

Effect of the Laccase Gene, *CNLAC1*, on Virulence of *Cryptococcus neoformans*

By S.D. Salas,* J.E. Bennett,* K.J. Kwon-Chung,‡ J.R. Perfect,§
and P.R. Williamson||

From *Clinical Mycology Section, and ‡Molecular Microbiology Section, Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Disease, Bethesda, Maryland 20892; the §Department of Medicine, Duke University Medical Center, Durham, North Carolina 27710; and ||Section of Infectious Diseases, University of Illinois at Chicago Medical Center, Chicago, Illinois 60612

Summary

To assess the relationship between melanin production by *Cryptococcus neoformans* and virulence on a molecular basis, we asked: (a) is *CNLAC1*, the laccase structural gene of *C. neoformans*, expressed in vivo?; (b) can mouse virulence be restored to *cnlac1* (Mel^-) mutants by complementation with *CNLAC1*?; and (c) will targeted gene deletion of *CNLAC1* decrease virulence for mice? Melanin is produced when cryptococcal laccase catalyzes the oxidation of certain aromatic compounds, including L-dopa, to quinones, which then polymerize to melanin. To assess *CNLAC1* transcription, RNA was extracted from *C. neoformans* in cerebrospinal fluid of infected rabbits. Reverse transcriptase-polymerase chain reaction detected *CNLAC1* transcript, indicating that laccase may be produced in the infected host. To assess the effect of *CNLAC1* deletion on virulence, a Mel^- mutant (10S) was obtained by disruption of the 5' end of the gene. After multiple backcrosses with a parental strain to remove unintended genetic defects introduced by the transformation process, a Mel^- progeny was tested and found to be much less virulent for mice than a Mel^+ progeny. Another Mel^- strain (*mel2*), obtained from J.C. Edman (University of California at San Francisco, CA), produced *CNLAC1* transcript but no detectable melanin. Characterization of this mutant revealed a base substitution in *CNLAC1* that changed a histidine to tyrosine in a putative copper-binding site. When this base change was introduced into *CNLAC1* by site-directed mutagenesis, it no longer transformed *mel2* to Mel^+ , indicating the importance of this histidine in laccase activity. Complementation of a *mel2*-derived mutant with *CNLAC1* restored the Mel^+ phenotype and increased virulence. These results support the concept that the *CNLAC1* gene product has a role in virulence.

Cryptococcus neoformans is an opportunistic fungal pathogen that causes life-threatening infections in ~6% of patients with AIDS (1). Generally accepted virulence factors in *C. neoformans* are capsule formation and the ability to grow at 37°C (2, 3). *C. neoformans* is unique among species of *Cryptococcus* in its virulence and in its ability to produce melanin when grown on appropriate substrates.

Melanin-negative mutants have been reported to be less virulent than melanin-producing strains (2–4). In one study (3), a hypovirulent, spontaneously occurring, melanin-negative strain “back-reverted” (i.e., produced melanin) upon recovery from mice brains. This melanin-positive “revertant” demonstrated increased virulence upon reinoculation into mice.

Definitive studies on the role of melanin in the virulence of *C. neoformans* have been hindered by the lack of melanin-negative mutants characterized at the molecular level.

Mutants are needed that differ from wild-type in a single defined locus responsible for melanin production. Recently, the *C. neoformans* laccase 1 (*CNLAC1*)¹ gene has been isolated (5). Cryptococcal laccase, the gene product of *CNLAC1*, is believed to catalyze the oxidation of certain catecholamines into quinones, such as decarboxydopachrome, which then spontaneously polymerize into dark brown melanin-like compounds. This pigment may protect the fungus against nitrogen- and oxygen-based oxidative attack by host cells (6). Laccases are part of the family of blue copper-containing oxidases that contain three types

¹Abbreviations used in this paper: CHEF, contour-clamped homogeneous electric field electrophoresis; *CNLAC1*, *Cryptococcus neoformans* laccase 1; CSF, cerebrospinal fluid; FOA, 5-fluororotic acid; ORF, open reading frame; RT, reverse transcriptase; YEPD, yeast extract peptone dextrose.

of copper centers; each coordinate covalently, bound to their respective histidine and cysteine moieties (7).

We describe here the effect on virulence when *CNLAC1* is altered by UV mutagenesis, restored by complementation, and disrupted by gene knockout. We also describe the effect on melanin phenotype of site-directed mutagenesis in a His₁₆₄ putative copper-binding site of *CNLAC1*.

Materials and Methods

Strains. *C. neoformans* melanin negative mutants *mel1–mel4* (8), were provided by J.C. Edman. *C. neoformans* strains American Type Culture Collection (ATCC) 34873 (B-3501), B-4476 5-fluoroorotic acid (FOA), B-4500, and B-4476 were in our stock. Strains B-4500 (α type) and B-4476 (*a* type) are an isogenic set derived from B-3501 and B-3502 described previously (9). The source and genotypes of the strains derived from B-4500 and B-4476 are diagrammed in Fig. 1. *Escherichia coli* strain DH10B (Life Technologies, Gaithersburg, MD) was the host strain for construction of libraries and ligated products.

Genetic Crosses. Strains of two opposite mating types were crossed on V-8 juice agar and grown at 30°C until basidiospore chains could be detected microscopically. Basidiospores were harvested by cutting agar plugs from the edge of filamentous growth, vortexing for 10 min in normal saline, and plating out serial dilutions onto selective media. The mating type of the basidiospore growth was determined by backcrossing to standard strains of known mating type, either B-4500 (α) or B-4476 (*a*).

The 10S mutant was backcrossed to B-4500 four times. Mating type *a* nonpigmented colonies on norepinephrine or Niger seed agar were picked for each cross for the first three crosses. On the final cross, mating type *a* Mel⁺ (10S-BUC) and Mel⁻ (10S-BU) were selected.

The 2E-TU and 2E-TUC mutants were mated to B-4476 four times. After each cross, multiple colonies of mating type α

progeny with the same MEL phenotype were picked. For the 2E-TUC crosses, Southern blots were performed and compared with known controls to verify that the transformed *CNLAC1* construct was carried over in the cross. After the fourth cross, PCR using a primer binding the mutated base was used as described below to verify that the backcrossed product, 2E-TUC-4, contained the defective *mel2* gene and not the functional B-4476 *CNLAC1* gene.

Media. YEPD medium contained 1% yeast extract, 2% Bacto-peptone, and 2% glucose. For the detection of melanin phenotype, colonies were grown on either Niger seed agar (10) or norepinephrine medium (10), containing 100 mg norepinephrine tartrate (Sigma Chemical Co., St. Louis, MO) per liter. For enzyme assay and RNA extraction, organisms were grown in medium that contained L-asparagine (1 g/liter), MgSO₄·7H₂O (0.5 g/l), KH₂PO₄ (3 g/l), thiamine (1 mg/l), and glucose (0.1%) at pH 6.5.

