Stress tolerance and pathogenic potential of a mannitol mutant of *Cryptococcus neoformans*

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***Cryptococcus neoformans* produces large amounts of the acyclic hexitol mannitol in culture and infected animals, but the functional and pathogenic significance of mannitol production by this fungus is not known. We exposed *C. neoformans* H99 (Cn H99) to UV irradiation ($1 \times \text{LD}_{50}$) and screened survivors for mannitol production. A mutant, Cn MLP (Mannitol Low Producer), synthesized less mannitol from glucose (2.7 vs 8.2 nmol per 10^8 cells min^{-1} at 37 °C) and contained less intracellular mannitol (1 vs 11 μmol per 10^6 cells at 37 °C) than did Cn H99. Cn MLP and Cn H99 were similar with respect to carbon assimilation patterns, rates of glucose consumption, growth rates at 30 °C, urease and phenoloxidase activities, morphology, capsule formation, mating type, electrophoretic karyotype, rapid amplification of polymorphic DNA (RAPD) patterns and antifungal susceptibility. However, Cn MLP was more susceptible than was Cn H99 to growth inhibition and killing by heat and high NaCl concentrations. Also, the LD_{50} values in mice injected intravenously were 3.7×10^6 c.f.u. for Cn MLP compared to 6.9×10^2 c.f.u. for Cn H99. Moreover, 500 c.f.u. Cn H99 intravenously killed 12 of 12 mice by 60 d, whereas all mice given the same inoculum of Cn MLP survived. Classical genetic studies were undertaken to determine if these differences were due to a single mutation, but the basidiospores were nonviable. These results suggest that the abilities of *C. neoformans* to produce and accumulate mannitol may influence its tolerance to heat and osmotic stresses and its pathogenicity in mice.**

Keywords: *Cryptococcus neoformans*, mannitol, heat stress, osmotic stress, pathogenicity

INTRODUCTION

Cryptococcus neoformans is a saprobic yeast that is distributed throughout the world in association with soil and pigeon manure or with eucalyptus trees. Inhalation of aerosolized *C. neoformans* cells initiates a pulmonary infection that is self-limited in most cases. However, *C. neoformans* can disseminate widely in people with compromised immunity, and it tends to cause serious central nervous system infections. Indeed, cerebral cryptococcosis is one of the most common serious fungal diseases among AIDS patients and other immunocompromised hosts (Chuck & Sande, 1989; Levitz, 1991; Kwon-Chung & Bennett, 1992). Because this disease is now so common, and also because better therapeutic approaches are needed, there has been renewed interest in understanding interactions between *C. neoformans* and mammalian hosts, and also attributes of *C. neoformans* that may contribute either directly or indirectly to virulence.

In an earlier study, Wong *et al.* (1990) showed that *C. neoformans* produced large amounts of the hexitol mannitol in culture and in infected animals, and also that mannitol was a quantitative marker for experimental cryptococcal meningitis in rabbits. Furthermore, the authors hypothesized that mannitol may contribute to virulence or to the pathogenesis of cryptococcosis in at least two ways. Firstly, large amounts of mannitol accumulating within heavily infected brain tissues may increase the tonicity of those tissues, thereby contributing to cerebral oedema formation. Secondly, high concentrations of mannitol may scavenge free hydroxyl radicals, thereby protecting *C. neoformans* from oxidative killing by host phagocytes. One way to assess the physiological and pathogenic significance of mannitol production is to isolate and analyse appropriate *C. neoformans* mutants. The present study describes the isolation of a *C. neoformans* mutant that produces low amounts of mannitol and the physiological and pathogenic properties of this mutant.

METHODS

Strain and media. *C. neoformans* H99 (Cn H99) is a serotype A, mating type α (*MAT* α) strain that was originally isolated from human cerebrospinal fluid and has been used extensively in a rabbit model of experimental cryptococcal meningitis (Perfect *et al.*, 1980; Wong *et al.*, 1990). *C. neoformans* MLP (Cn MLP) was derived from Cn H99 as described below. *C. neoformans* strains B3501 (*MAT* α) and B3502 (*MAT* α) are serotype D strains that have been used extensively in genetic studies (Kwon-Chung & Bennett, 1992). *C. neoformans* ATCC 34689 [serotype A, *MAT* α (also known as NIH38)] and *C. neoformans* ESJ 450 [serotype A, *MAT* α , melanin⁺ (from Eric Jacobsen, Richmond, VA, USA)] were also used in genetic experiments.

