicillin ml^{-1}. Electrocompetent *E. coli* cells (ElectroMAX DH10B, Gibco-BRL) were transformed by electroporation according to instructions supplied by the manufacturer (Bio-Rad Laboratories).

Observation of polysaccharide capsule. A small loopful of cells, obtained from colonies grown on YEPD agar for 48 h at 25 °C or 30 °C, were mounted on India ink to observe the polysaccharide capsule.

DOPA and norepinephrine agar assay for melanin-like pigment formation. Yeast cells were patched on DOPA and norepinephrine agar (0·5 mM) and incubated for up to 2 weeks at 25 °C or 30 °C (Kwon-Chung *et al.*, 1982). Production of light tan, brown to black pigment was regarded as positive for laccase activity and the cultures that tested positive

Table 1. Heterobasidiomycetous yeast genera tested for production of melanin-like pigment

Genus	No. of species	No. of strains
Bensingtonia	3	3
Bullera	4	4
Bulleromyces	1	2
Cryptococcus (non-*neoformans*)	18	49
Cystofilobasidium	2	2
Fibulobasidium	1	2
Filobasidiella	1	157
Filobasidium	4	20
Fellomyces	1	1
Holtermannia	1	2
Leucosporidium	2	2
Malassezia	1	1
Phaffia	1	2
Rhodotorula	9	15
Rhodosporidium	5	5
Sporidiobolus	2	6
Sporobolomyces	5	5
Tremella	5	14
Trichosporon	15	21
Trimorphomyces	1	3
Ustilago	1	1

Table 2. Heterobasidiomycetous yeasts (excluding the genera *Filobasidiella* and *Cryptococcus*) which produced beige to tan coloured colonies on DOPA and/or norepinephrine (NOR) agar

Species	CBS no.	DOPA*	NOR*
Filobasidium floriforme	6240, 6241, 6242, 7634, 7635, 7636	—	f/d
F. capsuligenum	4736	—	f/d
F. globisporum	7642	—	f/d
F. uniguttulatum	1727	f	—
Holtermannia corniforme	7088	f/d	—
Tremella encephala	6968, 8207, 8217, 8218, 8220, 8235	f	f/d
T. foliacea	6969, 7215,	f	f/v
T. fusiformis	6970, 6971	—	f/v
T. globospora	6972,	f	f/d
T. subanomala	6976	f	f/d
Trimorphomyces	200.94	f	—
papilionaceus	443.92	f/d	f/d
	445.92	f/d	—

* f, faint; v, variable; d, delayed.

with both DOPA and norepinphrine were considered to be true laccase-positive.

DNA analysis. Rapid DNA preparation was as previously described (Fujimura & Sakuma, 1993) and for larger quantities, the method described by Varma & Kwon-Chung (1991) was used. For Southern analysis, DNA samples were digested with *Eco*RI and fractionated in agarose gels. DNA was transferred to Hybond-N nylon membranes and hybridized to labelled DNA probes using standard protocols. Random hexamer priming was used to label the DNA probes to specific activities $> 10^8$ d.p.m. μg^{-1} (Feinberg & Vogelstein, 1983).

Polymerase chain reaction (PCR). PCRs were performed with the MJ Research thermocycler using Boehringer Mannheim's *Taq* DNA polymerase and nucleotide mix, or PCR Supermix (Gibco-BRL). The amplification conditions used with each set of primers are described below.