Enzyme Assay. Whole cell laccase activity was measured using epinephrine tartrate (Sigma Chemical Co.) as a substrate by a modification of the method of Williamson (5). Briefly, *C. neoformans* was grown to mid-log phase at 30°C in 50 ml of asparagine medium. The cells were harvested by centrifugation, washed, re-suspended in the same medium without glucose, and incubated for 7 h on a shaker at 30°C. Cells were then collected by centrifugation and washed with 0.05 M sodium phosphate buffer, pH 6.5. A graph of cell concentration measured by hemocytometer, compared with absorbance at 600 nm (*A*₆₀₀) on a spectrophotometer (model DU-64; Beckman Instruments, Columbia, MD), was plotted to standardize cell counts. Each reaction mixture contained 0.05 M sodium phosphate buffer, pH 6.5, 1 mM epinephrine, and 10⁸ cells. Color production in the supernatant was measured after 30-min incubation at 25°C. 1 U of enzyme was defined as 0.001 *A*₄₇₅. All readings were corrected by subtracting *A*₄₇₅ of duplicates in which the reaction was stopped at zero time with 10 mM NaCN.

Transformation of *C. neoformans*. The electroporation method described by Edman and Kwon-Chung (11) was used to transform cells of *C. neoformans*.

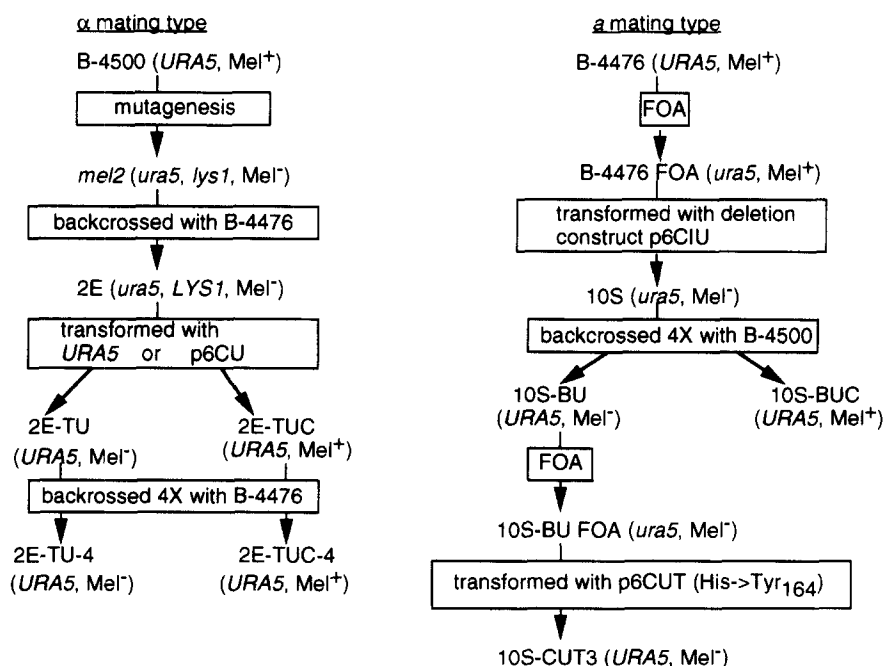


Figure 1. Derivation, genotype, and melanin phenotype of *C. neoformans* strains used in study.

Growth Rate at 37°C. Growth rates at 37°C were determined using the method described by Rhodes et al. (4).

Plasmid Constructions. PCR was used to amplify a functional cryptococcal *URA5* gene (1.2 kb) from the plasmid pURA5g2 (11), kindly provided by J. C. Edman. Briefly, the plasmid (200 ng) was added to a mixture that contained 100 mM Tris, 500 mM KCl, pH 8.3, 1.5 mM MgCl₂, 0.2 mM each deoxyribonucleoside triphosphate (Boehringer Mannheim Corp., Indianapolis, IN), and 1.1 mM each of two oligonucleotide primers containing *SpeI* restriction sites at the 5' end. Primer 1 sequence was (5'-GCCGCCACTAGTACGCCTGCCTGTACTTAA-3') and primer 2 sequence was (5'-GCCGCCACTAGTGGACATGATGATTGGAGT-3'). The mixture was subjected to 25 rounds of amplification; each round was annealed for 2 min at 45°C, and extended at 72°C for 2.5 min. The PCR fragment was then digested with *SpeI*, and inserted into the multiple cloning site (MCS) portion of pG51 (5) just upstream of the 6-kb genomic fragment of *CNLAC1* from strain B-3501 to yield plasmid p6CU (Fig. 2 A). A second PCR product of *URA5* was amplified using the same conditions and primer-binding regions as above but with nucleotides (5'-GCCGCCCATGG-3') at the 5' end of each primer yielding a *NcoI* recognition site. This PCR construct was then cleaved with *NcoI* and ligated into pG51 to interrupt the open reading frame (ORF) and yield plasmid p6CIU.

A 520-bp area of the *CNLAC1* gene in strain B-4500 was cloned so that the sequence could be compared with that of the

Mel⁻ mutant, 2E. This region contained a putative histidine to tyrosine mutation. A PCR product of this *CNLAC1* sequence was amplified using the same conditions except the template was B-4500 genomic DNA (200 ng); primer 1 was (5'-GCCGCCACTAGTGGCGATACTATTATCGTA-3') and primer 2 was 5'-GCCGCCACTAGTTTCTGGAGTGGCTAGAGC-3'. This PCR product was cleaved with *SpeI* and ligated into the MCS portion of pBluescript SK (Stratagene Inc., La Jolla, CA) to yield p45His. All plasmids are summarized in Table 1.

Site-directed Mutagenesis of *CNLAC1*. PCR point mutagenesis of the DNA encoding a histidine copper-binding site in *CNLAC1* was performed according to the methods described by Zhao et al. (12). Two unique restriction sites within the *CNLAC1* ORF of p6CU, *BstBI* and *NcoI*, were identified (Fig. 2 A). The *NcoI* site was located 13 bases from the DNA encoding for the histidine of interest. PCR primers were constructed that contained these unique restriction sites and the His→Tyr mutation. PCR was performed under the same conditions except that the template was p6CU, annealing temperature was 50°C, primer 1 was (5'-CCTGCTCATATTCGAAGACGC-3'), and primer 2 was (5'-CGTCGGCCATGGAATTGGAGTAATAGGAGTGCC-3'). The latter primer contained the mutated base (boldface type). The 275-bp PCR product was then cleaved sequentially with *BstBI* and *NcoI* and ligated into the unique *BstBI*/*NcoI* sites of p6CU to yield the mutated plasmid p6CUT (Fig. 2 A).

Differential PCR. To identify DNA with the 2E point mutation (C→T) at codon #164, oligonucleotides were constructed with and without the mutated base in the terminal position. PCR was performed under the same conditions except that the annealing temperature was 62°C, primer 1 was (5'-GTTAGCCAAGCTTTTCTT-3'), and primer 2 was (5'-CATGGAATTGGAGTATG-3') or (5'-CATGGAATTGGAGTAATA-3'). Boldface type