Stock *C. neoformans* cultures were grown on YEPD agar (Difco) at 30 °C, and the plates were stored at 4 °C. Liquid media used in this study included: (i) YEPD broth; (ii) yeast nitrogen base (Difco) supplemented with 2% (w/w) glucose (YNB/glucose), 2% (w/w) mannitol (YNB/mannitol), 2% (w/w) fructose (YNB/fructose) and/or 0.5–3.0 M NaCl; and (iii) minimal glucose medium [10 mM ammonium hydrogen phosphate, 10 mM potassium dihydrogen phosphate, 10 mM sodium chloride, 1 mM magnesium sulphate, 1 × trace elements mix (Campbell, 1988), 150 mM glucose, 100 µg thiamine l⁻¹; pH 6.8]. V8 juice agar was used for mating studies (Kwon-Chung & Bennett, 1992).

Mutant isolation. Cn H99 was grown to exponential phase in minimal glucose medium at 32 °C, and the cell density was adjusted to 1 × 10⁶ ml⁻¹. A 10 ml aliquot was exposed to 1 × LD₅₀ UV irradiation using a germicidal lamp in a culture hood, and 8 ml of the irradiated culture was used to inoculate 100 ml fresh medium. The culture was grown to stationary phase at 32 °C, and aliquots were plated on minimal glucose agar. Cells from individual colonies were subcultured in a 96-well microtiter plate, the wells of which contained 0.25 ml minimal glucose medium. The plates were incubated at 32 °C for 72 h, during which time the cells settled to the bottom of the wells. The culture supernatants (0.05 ml) were transferred to individual wells of a fresh 96-well plate and screened for the presence of mannitol (Niehaus & Flynn, 1994) by adding 0.1 ml mannitol assay mixture consisting of 40 mM sodium phosphate (pH 9.0), 0.5 mM NAD, 0.5 mM phenazine methosulfate (PMS), 0.75 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 2 U of a NAD-dependent mannitol dehydrogenase purified from *Saccharomyces cerevisiae* transformed with *C. neoformans* plasmid pMDH1 (Niehaus *et al.*, 1994). The plates were incubated in the dark for 10 min at 30 °C. The presence of as little as 50 µM mannitol led to reduction of MTT and an intense blue colour.

Mannitol and glucose measurements. Mannitol production and glucose consumption by *C. neoformans* were studied as described by Niehaus & Flynn (1994). Briefly, overnight cultures in minimal glucose medium were harvested, the cells were washed and the cell density adjusted to 1 × 10⁸ ml⁻¹. The cells were then suspended in 10 mM potassium phosphate buffer (pH 6.8) containing 100 mM glucose. Samples were removed at 30 min intervals for 3 h. Measurements at 37 °C were made after the cultures had been allowed to acclimatize in pre-warmed media for 2 h. Mannitol in the culture supernatants was measured enzymically. Glucose was quantified by the glucose-peroxidase method (Sigma).

Intracellular mannitol was quantified as described by Wong *et al.* (1990). Briefly, mannitol was measured by capillary gas chromatography in filtrates of exponential phase cultures and in supernatants obtained after the cultures had been heated to 10 °C for 10 min to release intracellular mannitol. The difference

in concentration between the heated and unheated samples was considered to be intracellular mannitol.