Isolation of the *CNLAC1* gene homologue. Two oligonucleotide primers (Table 3) compatible with the conserved copper-binding sites of *CNLAC1* gene (Williamson, 1994) and the laccase genes of three Homobasidiomycete species (Mikuni & Morohoshi, 1997; Perry *et al*., 1993; Giardina *et al*., 1995) were designed and used for *in vitro* amplification from B-3501 (serotype D strain). The PCR cycle was initiated by 4 min at 94 °C, followed by 29 cycles of denaturation (1 min; 94 °C), annealing (1 min; 50 °C) and polymerization (1 min; 72 °C). The reaction was terminated by an extended polymerization step (10 min; 72 °C). The *CNLAC1* PCR product was used as the probe to isolate genomic fragments from a size-selected library. The *CNLAC1* fragments obtained from a size-selected library of *F. neoformans* serotypes A (H99, a reference strain widely used for molecular genetic study), B (B-3939, the type culture of *C. neoformans* var. *gattii*), C (NIH34, a serotype C reference strain used for production of rabbit anti-C serum), were each cloned into a Bluescript SK+ vector (Stratagene) and subjected to nucleotide sequence analysis. The PCR fragment obtained from *C. podzolicus* (CBS 6442) was cloned into the same vector and sequenced.

Detection of *CAP59* sequence. Genomic DNAs from strains producing a large GXM capsule were digested with various restriction enzymes and analysed by Southern hybridization under varying stringency conditions using the *CAP59* cDNA probe obtained from strain B-3501 (serotype D) (Chang & Kwon-Chung, 1994). Attempt was also made to amplify the sequence by PCR using primers derived from the *CAP59* sequence of B-3501 (serotype D) (Table 3). The *in vitro* amplification procedure was the same as that for the *CNLAC1*.

Nucleotide sequence analysis. Nucleotide sequences were obtained using a Sequenase 2.0 kit (USB Cleveland) and the ABI PRISM (Perkin-Elmer) sequencer. Sequence analysis, comparisons and database searches were performed with MacVector 3.0 (Oxford Molecular Group) and various other programs of the Genetics Computer Group.

RESULTS AND DISCUSSION

Laccase activity

Numerous heterobasidiomycetous species belonging to 21 genera and having yeast anamorphs were screened

Table 3. Oligonucleotide primers used for the cloning of *CNLAC1* and *CAP59* homologues

	Oligonucleotide sequence (5′–3′)	Remarks
1	GACTAGAATTCGTATTGGTGGCACTTC	*CNLAC1* sense primer, derived from strain B-3501 serotype D (Williamson, 1994)
2	GCTAAAAGCTTCGTGCAGGTGGTAAGG	*CNLAC1* antisense primer, derived from strain B-3501 serotype D (Williamson, 1994)
3	AGAATTCCACCAGGCATATTTCG	*CAP59* sense primer, derived from strain B-3501 serotype D (Chang & Kwon-Chung, 1994)
4	ATCAAAGCTTGCAACGTCTCCATATCCTC	*CAP59* antisense primer, derived from strain B-3501 serotype D (Chang & Kwon-Chung, 1994)

for formation of the melanin-like pigment on DOPA and norepinephrine media (Table 1). Of the two substrates, the latter was found to be superior in reproducibility, stability and specificity for laccase activity judged by the results obtained from *F. neoformans* strains. Table 2 lists the species belonging to four of the 21 genera, excluding *Filobasidiella* and *Cryptococcus*, that reacted with DOPA and norepinephrine. Although the intensity varied, all strains of *F. neoformans* produced melanin. Colony colour of the strains listed in Table 2, varied from beige to light tan (f for faint) and was not comparable to the dark brown to black colour of *F. neoformans* colonies. All the strains, other than *F. neoformans*, that exhibited strong laccase activity belonged to *C. podzolicus* (Table 4). Of the 15 *C. podzolicus* isolates tested, only the type strain (CBS 6819) was clearly laccase-negative and may harbour mutations in genes involved in melanin synthesis. All the laccase-positive isolates of *C. podzolicus* used in this study were isolated from soil and have previously been identified as *C. laurentii*. However, sequence studies of the D1/D2 domains of the large subunit and the internal transcribed spacer region (ITS) of rDNA clearly showed that they are *C. podzolicus* (T. Boekhout and others, unpublished).