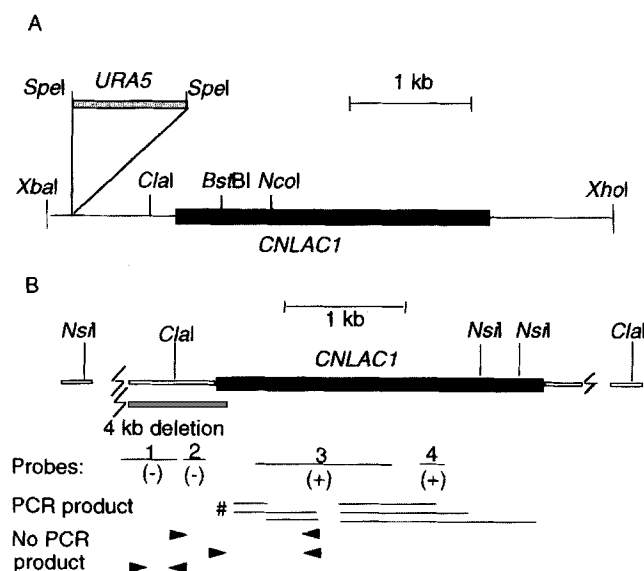


Figure 2. (A) Map of complementation plasmid, p6CU. A 1.2-kb PCR fragment containing *URA5* was inserted adjacent to a 6-kb genomic fragment containing *CNLAC1* at a *SpeI* site in pG51. The ORF of *CNLAC1* has 1-kb flanking sequences at both ends. Diagram does not include Bluescript SK sequence. (B) Wild-type *CNLAC1* gene and changes found in 10S. Deleted area is shown as hatched bar at the 5' end of *CNLAC1*. Probes that did or did not hybridize to a *ClaI* digest of 10S genomic DNA on Southern blot are designated (+) and (-), respectively. Probe 1 is 1.6-kb PCR product located 5' to the gene; probes 2 and 4 are end-labeled oligonucleotides; and probe 3 is a 876-bp *CNLAC1* internal cDNA fragment. (solid bars) PCR products. (#) Two PCR products near breakpoint of deletion that were larger than expected (see text). (Arrowheads) PCR primers that were unsuccessful in producing products using 10S DNA as template.

Table 1. Plasmid Characteristics

pURA5g	pBSK II with genomic fragment containing <i>URA5</i> inserted at <i>EcoRI</i> (11)
pG51	pBSK II with genomic fragment containing <i>CNLAC1</i> inserted at <i>EcoRI</i> (5)
p6CU	pG51 with a 1.2-kb PCR product containing <i>URA5</i> inserted at <i>SpeI</i>
p6CIU	pG51 with <i>CNLAC1</i> interrupted by <i>URA5</i> (1.2-kb PCR product) at <i>NcoI</i>
p5mel2	pBSK II with a 2E-derived 5.0-kb genomic fragment containing <i>CNLAC1</i> inserted at <i>ClaI</i>
p45His	pBSK II with a B-4500-derived 520-bp PCR product containing internal genomic sequence of <i>CNLAC1</i> inserted at <i>SpeI</i>
p6CUT	p6CU with the site-directed mutagenesis-derived 275-bp sequence containing the His ₁₆₄ mutation inserted at <i>BstBI</i> and <i>NcoI</i>
p2-4476	pBSK II with a B-4476-derived 2-kb genomic fragment (located 5' to <i>CNLAC1</i>) inserted at <i>ClaI</i>

represents primer with the mutated base. For size-selected libraries, template for PCR was obtained from genomic DNA (13) of strain 2E-TUC-4 which was digested with *Cla*I, cut out from electrophoretic gel, and purified according to instructions in the GENE CLEAN kit (BIO 101, Inc., Vista, CA). For PCR of B-3501, 2E-TU-4, and 10S-CUT3, genomic DNA (13) was used as template.

DNA Isolation, Genomic Library, DNA Sequencing, and Southern Analysis. Genomic DNA was isolated by the method of Varma and Kwon-Chung (13). A 5-kb *Cla*I size-selected library was prepared from *C. neoformans* strain 2E (Fig. 1) and inserted into pBluescript SK II+ (Stratagene Inc.) according to standard methods (14). Genomic clones were screened with the radiolabeled 876-bp *Acc*I fragment of the *CNLAC1* cDNA vector pc21 (5), using a random-priming kit (Stratagene Inc.) and [³²P]dCTP (Amersham Corp., Arlington Heights, IL). From the library, a 5.0-kb genomic clone containing *CNLAC1*, p5mel2, was selected. All plasmid DNA were sequenced by a dideoxy chain termination method according to the instructions of the Sequenase kit (United States Biochemical Corp., Cleveland, OH). Southern blot hybridizations using various restriction enzymes were performed according to standard methods (14). *CNLAC1* was detected in the blots by hybridization with the *Acc*I radiolabeled 876-bp cDNA fragment. The *URA5* gene probe consisted of a [³²P]dCTP random-labeled (Stratagene Inc.) 1.2-kb PCR fragment without the GCCGCC + *Spe*I sites described above. End-labeled probes of oligonucleotide 1 (Fig. 2 B, probe 2) [5'-TACTCGTGCCGGGCCTTG-3'] and oligonucleotide 2 (Fig. 2 B, probe 4 [5'-CTAGGGGTTCAACAATAT-3']) were constructed by standard techniques (14), using T4 polynucleotide kinase (New England Biolabs, Beverly, MA) and [³²P]ATP (Amersham Corp.). For isolation of DNA sequence 5' to *CNLAC1*, a 2–3-kb *Cla*I size-selected library was prepared from strain B-4476 FOA and inserted into pBluescript SK. Genomic clones were screened with a ³²P end-labeled oligonucleotide containing sequence located 800 bp upstream to the beginning of the ORF of *CNLAC1*: (5'-CAGTCAGTATCGACGGCA-3'). From this genomic library, a clone with a 2-kb insert, p2-4476, was selected.

DNA Fingerprinting. Genomic DNA was digested with *Acc*I and hybridized with the UT-4p probe according to the methods described by Varma and Kwon-Chung (15).

Contour-clamped Homogeneous Electric Field Electrophoresis. This was performed according to the modified methods of Wickes et al. (16).

RNA Isolation, Northern Analysis, and Reverse Transcriptase-PCR. RNA was extracted from cells grown at 30°C in asparagine broth medium. At mid-log phase (based on A_{600}), cells were washed with sterile H₂O, then transferred to asparagine broth media without glucose (see Media) for 2 h. Total RNA was isolated using the Fast RNA kit-Red (BIO 101, Inc.). Northern (RNA) blot analysis was performed by standard methods (14). Blots were hybridized with the *CNLAC1* cDNA probe. Total RNA was assessed with a radiolabeled *Candida albicans* 18S rDNA sequence that exhibits 89% homology to the 18S rRNA sequence of *C. neoformans* (EMBL/GenBank/DDBJ accession number L05428) and was a generous gift of A. Geber, Food and Drug Administration, Rockville, MD. For some of the Northern blots, total RNA was assessed with a *C. neoformans* actin probe (EMBL/GenBank/DDBJ accession number U10867). This actin probe was generated by PCR using the same conditions except that the annealing temperature was 57°C, template was cDNA of strain H99, primer 1 was (5'-TCGCCGCCTTGGTCATTG-3') and primer 2 was (5'-CGTATTCGCTCTTCGCGA-3'). For a

northern and Southern blot of the 5' region of *CNLAC1*, a 1.6-kb probe (Fig. 2 B, probe 1) containing PCR-generated (same conditions) sequence extending 5' to *CNLAC1* using p2-4476 as template, primer 1 was (5'-ATCTAGGGCTCTTGAAAC-3'), and primer 2, (5'-TTGACTCGACGATCTTGG-3').

Reverse transcription reactions were performed using SuperScript Preamplification System for First Strand cDNA Synthesis (GIBCO BRL, Gaithersburg, MD). Quantity of total RNA was estimated by ethidium bromide staining. Total RNA was pre-treated with DNase I according to kit protocol (GIBCO BRL). Amplification of the target cDNA by PCR (annealing temperature 50°C) was performed using the following internal *CNLAC1* primers: (5'-GGCGATACTATTATCGTA-3') and (5'-AAGATGCCACCCAATATG-3'). Positive controls using B-3501 RNA were run with "test" samples.