Other properties. The presence or absence of polysaccharide capsules and their size were assessed by microscopic examination of yeast cells in India ink wet mounts. Phenoloxidase production was tested on caffeic acid plates (Kwong-Chung & Bennett, 1992). Growth at 37 °C was tested on YEPD agar plates. The ability to assimilate various substrates was tested using the API 20C clinical yeast system (API Laboratories) or in YNB/glucose, YNB/mannitol or YNB/fructose. Standard procedures were used to test for urease production (Kwong-Chung & Bennett, 1992). Mating type was determined by crossing Cn MLP with tester strains *C. neoformans* B-3501 and B-3502 on V8 agar, incubating the plates at room temperature for 10–14 d, and examining wet mounts for typical hyphae, basidia and basidiospores (Kwon-Chung & Bennett, 1992). Susceptibility to amphotericin B and fluconazole was tested in RPMI media by the macro broth dilution method at the Fungus Testing Laboratory, University of Texas Health Sciences Center, San Antonio, TX, USA.

C. neoformans chromosomes were separated by field inversion gel electrophoresis (FIGE), according to the method described by Cushion *et al.* (1993) for *Pneumocystis carinii* chromosomes. Cn H99 and Cn MLP were grown to late exponential phase in yeast nitrogen base/glucose medium. They were then spheroplasted with lysing enzyme from *Trichoderma harzianum* (Sigma) and the spheroplasts were embedded in 1% (w/w) agarose. The gels contained 1% SeaKem agarose (FMC Bioproducts) prepared in 0.5 × TBE (45 mM Tris/HCl, 45 mM boric acid, 1.25 mM EDTA, pH 8.0). The electrophoresis buffer system was 0.5% TBE supplemented with 0.1 M glycine. Gels were run at 4 °C for 48 h at 5.3 V cm⁻¹ with 50 s forward and 25 s reverse pulse times. The gels were stained with ethidium bromide and photographed. *S. cerevisiae* chromosomes were included as size markers.

The technique of rapid amplification of polymorphic DNA (RAPD) was used to detect polymorphisms in the DNA fingerprints of Cn H99 and Cn MLP (Welsh & McClelland, 1990). Genomic DNA was isolated as described by Moore & Edman (1993). Amplification reactions were performed in volumes of 25 µl containing 10 mM Tris/HCl (pH 8.3), 1.5 µM magnesium chloride, 200 µM of each deoxynucleoside triphosphate, 0.5 µM oligonucleotides (10-mer Kit A, Operon Technologies), 100 ng genomic DNA, and 0.25 U Taq DNA polymerase (Epicenter Technologies) overlaid with 35 µl mineral oil. Amplification was performed in a Perkin Elmer Cetus DNA thermal cycler programmed as follows: initial denaturation at 95 °C, 1 min annealing at 35 °C, 2 min polymerization at 72 °C with final extension for 10 min at 72 °C. Amplified products were separated by electrophoresis in 1.5% SeaKem agarose in 40 mM Tris acetate/1 mM EDTA (pH 8.5), and then visualized by UV fluorescence after ethidium bromide staining.

Stress tolerance. Since intracellular polyols protect other fungi from heat and osmotic stresses (Trollmo *et al.*, 1988; Larsson *et al.*, 1993; Mager & Varela, 1993; Wang *et al.*, 1994; Lewis *et al.*, 1995), we compared the abilities of Cn H99 and Cn MLP to tolerate these stresses. The ability to tolerate heat stress was assessed by culturing Cn H99 and Cn MLP overnight in YNB/glucose at 30 °C, diluting to an OD₆₀₀ value of 0.1 in fresh medium, and shaking the flasks at 180 r.p.m. at 30, 37 or 42.5 °C. Growth was assessed by measuring OD₆₀₀ at suitable intervals. In cultures that did not grow, c.f.u. were quantified by plating serially-diluted samples on YEPD agar and incubating at 30 °C for 48 h.

The ability to tolerate osmotic stress was assessed by diluting overnight cultures (YNB/glucose, 30 °C) of Cn H99 and Cn

MLP into fresh YNB/glucose supplemented with 0, 0.5, 0.9, 2.0, 2.5 or 3.0 M NaCl and shaking at 180 r.p.m. at 30 °C. At intervals thereafter, growth and/or c.f.u. were measured as described above.