Isolation of *CNLAC1* homologous sequences

DNAs were prepared from representative strains of *Filobasidium floriforme*, *F. uniguttulatum*, *Tremella. encephala*, *T. foliacea* and *T. globospora* in addition to *F. neoformans* and *C. podzolicus*. Southern analysis was performed using an 800 bp *CNLAC*1 cDNA probe (C. *neoformans* serotype D) (Williamson, 1994) under various stringency conditions. No detectable hybridization signals were observed with those species that produced beige to tan colonies on DOPA or norepinephrine medium. Low-stringency washes (2× SSC: 0·1% SDS, 20 min, room temperature) and extended exposure for up to 2 weeks at −70 °C still revealed no hybridization signals. Intense hybridization signals were observed with DNAs from strains of *F. neoformans* while those with varying intensity were detected with DNAs obtained from various *C. podzolicus* isolates. Southern hybridizations representing negative and positive results are shown in Fig. 1. Size-selected genomic libraries were constructed to isolate conserved regions of the laccase gene from strains of A, B and C serotypes of *F. neoformans*. PCR, using primers compatible with the conserved laccase copper-binding sites (Table 3), enabled the isolation of homologous sequences from *C. podzolicus* while the same primers failed to yield amplicons from species that produced beige to tan colonies. The PCR fragment of the *CNLAC1* amplified from *C. podzolicus* CBS 6442 was sequenced in order to compare it with those of *F. neoformans*. Sequence comparisons of the conserved region indicated a high degree of similarity between the *CNLAC1* fragments isolated from four *F. neoformans* strains and *C. podzolicus* CBS 6442. A pair-wise sequence comparison was performed between *C. podzolicus*, the strains of four *F. neoformans* serotypes and three species of Homobasidiomycetes: *Pleurotus ostreatus* (Giardina

Table 4. *Cryptococcus* species (excluding *C. neoformans*) tested for the production of melanin-like pigment on DOPA or norepinephrine (NOR) agar

Species	CBS no.	DOPA*	NOR*
C. aerius	155	−	−
C. ater	4685	−	−
	5809	−	−
	5591	−	−
C. bhutanensis	6294	f/d	−
C. consortionis	7159	−	−
C. dimennae	5570	−	−
C. curvatus	570	−	−
C. flavus	331	−	−
	8296	−	−
C. friedmannii	7160	−	−
C. heveanensis	569	−	−
C. humicolus	571	−	−
	4281	−	−
C. hungaricus	4214	−	−
C. laurentii	139	−	f/d
	973	−	−
	2174	−	−
	5746	−	−
	4919	−	−
	6473	−	d
	6474	−	−
	6475	−	−
	6476	−	−
	7140	−	−
	7235	−	−
	8359	−	−
	8360	−	−
C. macerans	2206	−	−
C. magnus	140	−	−
C. penaeus	2409	−	−
C. podzolicus	6819	−	−
	7717	−	d
	6442	+	+
	6445	+	+
	6446	+	+
	6484	+	+
	6485	f	+
	6486	+	+
	6487	+	+
	6488	+	+
	6490	+	+
	6492	f	+
	6493	+	+
	6494	+	+
	6496	+	+
C. terreus	1895	−	f/d
C. vishniacii	8142	−	−
	8143	−	f/d

* f, faint; d, delayed.

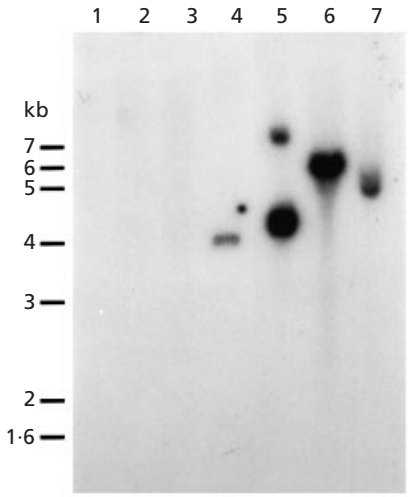

Fig. 1. Southern blot hybridization of genomic DNAs obtained from various species of heterobasidiomycetous yeasts with the *CNLAC1* cDNA probe of B-3501. Lane 1, *T. globospora* (CBS 6972); lane 2, *T. foliacea* (CBS 6969); lane 3, *T. encephala* (CBS 8207); lane 4, *C. podzolicus* (CBS 6442); lane 5, *F. neoformans* var. *neoformans* (B-3501); lane 6, *F. neoformans* var. *neoformans* (H99); lane 7, *F. neoformans* var. *bacillispora* (NIH34). The hybridization and washes were performed under low-stringency conditions (45 °C; 2× SSC/0·1% SDS) and the filter was exposed for 7 d at −70 °C.