Detection of *CNLAC1* Transcript in Experimental Rabbit Meningitis. The immunocompromised rabbit meningitis model described by Perfect et al. (17) was used. Cells of *C. neoformans* strain H99 (17) were propagated on Sabouraud agar plates for 4 d at 37°C, collected on cotton swabs, and suspended in PBS, pH 7.4, at a concentration of 1.6×10^8 cells per ml. One-half ml of this cell suspension was injected intrathecally into two New Zealand White male rabbits that had received an intramuscular injection of 2.5 mg cortisone acetate per kg (50 mg/ml; Merck, Sharp, & Dohme, West Point, PA) 24 h earlier and then daily for 2 wk. H99 cells were collected from cerebrospinal fluid (CSF) on days 1, 2, 3, 7, and 10 after inoculation. At the time of CSF removal, cells were pooled from the two rabbits, plated for quantitative viability on YEPD agar, and centrifuged. The sediment was washed once in diethyl pyrocarbonate-treated water, quick frozen in dry ice/acetone bath, and stored at -70°C. Total cryptococcal RNA was extracted using the Fast-RNA kit-Red on H99 cells from the inoculum, CSF from day 1, and pooled CSF from days 2–10. Actin sense primer (5'-TCGCCGCCTTGGTCATTG-3') and antisense primer (5'-CGTATTCGCTCTTCGCGA-3') were used in the reverse transcriptase (RT)-PCR reaction to confirm the presence of RNA.

Virulence Studies. *C. neoformans* cells were suspended in saline after 48-h growth on YEPD agar at 30°C. Cell counts were estimated by hemocytometer, and confirmed by colony counts on YEPD agar. 6–10-wk-old female BALB/c mice were inoculated with 0.1 or 0.2 ml of yeast cell suspensions in the lateral tail vein. Groups of 15 or 20 mice were inoculated with each yeast strain and monitored daily for survival. Mice unable to reach food or water were killed and counted as dying that day. At death, a sterile loop was "swabbed" onto mice brain tissue, then plated onto Niger seed agar. Mice brain cultures were grown at 30°C. Melanin phenotype of isolated colonies from these cultures were recorded at 7 d.

Statistical Analysis. Comparison between melanin-positive and -negative strain survival curves were performed using the Kal-mogorov-Smirnov two-sample test (18).

Results

Deletion of *CNLAC1*. An interrupted *CNLAC1* gene fragment was constructed by insertion of a 1.2-kb *URA5* fragment within the ORF of *CNLAC1* in a 6-kb genomic fragment to form p6CIU. The 7.2-kb interrupted fragment was then excised from p6CIU with *Xba*I and *Xho*I, and the purified fragment was used to transform *C. neoformans* strain B-4476 FOA (Mel⁺, *ura5*) to attempt a

one-step gene disruption (19). 115,000 transformants were obtained. 10 (Mel^-) transformants were screened by Southern blots using a ^{32}P -labeled, 876-bp internal fragment of *CNLAC1* used previously (5). The initial Southern blots of one of the Mel^- transformants, strain 10S, showed that it had an episomal element that contained the transformed construct (data not shown). This plasmid was lost upon repeated subculture on YEPD and 10S reverted back to uracil auxotrophy. However, it retained a stable Mel^- phenotype, and repeat Southern blot analysis showed disruption of *CNLAC1* (Fig. 3 A). Genomic DNA from strains B-4476 FOA and 10S were digested with *Cla*I and the Southern blot was probed with *CNLAC1*. B-4476 FOA had a single 5-kb band (Fig. 3 A, lane 3), and 10S had a single larger (9-kb) band (Fig. 3 A, lane 4). This suggested that a deletion had occurred in 10S. Uncut genomic 10S DNA probed with *CNLAC1* showed no evidence for episomal elements (data not shown).

Further Southern analysis and PCR mapping were done to define the location of the deletion in 10S. *Nsi*I digests of genomic 10S and B-4476 FOA DNA were probed with internal *CNLAC1* sequences (Fig. 2 B, probe 3), yielding a 7.5-kb band with B-4476 FOA and a 3.5-kb band with 10S. This suggested a 4-kb deletion 5' to the two *Nsi*I sites within *CNLAC1* (Fig. 2 B). Southern analysis of *Cla*I digests of these two strains were probed with endlabeled oli-

gonucleotides (Fig. 2 B, probes 2 and 4). Probe 4 gave a positive signal in both strains, but probe 2 was positive in the wild type only, confirming a deletion in the area near the *Cla*I site. The loss of the *Cla*I site known to be in this region could explain the size difference seen on the Southern blot shown in Fig. 3 A. This blot was also probed with a 1.6-kb PCR fragment (Fig. 2 B, probe 1) obtained with B-4476 FOA template. The 3' end of probe 1 was located at base -335 from the *CNLAC1* ORF. B-4476 FOA, but not 10S, hybridized with probe 1, again confirming a large deletion in the 5' sequences of 10S. PCR further localized the deletion breakpoint between bases 26 and 68. PCR primers located 3' to base 68 failed to yield products (Fig. 2 B). Successful PCR primers 5' to the breakpoint gave appropriate sized products except when the product included the area between bases 69 and 368. PCR products spanning this area were 450 bases too large (Fig. 2 B). Although these bases were not sequenced, this area does not include the *Nco*I site where *URA5* was inserted in p6CIU. Loss of uracil prototrophy during initial subculture indicated that no functioning copy of *URA5* remained. Fig. 2 B presents our findings diagrammatically and indicates the area of the 4-kb deletion.

By Northern blot analysis, B-4476 FOA had *CNLAC1* transcript and 10S did not. A ribosomal probe confirmed equal loading of total RNA in both strains (data not shown). Since the deletion included areas outside the ORF, we performed a Northern blot analysis to look for any closely linked genes that may have also been deleted in this mutant. A Northern blot of B-4476 FOA and 10S using deleted genomic sequence upstream from *CNLAC1* (p2-4476) as a probe revealed no evidence for any additional gene disruptions (data not shown).

Before studying virulence, deletant strain 10S (mating type α) was backcrossed four times with parent strain B-4500 (mating type α) to create a genetically related pair that differed primarily at the *MEL* locus. Among the mating type α progeny from the final cross, one Mel^+ (10S-BUC) and one Mel^- (10S-BU) strain were selected (Fig. 1).

Southern blots of digests from the backcrossed mutants yielded band patterns similar to their "parent" strains when probed with *CNLAC1*. 10S-BUC, Mel^+ had the same band pattern with *Cla*I and *Nde*I as its parent, B-4500 (Fig. 3 B). 10S-BU, Mel^- had a single 9.5-kb band with *Cla*I similar to its 10S parent (Fig. 3, A and B). DNA fingerprinting band patterns with *Acc*I genomic digests of 10S-BU and 10S-BUC were identical (data not shown).