Pathogenicity in mice. Cn H99 and Cn MLP cells were grown to exponential phase in YNB/glucose at 30 °C and the cells washed and resuspended in sterile 0.9% NaCl. Viable cells were counted by trypan blue exclusion in a haemocytometer and by c.f.u. counts on YEPD agar. Serial dilutions were made to obtain 10^3 , 10^5 and 10^7 viable cells per 0.1 ml sterile saline. Groups of six male Swiss mice (6–8 weeks old, Harlan Sprague Dawley) were injected with 0.1 ml of each cell suspension via the lateral tail vein. The animals were observed for survival over a 30 d period. All dead animals were necropsied, and their brains cultured on YEPD agar. The experiment was repeated once, the results were pooled and LD₅₀ values calculated (Reed & Muench, 1938). Also, groups of 12 mice were injected intravenously with 500 c.f.u. Cn strains H99 or MLP and the animals observed for survival for 60 d.

Genetic studies. To determine if differences between *C. neoformans* strains were due to single or multiple mutations, these *MAT α* strains were crossed with the wild-type *C. neoformans* *MAT α* strains ATCC 34689 (serotype A), ESJ 450 (serotype A) and B3502 (serotype D). The V8 agar mating plates were incubated at room temperature for 10–14 d, then individual basidiospores were isolated with a micromanipulator (Narshige USA), plated on YEPD agar and incubated at 30 °C.

RESULTS

Isolation of a mannitol low-producing mutant (Cn MLP)

A total of 5×10^3 colonies of Cn H99 that survived UV exposure were tested for mannitol production; of these, one showed no colour reaction in the enzyme assay for mannitol. This isolate was subcultured on minimal

glucose agar. Twenty randomly-selected colonies were retested and were negative in the assay for mannitol. This strain was designated Cn MLP (Mannitol Low Producer).

Properties of Cn MLP

Cn MLP produced significantly less mannitol than did Cn H99 at 32 °C and 37 °C (Table 1) and the intracellular mannitol content of exponential phase Cn MLP cells was less than 10% that of Cn H99 cells (1 vs 11 mol per 10^6 cells, 37 °C). In contrast, Cn H99 and Cn MLP were similar with respect to multiple phenotypic and genotypic properties (Table 1). The rates of glucose consumption by the two strains were not significantly different. Both strains had similar biochemical profiles: both assimilated fructose, mannitol, L-arabinose, inositol and sorbitol; neither assimilated glycerol or lactose as sole carbon source; and both produced urease. Also, several factors previously shown to be associated with virulence were similar in the two strains. Both strains grew well on YEPD agar at 37 °C, their mean generation times at 30 °C or 37 °C did not differ significantly, they produced equivalent amounts of melanin from caffeic acid, Cn MLP produced larger polysaccharide capsules at 30 °C and 37 °C, and the MICs of amphotericin B and fluconazole were within one twofold dilution (Table 1).

The electrophoretic karyotypes of Cn H99 and Cn MLP showed identical 8-chromosomal band patterns in ethidium-bromide-stained FGE gels (data not shown). In RAPD analyses with the 20 primers in the OPA kit, there were no distinct polymorphic bands between the two strains (data not shown).

When Cn MLP was crossed with *C. neoformans* B3502 (*MAT α*), characteristic hyphae with clamp connections,

Table 1. Comparison of morphological and physiological features of *C. neoformans* strains H99 and MLP

Feature	Growth temperature (°C)	Cn H99	Cn MLP
Mannitol production*	32	11.0 ± 3.0	3.0 ± 1.0
	37	8.2 ± 1.3	2.7 ± 0.3
Glucose consumption†	32	78.0 ± 9.0	80.0 ± 4.0
	37	91.7 ± 14.4	83.3 ± 14.4
Generation time‡	30	186 ± 11	165 ± 15
	37	204 ± 14	215 ± 45
Melanin formation		+	+
Mating type		α	α
Capsule size (µm)§	30	1.02 ± 0.41	1.35 ± 0.48
	37	1.12 ± 0.43	1.41 ± 0.57
MIC amphotericin B	25	0.50	0.25
MIC fluconazole	25	2.0	4.0

* Given in nmol per 10^8 cells min⁻¹, mean ± SD, *n* = 4, *P* < 0.001.

† Given in nmol per 10^8 cells min⁻¹, mean ± SD, *n* = 3, *P* > 0.40.