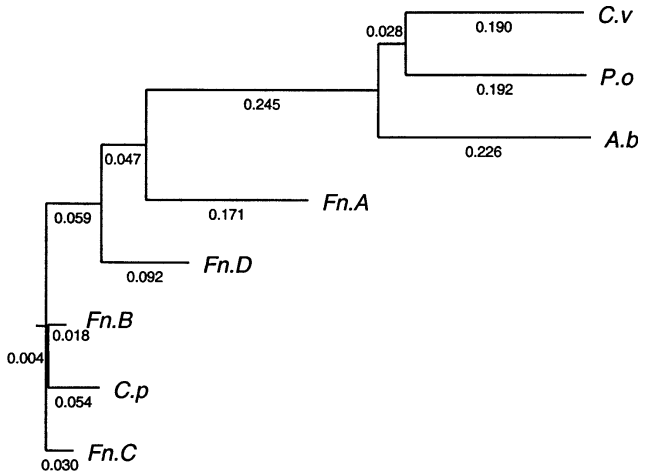

Fig. 2. Dendrogram derived from conserved sequence comparison using the CLUSTER W ALIGNMENT program. *A.b*, *A. bisporus* (GenBank accession L10063), *P.o*, *Pleorotus ostreatus* (GenBank accession Z34847), *C.v*, *Coriolus versicolor* (EMBL D284235). GenBank accession numbers of the four *F. neoformans* serotypes and *C. podzolicus* are: *Fn.A*, AF337642; *Fn.D*, L22866; *Fn.B*, AF337643; *Fn.C*, AF337644; *C.p*, AF337645. The numbers on the branches of the dendrogram indicate changes per residue.

et al., 1995), *Coriolus versicolor* (Mikuni & Morohoshi, 1997) and *Agaricus bisporus* (Perry *et al*., 1993). It revealed the highest homology (94%) between strains of serotype B and C followed by serotype B/C and *C. podzolicus* (92%), serotype B/C and serotype D (82%), serotype A and serotype D (70%), serotype A and serotype C (68%), serotype A and serotype B (67%), and serotype A and *C. podzolicus* (67%). The sequences of the copper-binding region conserved in three species of Homobasidiomycetes were considerably diverged from those of *C. podzolicus* and *F. neoformans* (% homology less than 37%) (Fig. 2). The higher homology observed between sequences of serotype D and serotypes B/C compared to that between serotypes D and A was unexpected since it was not the case with either the partial gene sequence of *CAP59* (Nakamura *et al*., 2000) or the sequence of a PCR fragment (462 bp) amplified from a region of *CNLAC1* gene partially overlapping with ours in different strains of serotype B (Xu *et al*., 2000). However, a closer genetic relationship of serotype D to serotype B strains than to serotype A isolates of *F. neoformans* has been observed when partial large-subunit rRNA sequence was analysed (Gueho *et al*., 1993). Franzot *et al*. (1999) also observed significant differences in *URA5* sequences and the DNA fingerprinting patterns between strains of serotype A and D and indicated that serotype A strains warrant a separate varietal status: *C. neoformans* var. *grubii*. Subsequent analysis of other genetic markers, such as the intergenic spacer sequence associated with rDNA or amplified fragment length polymorphisms, however, did not support a separate varietal status for serotype A (Diaz *et al*., 2000; Boekhout *et al*., 2001). These findings indicate that the phylogenetic relationship between strains based on a single gene sequence can vary depending on the strains of *F. neoformans* used and hence can lead to confusing results. They also indicate the existence of considerable genetic heterogeneity among strains of the same serotype of *F. neoformans*.