Enzyme assay for diphenol oxidase activity using epinephrine as substrate was performed on the 10S mutants (Fig. 4 B). Control B-3501, Mel^+ had 200 U/ 10^8 cells at 30 min. The Mel^+ strain obtained by backcrossing (10S-BUC), and melanin-negative strain 10S-BU had <10 U/ 10^8 cells. B-4500, the parental strain of 10S-BUC, also had no measurable enzyme activity. B-4500 and 10S-BUC produced brown pigment when grown on Niger seed agar at either 30°C or 37°C, whereas 10S-BU did not. The observation that 10S-BUC cells produced brown colonies on

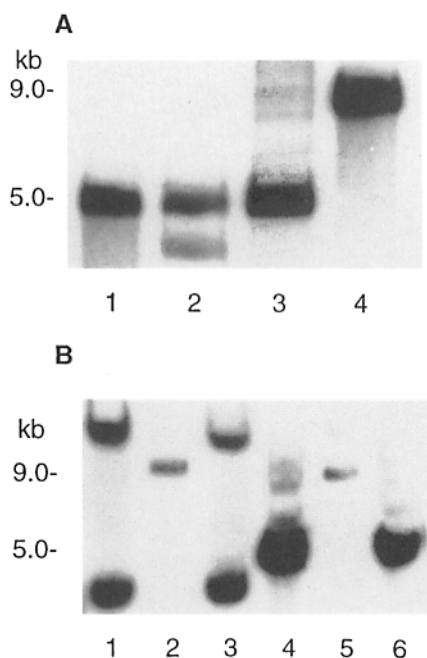


Figure 3. Southern blots. (A) Southern blots of *Cla*I genomic digests probed with *CNLAC1*. Lane 1 is strain 2E; lane 2, 2E-TUC. 2E-TUC has a 5-kb band that is the same size as 2E, and a 4-kb band that is from the transformed B-3501's DNA. Lane 3 is B-4476 FOA; lane 4, 10S. (B) Southern blots of 10S backcrossed strains probed with *CNLAC1*. Lanes 1-3 are *Nde*I digests: lane 1 is strain B-4500; lane 2, 10S-BU; and lane 3, 10S-BUC. Lanes 4-6 are *Cla*I digests of the same three strains in the same order.

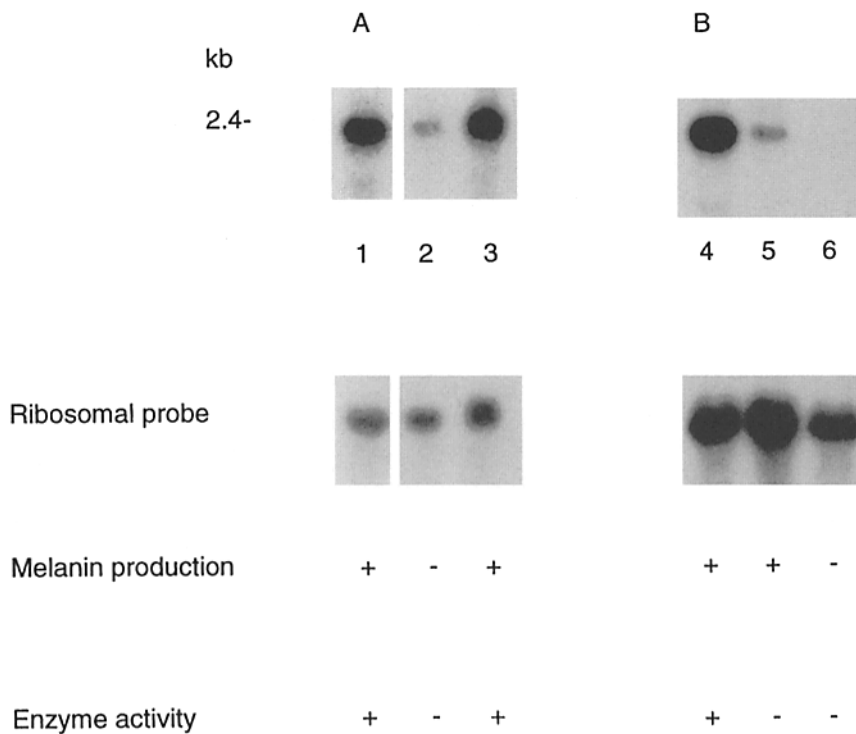


Figure 4. Northern blots, melanin production, and diphenol oxidase activity. (Top) Northern blots of total RNA probed with *CNLAC1*. (A) Lane 1, 3501 (control); lane 2, *mel2* mutant (2E); lane 3, *mel2* post-*CNLAC1* complementation (2E-TUC). (B) Lane 4, 3501; lane 5, 10S-BUC; and lane 6, 10S-BU. (Middle) Same blots probed with a ribosomal probe to quantify RNA. (Bottom) Melanin production on Niger seed agar and diphenol oxidase activity.

Niger seed medium but did not convert DOPA to dopachrome is consistent with our observation that the former is a more sensitive test for laccase activity.

Northern blot analysis of 10S-BU, 10S-BUC, and control strain B-3501 revealed that the melanin-positive strains had *CNLAC1* message (Fig. 4 B). Strain 10S-BUC had less message than B-3501. Melanin-negative strain 10S-BU had no detectable message (Fig. 4 B). The blots were probed with radiolabeled *CNLAC1*. Rehybridizing the blots with the ribosomal probe showed equal amounts of total RNA (Fig. 4 B). In addition, *CNLAC1*-deleted strain 10S-BU had no detectable *CNLAC1* message by RT-PCR.

We studied growth rates and virulence of the 10S backcrossed strains. Generation times at 37°C were for 10S-BU (*Mel*⁻), 2.4 h and for 10S-BUC (*Mel*⁺), 2.8 h. Hemocytometer counts were used instead of optical density to calculate doubling times because 10S-BUC had a larger cell size. The slight size differences showed that, despite backcrossing both 10S-BU and 10S-BUC against the same parental strain, differences likely were present in addition to laccase activity. Both strains were equally encapsulated when viewed by India ink microscopy. Each strain was inoculated into 15 female BALB/c mice (10 wk old) intravenously (3×10^6 cells/mouse). Their brains were cultured on bird seed agar with chloramphenicol and biphenyl (10) and incubated at 30°C for 7 d. Melanin phenotype was stable in vivo, in that the presence or absence of brown pigment was the same as the inoculum. No colonies of the other phenotype were detected. The melanin-positive strain (10S-BUC) was statistically ($P < 0.0001$) more viru-

lent than 10S-BU, as judged by cumulative mortality at 100 d (Fig. 5).