‡ Cultures grown in YNB/glucose, mean ± SD, *n* = 4, *P* > 0.1.

§ Cultures in stationary phase, YNB glucose medium, mean ± SD, *n* = 4, *P* < 0.05.

|| Given in µg ml⁻¹, macro broth dilution method, RPMI media, 25 °C, 48 h.

Fig. 1. Effects of heat stress on Cn H99 and Cn MLP. Cultures of Cn H99 (○) and Cn MLP (●) grown overnight at 30 °C in YNB/glucose were diluted in fresh YNB/glucose and shaken at 180 c.p.m. at 30 °C (a), 37 °C (b) or 42.5 °C (c). Growth was assessed by measuring OD₆₀₀ at the intervals shown. Since neither strain grew at 42.5 °C, cell densities (c.f.u. ml⁻¹) were measured and used to calculate percentage survival over time. (a) and (b) are representative of three experiments; the data in (c) are means ± SD for three experiments.

Fig. 2. Effects of osmotic stress on Cn H99 and Cn MLP. Cultures of Cn H99 (○) and Cn MLP (●) grown overnight at 30 °C were diluted in YNB/glucose plus 0.5 M (a), 2.0 M (b) or 3.0 M (c) NaCl and shaken at 180 c.p.m. at 30 °C. Growth and percentage survival over time were measured as described in the legend of Fig. 2. (a) and (b) are representative of three experiments; the data in (c) are means ± SD for three experiments.

basidia and basidiospores were observed. In contrast, efforts to cross Cn MLP with *C. neoformans* B3501 (*MAT α*) did not yield the perfect stage.

Effects of heat and osmotic stresses

Cn H99 and Cn MLP had similar growth kinetics when cultured in YNB/glucose at 30 °C, but Cn MLP tolerated exposure to higher temperatures and higher NaCl concentration less well than did Cn H99. When the cultures were shifted from 30 °C to 37 °C, Cn MLP had a longer lag phase than did Cn H99; thereafter, the two strains had similar generation times and final cell densities. Neither strain grew when the cultures were shifted from 30 °C to 42.5 °C, but serial c.f.u. determinations showed that Cn MLP was killed more rapidly than was Cn H99 (Fig. 1). Similarly, when cultures of Cn H99 and Cn MLP were

shifted into YNB/glucose plus 0.5–2.5 M NaCl, both strains showed longer initial lag phases and lower final cell densities at progressively higher NaCl concentrations. However, the growth inhibitory effects of high NaCl concentrations were greater with Cn MLP than with Cn H99 (Fig. 2). In addition, though neither Cn H99 nor Cn MLP grew in YNB/glucose plus 3 M NaCl; serial dilution and c.f.u. determinations showed that Cn H99 survived for 8 h in 3 M NaCl, whereas Cn MLP did not (Fig. 2).

Pathogenicity in mice

Both Cn H99 and Cn MLP caused lethal infections in mice, but Cn MLP was 5000-fold less pathogenic. The LD₅₀ values in intravenously-infected mice were 6.9×10^2 c.f.u. Cn H99 and 3.7×10^6 c.f.u. Cn MLP. That deaths were due to *C. neoformans* infection was confirmed by

Fig. 3. Comparative survival of mice over time. Groups of 12 mice each received 5×10^2 c.f.u. Cn H99 (○) or Cn MLP (◇) intravenously and survival in the two groups was compared over time.

positive brain cultures in all cases. Also, several Cn MLP isolates from brain tissues were retested for mannitol production, and none had reverted to wild-type. In a separate experiment, 12 of 12 mice challenged intravenously with 5×10^2 c.f.u. Cn H99 had died by 51 d; in contrast, 12 of 12 mice challenged with an equal inoculum of Cn MLP survived for 60 d (Fig. 3).