The *CAP59* homologues were not detected in the genomes of GXM polysaccharide capsule forming yeasts other than various strains of *F. neoformans*

Of the yeasts listed in Table 1, large polysaccharide capsules were observed in species of *Filobasidiella*, *Cryptococcus*, *Bulleromyces*, *Bullera*, *Filobasidium*, *Tremella* and *Trimorphomyces*. The species forming large capsules mostly belonged to the Tremellales and Filobasidiales (Fell *et al*., 2000). Since the major polysaccharide produced by species of *Filobasidiella*, *Filobasidium*, *Tremella* and *Cryptococcus* is known to be GXM (Abercombie *et al*., 1960; Cherniak *et al*., 1988; Ikeda *et al*., 1991; Slodki, 1966), we assumed that the genes involved in polysaccharide formation were conserved among these yeasts. It was expected, therefore, that Southern blot analysis would detect the DNA sequences which hybridize to the *CAP59* cDNA probe of serotype D from *F. neoformans*. An intense signal was observed for all four serotypes of *F. neoformans*. Southern blot analysis of genomic DNAs from species of *Tremella*, *Filobasidium* and *Cryptococcus*, however, showed a faint or no signal even under conditions of low stringency and prolonged exposure (7 d) at −70 °C. Southern blot analysis of DNAs from selected strains of

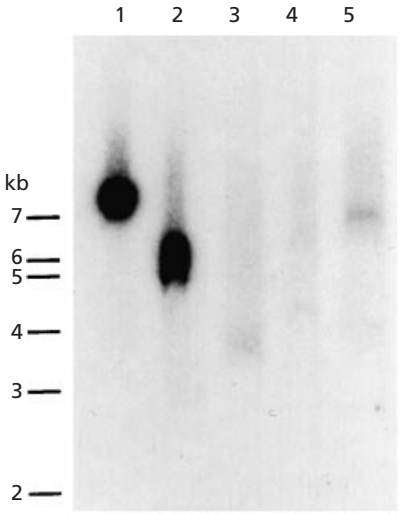

Fig. 3. Southern blot hybridization of genomic DNAs obtained from various species of Heterobasidiomycetes probed with radiolabelled *CAP59* cDNA of B-3501 (serotype D). Lane 1, *F. neoformans* var. *neoformans* (B-3501, serotype D); lane 2, *F. neoformans* var. *bacillispora* (NIH34, serotype C); lane 3, *Tremella foliacea* (CBS 6969); lane 4, *Filobasidium uniguttulatum* (CBS 1727); lane 5, *C. podzolicus* (CBS 6442). Experimental conditions were the same as those for the *CNLAC1* probe.

F. neoformans serotype A, *F. neoformans* serotype C, *Tremella foliacea*, *Filobasidium uniguttulatum* and *Cryptococcus podzolicus* is shown in Fig. 3. Besides *F. neoformans*, only *Cryptococcus podzolicus* (CBS 6474) showed a signal but it was extremely faint. While a faint hybridization signal was obtained with *C. podzolicus* DNA under low-stringency conditions, PCR primers 3 and 4 (Table 3) that effectively amplify the *CAP59* sequence from all four serotypes of *F. neoformans* failed to generate any product. This suggested that the signal with *C. podzolicus* DNA was probably the result of nonspecific hybridization due to low stringency conditions. These yeasts may have functional homologues of *CAP59* that have little nucleotide sequence similarity. The other explanation may be that the *CAP59* pathway in *F. neoformans* is not conserved in other basidiomycetous yeasts. Though the function of *CAP59* in *F. neoformans* is unknown, the presence of a transmembrane domain suggests it to be involved in secretion (Chang & Kwon-Chung, 1994).