Complementation of a Melanin-negative Mutant, mel2. Torres-Guerreo et al. (8), described seven melanin-negative mutants (*mel1-mel7*) of *C. neoformans* having independent loci produced by UV and ethyl methane sulfonate treat-

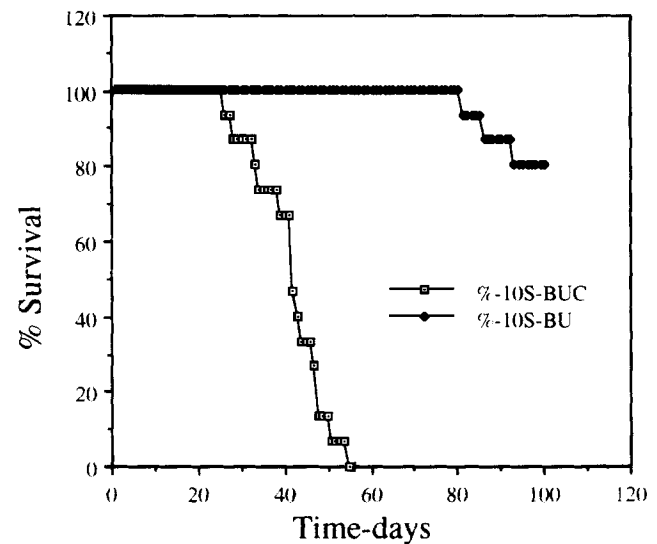


Figure 5. Virulence of the 10S mutants in mice. Percent survival of mice plotted versus time (days). Strains 10S-BUC, *Mel*⁺, and 10S-BU, *Mel*⁻, at infecting dosage of 3×10^6 cells/mouse.

ment of B-4500. *URA5* auxotrophs of strains *mel1*–*4*, were transformed in the present study with a 7.2-kb fragment of *CNLAC1/URA5* that had been excised from p6CU with *XbaI/XhoI* (Fig. 2 A). The *mel2* (*ura5*, *lys1*) mutant was the only strain of the four that was successfully complemented, with 94% of 126 *URA5* transformants producing brown pigment on norepinephrine media as compared with a 0% spontaneous reversion frequency. Transformation of *mel1*, *mel3*, and *mel4*, with the same *CNLAC1* construct produced only a few brown colonies (0, 1.6, and 2.5%, respectively), which was not significantly above the spontaneous reversion frequency of each mutant (0, 0.1, and 3.8%, respectively). Unlike *mel1* and *mel3* strains, *mel2* and *mel4* were not copper suppressible (8).

The *mel2* mutant described above was restored to lysine prototrophy by backcrossing once to B-4476 to produce strain 2E (*mel2*, *ura5*, *LYS1*). Complementation of strain 2E with *CNLAC1/URA5* from p6CU was accomplished and a melanin-positive transformant, 2E-TUC (*Mel*⁺, *URA5*, *LYS1*), was selected for further study.

Southern blot analysis revealed that strain 2E-TUC had an integrated copy of the transformed construct. As shown in Fig. 3 A, a *Clal* digest of 2E-TUC DNA probed with *CNLAC1* showed a 4-kb band in addition to the 5-kb nonfunctional copy in 2E. Southern blots of the pretransformed 2E strain yielded a single 5-kb band (Fig. 3 A). Southern blot analysis of uncut DNA from 2E-TUC showed only one band corresponding to uncut DNA, consistent with an integrated, rather than an episomal copy of *CNLAC1* (data not shown). For additional studies, we needed to convert the *Mel*⁻ strain 2E to uracil prototrophy. 2E was transformed with a 1.2-kb PCR product containing the *URA5* gene to create strain 2E-TU (*Mel*⁻, *URA5*). Southern blot hybridized with the *URA5* probe confirmed that it contained an extra copy of the *URA5* gene (data not shown). Southern blots of uncut 2E-TU DNA yielded no “small” (<12 kb) molecular weight bands (data not shown). These data indicated that the complemented strains of 2E had ectopically integrated copies of the transforming DNA.

Northern blot analysis revealed that the *Mel*⁻ mutant 2E produced a small amount of *CNLAC1* transcript (Fig. 4 A). Small quantities of *CNLAC1* message have also been seen in the parental wild-type strain, B-4500 (data not shown). The quantity of message in the complemented strain 2E-TUC was increased in comparison to 2E (Fig. 4 A). Rehybridized blots with the ribosomal probe revealed equal amounts of total RNA.

Phenoloxidase activity of these strains was measured using epinephrine as substrate. Control strain B-3501 on this assay had 335 U/10⁸ cells. The *Mel*⁺ complemented strain 2E-TUC had 91 U/10⁸ cells. The melanin-negative strain 2E had <10 U/10⁸ cells.

Characterization of the mel2 Mutation. Since message was detected by Northern analysis in the *mel2*-derived mutant (2E), a point mutation in *CNLAC1* was suspected. To characterize this mutation, a 5-kb *Clal* genomic clone of

2E, p5mel2, was sequenced in both directions. When the nucleotide sequence of 2E was compared with strain 3501, six differences in the predicted amino acid sequence were found. Whereas most of these mutations may represent heterogeneity between strains, one amino acid change (*His*₁₆₄→*Tyr*) was located within the highly conserved putative copper-binding site. *His*₁₆₄ may be critical for type III copper binding in this laccase, and could explain the complete absence of pigment despite successful transcript production. To further investigate, a 580-bp PCR fragment, p45His, from B-4500 (wild-type strain of 2E) was subcloned. The sequence of p45His did not contain the C→T base change at codon #164. These data suggested the importance of the *His*→*Tyr* mutation in the *mel2* mutant.

Site-directed Mutagenesis of CNLAC1. To confirm the role of the histidine to tyrosine point mutation at amino acid #164 in the *Mel*⁻ phenotype, site-directed mutagenesis of *CNLAC1* DNA was performed. This point mutation was duplicated in p6CU, to produce p6CUT. The entire 270-bp, PCR-generated cassette of p6CUT was checked by sequence analysis to verify the correct nucleotide substitution and to rule out any PCR-induced base substitutions. The mutated plasmid was also checked by restriction mapping to verify sizes (data not shown). Linear p6CU had been able to restore melanin positivity in the *Mel*⁻ mutants 10S-BU FOA and 2E. 10S-BU FOA is a *URA5* auxotroph selected by plating 10S-BU on FOA media (11). Complementation of the *CNLAC1* deletant, 10S-BU FOA, was attempted with the linear (Fig. 2 A) 7.2-kb fragments of *CNLAC1/URA5* from p6CU and p6CUT. 75% of 7,000 p6CU transformants were melanin positive, compared with none of the 4,000 p6CUT transformants. The *Mel*⁻ transformants with ectopic integration of p6CUT were randomly selected by passage on YEPD media and subsequent Southern blots (data not shown). One stable transformant, 10S-CUT3, was selected for further analysis. Next, primers were selected with and without the mutated nucleotide as the terminal base of the antisense primer. PCR reactions with these primers using pCUT transformant (10S-CUT3) DNA as template, confirmed that the integrated mutated DNA was present (data not shown). Transformant 10S-CUT3 had *CNLAC1* message present by RT-PCR (data not shown). This strain remained melanin negative on Niger seed agar despite prolonged incubation (10 d).