Genetic analyses

Since preliminary mating experiments showed that Cn MLP was a haploid and heterothallic *MAT α* strain, we attempted to use classical genetic approaches to ascertain if the multiple phenotypic abnormalities in Cn MLP (i.e. low mannitol production, stress intolerance and loss of pathogenicity) were due to a mutation in a single gene. To accomplish this, we first crossed Cn MLP with the *MAT α* *C. neoformans* strains ATCC 34689, ESJ 450 or B3502. All three crosses generated typical hyphae, basidia and basidiospores. However, more than 40 individual basidiospores isolated from these crosses were nonviable when subcultured on YEPD. Therefore, we attempted to generate a *MAT α* derivative of Cn H99 for use as a mating partner of Cn MLP. When Cn H99 was crossed with *C. neoformans* strains ATCC 34689 and ESJ 450, typical hyphae, basidia and basidiospores were obtained. Once again, however, none of the individual basidiospores isolated from these crosses were viable when subcultured on YEPD.

DISCUSSION

The overall objective of this study was to ascertain the functional and pathogenic significance of mannitol biosynthesis by the human pathogen *C. neoformans*. Since there is no obvious selection for low mannitol production, we screened 5000 colonies derived from UV-mutagenized Cn H99 cells for the ability to produce mannitol from glucose and found a single mannitol low producer. In

addition to producing low amounts of mannitol, this mutant (Cn MLP) was more susceptible to heat and osmotic stresses and less pathogenic in mice than was its parent.

Acyclic polyols such as glycerol, D-arabinitol and mannitol are produced in abundance by a wide variety of fungi (Jennings, 1984). It has been proposed that polyols function in fungi as energy stores, translocatory compounds, intracellular reducing equivalents, and as intracellular osmolytes and stress protectants. The evidence supporting most of these proposed functions is indirect, but recent studies have provided clear experimental evidence that glycerol functions in *Saccharomyces* spp. as an intracellular osmolyte. Wild-type *Saccharomyces* spp. synthesize and accumulate glycerol intracellularly when subjected to osmotic stress, and several groups have recently shown that mutations or deletions in the genes encoding the glycerol-3-phosphate dehydrogenases of *S. cerevisiae* (Larsson *et al.*, 1993; Albertyn *et al.*, 1994) or *Saccharomyces diastaticus* (Wang *et al.*, 1994) resulted in inability to synthesize glycerol and grow in hyperosmolar media. Also, two groups have shown that induction of glycerol biosynthesis by osmotic stress conferred cross-protection against heat stress in *S. cerevisiae* (Trollmo *et al.*, 1988; Lewis *et al.*, 1995).

With this background, it was not surprising that Cn MLP was more susceptible to heat and osmotic stresses than was its wild-type parent Cn H99. Although the two strains had similar growth kinetics when cultured in YNB/glucose at 30 °C, Cn MLP exhibited graded abnormalities in growth kinetics when it was shifted from 30 °C to 37 °C or from YNB/glucose into YNB/glucose plus 0.5–2.5 M NaCl. The growth inhibitory effects of osmotic stress on Cn MLP were similar to those observed in the *S. cerevisiae* and *S. diastaticus* glycerol mutants described above. Cn MLP was also more susceptible than was Cn H99 to killing by exposure to 42.5 °C or to higher NaCl concentrations. As far as we know, fungal polyol mutants have not previously been tested for the ability to survive extreme heat or osmotic stresses. However, Edgley & Brown (1983) reported that a high proportion of *S. cerevisiae* cells exposed to 1.7 M NaCl were killed within a few hours, after which c.f.u. increased gradually as the survivors synthesized glycerol and accumulated it intracellularly. In contrast, *Saccharomyces rouxii* was resistant to killing by exposure to 1.7 M NaCl. Similarly, Larsson & Gustafsson (1993) showed that a high proportion of *Debaryomyces hansenii* cells were killed in 5–7 hours after exposure to 2.7 M NaCl. *S. rouxii* contains large amounts of intracellular glycerol and D-arabinitol (Edgley & Brown, 1983) and *D. hansenii* large amounts of intracellular D-arabinitol (Larsson & Gustafsson, 1993), even in the absence of salt stress. In contrast, *S. cerevisiae* accumulates glycerol intracellularly only after its synthesis is induced by appropriate environmental stimuli (Edgley & Brown, 1983). Thus, the marked differences we observed in the abilities of Cn H99 and Cn MLP to tolerate environmental stresses support the hypothesis that high intracellular mannitol levels protect *C. neoformans* from the deleterious effects of heat and osmotic

stresses, just as high intracellular glycerol and/or D-arabinitol levels protect other fungi from similar stresses.