Tremella lineage of *CNLAC1* among the capsulated yeasts

Although the DNA sequence of the laccase gene in *C. podzolicus* shows high homology to that of *F. neoformans*, a phylogenetic tree constructed by Fell *et al.* (2000) on the large rDNA subunit did not show them to be in the same cluster. Both *C. podzolicus* and *F. neoformans*, however, were in the Tremellales clade of the hymenomycetous yeasts (Fell *et al.*, 2000). This suggests that *CNLAC1* has evolved independently and is preserved in multiple internal clusters among species with the *Tremella* lineage and thus the sequence is inappropriate for the construction of phylogenetic trees. Considering the higher than 90% sequence homology between *F. neoformans* and *C. podzolicus*, it is likely that the *CNLAC1* homologues of these two species have originated from a common ancestor. The lack of *CNLAC1* homologue in *Tremella* species that produced faintly brown colonies on DOPA or norepinephrine agar, such as *T. foliacea* and *T. encephala*, was unexpected. It is possible that these *Tremella* species possess a laccase gene that has undergone enough genetic drift not to be detected by Southern hybridizations or PCR techniques. The laccase gene sequences of homobasidiomycetous species showed no close relationship with those of *F. neoformans* and *C. podzolicus* (Fig. 2).

Uniqueness of *F. neoformans* among tremellaceous yeasts

F. neoformans possesses distinct features among members of the Tremellales. It is the only yeast in this order that produces a slender capitate holobasidium with basipetally formed chains of basidiospores (Kwon-Chung, 1975) and it is the only species to cause a systemic fatal infection in humans. As far as we know, it is the only species of the Tremellales that grows optimally at temperatures above 30 °C. The optimum and maximum temperatures for growth of *F. neoformans* are 32 °C and 40 °C respectively (Kwon-Chung & Bennett, 1992). Some of the melanin-forming strains of *Cryptococcus* species isolated from clinical specimens and identified as *C. laurentii* may have been *C. podzolicus*; the aetiological significance of *Cryptococcus* species excluding *C. neoformans*, however, remains doubtful (Krajden *et al.*, 1991). Although the polysaccharide capsule and *CNLAC1*, two important virulence factors of *F. neoformans*, are present in most strains of *C. podzolicus*, the species lacks thermotolerance, the major factor required for successful growth in the tissues of warm-blooded hosts.

All strains of *C. podzolicus* tested in this study failed to grow at temperatures higher than 30 °C.

Some of the melanin-producing strains previously identified as *C. laurentii* on the basis of their carbon assimilation profiles showed poor growth at 37 °C (Kwon-Chung *et al.*, 1992). We attempted to produce experimental infections with such strains but found them unable to survive long enough to cause infection in mouse tissue (data not shown).

The yeasts of *Tremella* used in this study grew optimally at 25 °C but failed to grow at temperatures above 30 °C. Yeast cells of *F. neoformans* var. *neoformans* are most commonly isolated from pigeon droppings throughout the world (Kwon-Chung & Bennett, 1992). The pigeon body temperature is at least 40 °C and *F. neoformans* yeast cells ingested by pigeons survive in the digestive system for a prolonged time (Swinne-Desgain, 1976). The evolution of thermotolerance in this species coupled

with polysaccharide capsule formation and *CNLAC1* activity may have rendered *F. neoformans* a successful pathogen of warm-blooded animals while other heterobasidiomycetous yeasts remained saprophytic.

Conclusion

F. neoformans is unique among pathogenic yeasts due to the presence of major virulence factors, such as the polysaccharide capsule and the formation of melanin. Although *F. neoformans* is known to have *Tremella* lineage and the polysaccharide capsule is a common property produced by many yeasts in the Tremellales, the genetic control of capsule synthesis appears to have diverged. Determination of such genetic diversity will require the molecular dissection of the GXM-capsule-forming genes in the other fungi. The sequence homologous to the *CNLAC1* gene that encodes the laccase of *F. neoformans* was found only in *C. podzolicus*, although several other capsule-forming yeasts reacted weakly to substrates of laccase. The *CNLAC1* homologue of *C. podzolicus* was most closely related to serotypes B/C of *F. neoformans*, suggesting a common origin. The lack of pathogenicity in *C. podzolicus* appears to be primarily due to its inability to grow at temperatures equal to or higher than 37 °C.

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