Virulence of the Mel⁺ and Mel⁻ Mutants Derived from mel2. Injection of mice with three different inocula of strains 2E-TU, (*Mel*⁻) and 2E-TUC, (*Mel*⁺) revealed no significant difference in virulence (data not shown). Generation times at 37°C were 4.0 h for 2E-TUC compared with 3.5 h in 2E-TU. Because of concern that the difference in growth rates could represent either an electroporation- or a mutagenesis-induced effect, both strains were backcrossed four times with wild-type strain B-4476. Fourth generation progeny from 2E-TU and 2E-TUC × B-4476 matings yielded strains 2E-TU-4, *Mel*⁻ and 2E-TUC-4, *Mel*⁺, respectively (Fig. 1). Southern blots of strains 2E-TU-4 and 2E-TUC-4

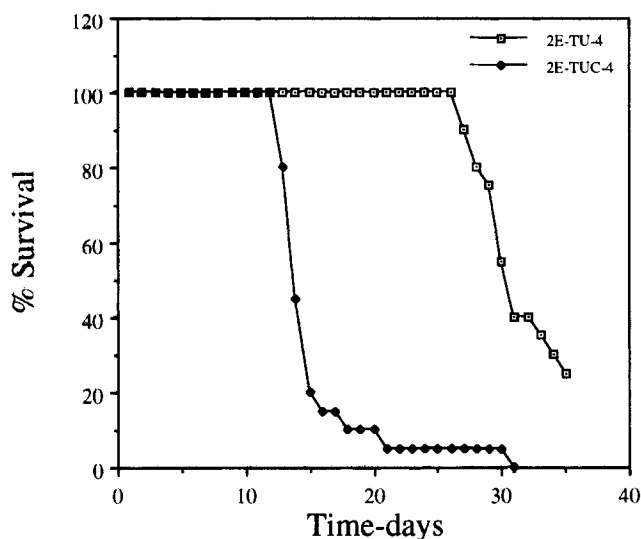


Figure 6. Virulence of the 2E mutants in mice. Percent survival of mice plotted against time (days). Strains 2E-TU-4, Mel⁻ and 2E-TUC-4, Mel⁺ at infecting dosage of 10⁶ cells/mouse.

using ClaI digests probed with *CNLAC1* yielded the same patterns as parental strains, 2E-TU (5 kb) and 2E-TUC (5 and 4 kb), respectively (Fig. 3 A). DNA fingerprinting of AclI genomic digests from 2E-TU-4 and 2E-TUC-4 were identical. Contour-clamped homogeneous electric field electrophoresis (CHEF) analysis of these strains yielded the same pattern. Probing the CHEF gel with the *CNLAC1* probe found the integrated copy of the gene to reside on the second smallest chromosome, tentatively identified as chromosome 11, whereas the native copy resided in chromosome 5, as previously described (5). To test whether strain 2E-TUC-4 retained the transformed and defective (2E) copies of the *CNLAC1* gene through the crosses, (i.e., did not acquire a functional copy from B-4476), we used differential PCR. PCR was performed using 3.5–4.5- and 4.5–5.5-kb ClaI-digested genomic DNA libraries of 2E-TUC-4 as template. Control genomic DNA was from 2E-TU-4 and B-3501. *CNLAC1* primers with and without the His₁₆₄ mutation were used. PCR product of the predicted size was found using template from 3501 and 2E-TUC-4 (3.5–4.5 kb) library with the unmutated primer only (data not shown). This confirmed that the *CNLAC1* gene from B-3501 was present in the 3.5–4.5-kb fragment of 2E-TUC-4's DNA. PCR product of the predicted size was found using either 2E-TUC-4 (4.5–5.5 kb) library or 2E-TU's DNA as template with the mutated primer only (data not shown). This confirmed that the *CNLAC1* gene with the original His₁₆₄ point mutation was retained through the crosses and resided in the 4.5–5.5-kb fragment of 2E-TUC-4's DNA.

Strain 2E-TUC-4, (Mel⁺), produced brown pigment on Niger seed agar when grown at 37°C. Growth rates at 37°C for strains 2E-TU-4 and 2E-TUC-4 were equal at 3.1 h. Both mutants had the same cell diameter and were equally encapsulated when viewed by India ink micros-

copy. 6-wk-old female BALB/c mice were infected intravenously with 10⁶ cells from 2E-TU-4 and 2E-TUC-4. 20 mice were inoculated from each strain. A statistically significant difference ($P < 0.0001$) in mice mortality between those infected with 2E-TU-4 and 2E-TUC-4 was seen (Fig. 6). Melanin phenotype was stable in the brain isolates.

RT-PCR of CNLAC1 Transcript in CSF of Infected Rabbits. To test for *CNLAC1* transcript, H99 cells grown on Sabouraud agar and H99 cells harvested from rabbit CSF (day 1, pooled days 2–10) samples were tested by RT-PCR. The preinoculation and both the RNA samples from CSF had *CNLAC1* message (data not shown). Actin controls confirmed the presence of RNA in all samples. The presence of *CNLAC1* transcript in vivo does not prove but supports the concept that sufficient laccase activity and substrate are present to melanize cryptococcal cell walls in the infected host.

Discussion

Several lines of evidence support the role of the *C. neoformans* laccase in virulence. The genetic studies cited earlier (2–4) do not distinguish between an effect of the *CNLAC1* gene product and other genes linked to the melanin phenotype. The ease with which melanin-negative mutants are obtained and even occur spontaneously is one indication that multiple genes are required for expression of this phenotype. Copper transport may be essential, judging from copper suppression of the melanin-negative phenotype in some mutants (8) and the presence of 4 mol of copper in the laccase (5). Indeed, site-directed mutagenesis of a single base in a copper-binding site of *CNLAC1* ablated laccase activity without preventing transcription of *CNLAC1*. Substrate transport may be important, judging by decreased catecholamine uptake in some melanin-negative mutants (20). However, the site of melanin production is unknown. Little enzymatic activity can be found in cytoplasm extracts. Laccase may be transported to the cytoplasmic membrane, or secreted into the periplasmic space and bound to the cell wall. The most dense melanin deposition is on the inner aspect of the cell wall (21). No membrane-binding domains were found in the laccase (5) and no extracellular enzyme has been detected.

Relation of cryptococcal laccase to virulence would be clearer if the function of the enzyme in host defense were known and if it were certain that the enzyme was produced in the central nervous system. It is odd that an enzyme suppressed by glucose or 37°C incubation (22) and produced in stationary phase would enhance virulence. Masson-Fontana silver stain of cryptococci in mouse, human, and rat brain show a variable but definite brown yeast cell wall. But this stain is not specific for melanin. *Cryptococcus laurentii*, which is Mel⁻, also stains with Masson-Fontana (23).

No specific antibody has been available for immunocytochemical detection of cryptococcal laccase in infected brain. RT-PCR of cryptococci in rabbit CSF was used here to show that *CNLAC1* transcript is produced in the infected host, although abundance of transcript was not de-

terminated. The composite evidence supports the existence of both laccase and L-dopa, a laccase substrate, in the infected host's brain. Melanin may be deposited in the cryptococcal cell wall in vivo as well as in vitro.

Melanin protects human skin and fungal plant pathogens from the effects of sunlight (24). *C. neoformans* that are melanized by growth in L-dopa medium are more resistant to killing by nitrogen- and oxygen-based oxidants (6). Melanin may protect *C. neoformans* from oxidative attack by host cells (21).

The relation of *CNLAC1* to virulence is best studied in a mutant in which this gene has been deleted. Infrequency of homologous integration in *C. neoformans* complicates this approach. Our 10S mutant had a disrupted copy of *CNLAC1*, no transcript detectable by RT-PCR, and no melanin production on Niger seed or norepinephrine medium. The sequence of molecular events leading to the *CNLAC1* deletion in 10S is unclear. A 4-kb upstream sequence was deleted during construction of this mutant. No transcript or any ORF has been detected from this deleted upstream area, although this does not assure that none exist. When the strain with the disrupted gene was backcrossed four times with a parental strain, a progeny carrying the disrupted gene was not lethal for mice whereas a progeny carrying the nondisrupted *CNLAC1* gene was virulent.