Whether mannitol biosynthesis and accumulation by *C. neoformans* also contributes to virulence is more difficult to assess. Once diminished mannitol biosynthesis by Cn MLP had been verified and quantified, we undertook extensive studies of the mutant's morphological, physiological and genotypic properties. We found that Cn MLP was similar to its wild-type parent in many respects, including all known *C. neoformans* virulence factors. Specifically, Cn MLP grew well at 37 °C, produced phenoloxidase from caffeic acid, produced polysaccharide capsules that were even larger than those of Cn H99, and was a haploid and heterothallic *MAT α* strain. Moreover, the generation times of Cn MLP and Cn H99 did not differ significantly at 30 or 37 °C.

Nevertheless, Cn MLP was much less pathogenic than Cn H99 when injected intravenously into mice. One possible explanation for this observation is that the diminished abilities of Cn MLP to synthesize and accumulate mannitol may have interfered with its abilities to adapt to the multiple environmental stresses that must be overcome in the process of invading and proliferating within a mammalian host. Also, high levels of mannitol within and around the fungal cells may protect wild-type *C. neoformans* from reactive oxygen intermediates produced by host phagocytes. This possibility is supported by our recent observations that (i) Cn MLP was hypersusceptible to *in vitro* killing by normal human neutrophils and by reactive oxygen intermediates generated chemically, and (ii) the hydroxyl radical scavengers mannitol and dimethyl sulfoxide protected both Cn MLP and Cn H99 from killing by neutrophils and by cell-free oxidants (V. Chaturvedi, B. Wong & S. L. Newman, unpublished).

To prove unequivocally that low mannitol production causes either increased susceptibility to heat or osmotic stress, or decreased pathogenicity in animals would require that we show that Cn MLP has only a single mutation and that this mutation interferes directly with mannitol biosynthesis. We used classical genetic approaches in an effort to exclude the possibilities that (i) low mannitol production, stress intolerance and lower pathogenicity were caused by multiple mutations, or (ii) these multiple abnormal phenotypes all resulted from another mutation and thus were unrelated causally to one another. Our efforts to obtain viable products of meiosis from crosses between Cn MLP and several wild-type *MAT α* Cn strains were unsuccessful. This was not entirely unexpected; other investigators have reported that crosses between *C. neoformans* serotypes A and D strains, and also crosses between serotype A strains are not easily accomplished (Kwon-Chung & Bennett, 1992; J. Perfect, personal communication). In addition, these efforts were limited by the need to isolate individual basidiospores by micromanipulation. It may be worthwhile to generate genetically marked derivatives of Cn MLP and a serotype A *MAT α* *C. neoformans* strain so that more efficient methods than single-spore micromanipulation can be used to identify viable products of meiosis in future genetic analyses.

An alternative approach would be to isolate additional mutants that produce low amounts of mannitol and to test these mutants for their abilities to tolerate environmental stresses and to cause disease in animals. Unfortunately, it was necessary to screen 5000 individual colonies biochemically to identify Cn MLP. Therefore, it would not be practical to generate a statistically meaningful sample of mannitol low producers for use in genetic linkage studies. A final possible approach would be to define at the biochemical level the basis of mannitol hypoproduction by Cn MLP. The mutation of interest may be in a gene that regulates expression of one or more enzymes responsible for mannitol biosynthesis, which have not yet been elucidated in *C. neoformans*.

In summary, we have shown that a *C. neoformans* mutant that produces low levels of mannitol is more susceptible to heat and osmotic stresses, and is less pathogenic in mice. Since we have studied only a single *C. neoformans* mutant and since classical genetic analyses of this mutant did not succeed, the results of this study do not establish a direct causal relationship between the properties of interest. Our future studies will focus on the biochemistry and molecular biology of mannitol biosynthesis in *C. neoformans* and on possible mechanisms by which mannitol biosynthesis and accumulation influence virulence.

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