Another approach used here was to obtain a mutagenized melanin-negative strain and transform this strain to a Mel⁺ phenotype. A melanin-negative strain (*mel2*) did produce small amounts of *CNLAC1* transcript of the appropriate size but was found to have several base changes within *CNLAC1*, as compared with the parent strain. One base change converted histidine to tyrosine in one of the copper-binding sites, a change that caused transformants with this base change to produce transcript but no melanin. *mel2* was backcrossed with a parental strain to select an F₁ progeny that was not a lysine auxotroph. The *LYS1* strain was transformed with the *URA5* gene and also with a plasmid containing both *URA5* and *CNLAC1*. Mel⁻ and

Mel⁺ transformants did not differ significantly in mouse lethality with either of three different inocula. However, these strains differed in generation time by 30 min. Further backcrossing of these two strains with B-4476, a wild-type strain of the opposite mating type, produced a Mel⁺ strain that had the same generation time but was much more rapidly lethal than the Mel⁻ strain. Both strains had the same pattern on CHEF gel and on Southern analysis of an *AccI* digest probed with UT-4p. Differential PCR showed that the Mel⁺ strain, 2E-TUC-4, contained both the mutagenized *CNLAC1* gene and the transformed *CNLAC1* gene from strain B-3501. Probing the CHEF gel showed that the two copies resided on different chromosomes. The Mel⁻ strain 2E-TU-4, had only a single copy of *CNLAC1* residing on chromosome 5, and this copy on PCR contained the single base change that is characteristic of the 2E mutation. These results show that the difference in virulence between the two strains should be attributed to the nonhomologous integration of *CNLAC1*. Failure to show this effect before backcrossing is likely due to strain differences introduced into the parental strain 2E by mutagenesis, or into either strain by electroporation, and only subsequently removed by backcrossing to the nonmutagenized parental strain, B-4476.

Whenever mutagenized cells are created or DNA is introduced into cells by electroporation, untoward effects may occur. We have observed morphologically abnormal *C. neoformans* cell shapes after DNA was introduced by electroporation. Our data suggest that backcrossing to a congenic parent after potentially mutagenic insults to cryptococcal cells may correct unwanted effects. Backcrossing may be necessary before studying the effects of gene knockout or complementation on the virulence of *C. neoformans*.

This study provides a decisive link between the *CNLAC1* gene product and virulence. Complementation of two different types of Mel⁻ mutants with *CNLAC1* increased mouse virulence.

We gratefully acknowledge the expert technical assistance of K. Marsden. *C. neoformans* melanin-negative mutants *mel1-mel4* were a generous gift of J.C. Edman.

J.R. Perfect was supported by Public Health Service grant AI 28388 and Veterans Administration Research Center in AIDS and HIV infection.

Address correspondence to Dr. John E. Bennett, Clinical Mycology Section, Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, Building 10, Room 11C304, 10 Center Drive, MSC 1882, Bethesda, MD 20892-1882.

Received for publication 11 March 1996 and in revised form 8 May 1996.

References

1. Pinner, R.W., R.A. Hajjeh, and W.G. Powderly. 1995. Prospects for preventing cryptococcosis in persons infected with human immunodeficiency virus. *Clin. Infect. Dis.* 21: S103-107.
2. Kwon-Chung, K.J., I. Polacheck, and T.J. Popkin. 1982. Melanin-lacking mutants of *Cryptococcus neoformans* and their virulence for mice. *J. Bacteriol.* 150:1414-1421.
3. Kwon-Chung, K.J., and J.C. Rhodes. 1986. Encapsulation and melanin formation as indicative of virulence in *Cryptococcus neoformans*. *Infect. Immun.* 51:218-223.
4. Rhodes, J.C., I. Polacheck, and K.J. Kwon-Chung. 1982. Phenoloxidase activity and virulence in isogenic strains of

- Cryptococcus neoformans*. *Infect. Immun.* 36:1175–1184.
5. Williamson, P.R. 1994. Biochemical and molecular characterization of the diphenol oxidase of *Cryptococcus neoformans*: identification as a laccase. *J. Bacteriol.* 176:656–664.
 6. Wang, Y., and A. Casadevall. 1994. Susceptibility of melanized and nonmelanized *Cryptococcus neoformans* to nitrogen- and oxygen-derived oxidants. *Infect. Immun.* 62:3004–3007.
 7. Reinhammer, B. 1984. Laccase. In *Copper Proteins and Copper Enzymes*. R. Lontie, editor. CRC Press, Inc., Boca Raton, FL. 4–10.
 8. Torres-Guerro, H., and J.C. Edman. 1994. Melanin-deficient mutants of *Cryptococcus neoformans*. *J. Med. Vet. Mycol.* 32:303–313.
 9. Kwon-Chung, K.J., J.C. Edman, and B.L. Wickes. 1992. Genetic association of mating types and virulence in *Cryptococcus neoformans*. *Infect. Immun.* 60:602–605.
 10. Kwon-Chung, K.J., and J.E. Bennett. 1992. Cryptococcosis. In *Medical Mycology*. Lea & Febiger, Malvern, PA. 397–446.
 11. Edman, J.C., and K.J. Kwon-Chung. 1990. Isolation of the *URA5* gene from *Cryptococcus neoformans* var *neoformans* and its use as a selective marker for transformation. *Mol. Cell. Biol.* 10:4538–4544.
 12. Zhao, L.J., Q.X. Zhang, and R. Padmanabhan. 1993. Polymerase chain reaction–based point mutagenesis protocol. *Methods Enzymol.* 217:218–227.
 13. Varma, A., and K.J. Kwon-Chung. 1991. Rapid method to extract DNA from *Cryptococcus neoformans*. *J. Clin. Microbiol.* 29:810–812.
 14. Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
 15. Varma, A., and K.J. Kwon-Chung. 1992. DNA probe for strain typing of *Cryptococcus neoformans*. *J. Clin. Microbiol.* 30:2960–2967.
 16. Wickes, B.L., T.D.E. Moore, and K.J. Kwon-Chung. 1994. Comparison of the electrophoretic karyotypes and chromosomal location of ten genes in the two varieties of *Cryptococcus neoformans*. *Microbiology*. 140:543–550.
 17. Perfect, J.R., S.D.R. Lang, and D.T. Durack. 1980. Chronic cryptococcal meningitis: a new experimental model in rabbits. *Am. J. Pathol.* 101:177–194.
 18. Siegel, S. 1956. *Non-parametric Statistics*. McGraw-Hill Book Co., New York. 127–136.
 19. Rothstein, R.J. 1983. One-step gene disruption in yeast. *Methods Enzymol.* 101:202–211.
 20. Polacheck, I., V.J. Hearing, and K.J. Kwon-Chung. 1982. Biochemical studies of phenoloxidase and utilization of catecholamines in *Cryptococcus neoformans*. *J. Bacteriol.* 150:1212–1220.
 21. Wang, Y., P. Aisen, and A. Casadevall. 1995. *Cryptococcus neoformans* melanin and virulence: mechanism of action. *Infect. Immun.* 63:3131–3136.
 22. Jacobson, E.S., and H.S. Emery. 1991. Temperature regulation of the cryptococcal phenoloxidase. *J. Med. Vet. Mycol.* 29:121–124.
 23. Kwon-Chung, K.J., W.B. Hill, and J.E. Bennett. 1981. New, special stain for histopathological diagnosis of cryptococcosis. *J. Clin. Microbiol.* 13:383–387.
 24. Wheeler, M.H., and A.A. Bell. 1988. Melanins and their importance in pathogenic fungi. *Curr. Top. Med. Mycol.* 2:338–387.