Identification of a novel gene, *URE2*, that functionally complements a urease-negative clinical strain of *Cryptococcus neoformans*

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A urease-negative serotype A strain of *Cryptococcus neoformans* (B-4587) was isolated from the cerebrospinal fluid of an immunocompetent patient with a central nervous system infection. The *URE1* gene encoding urease failed to complement the mutant phenotype. Urease-positive clones of B-4587 obtained by complementing with a genomic library of strain H99 harboured an episomal plasmid containing DNA inserts with homology to the *sudA* gene of *Aspergillus nidulans*. The gene harboured by these plasmids was named *URE2* since it enabled the transformants to grow on media containing urea as the sole nitrogen source while the transformants with an empty vector failed to grow. Transformation of strain B-4587 with a plasmid construct containing a truncated version of the *URE2* gene failed to complement the urease-negative phenotype. Disruption of the native *URE2* gene in a wild-type serotype A strain H99 and a serotype D strain LP1 of *C. neoformans* resulted in the inability of the strains to grow on media containing urea as the sole nitrogen source, suggesting that the *URE2* gene product is involved in the utilization of urea by the organism. Virulence in mice of the urease-negative isolate B-4587, the urease-positive transformants containing the wild-type copy of the *URE2* gene, and the urease-negative vector-only transformants was comparable to that of the H99 strain of *C. neoformans* regardless of the infection route. Virulence of the *URE2* disruption strain of H99 was slightly reduced compared to the wild-type strain in the intravenous model but was significantly attenuated in the inhalation model. These results indicate that the importance of urease activity in pathogenicity varies depending on the strains of *C. neoformans* used and/or the route of infection. Furthermore, this study shows that complementation cloning can serve as a useful tool to functionally identify genes such as *URE2* that have otherwise been annotated as hypothetical proteins in genomic databases.

Received 14 July 2006
Revised 8 August 2006
Accepted 16 August 2006

INTRODUCTION

C. neoformans is a significant human-pathogenic yeast that can be readily distinguished from other common clinical yeasts, such as species of *Candida*, by its ability to form a polysaccharide capsule and melanin, and to express urease activity. The polysaccharide capsule (Chang & Kwon-Chung, 1994, 1998, 1999; Chang *et al.*, 1996) and melanin (Kwon-Chung *et al.*, 1982; Liu *et al.*, 1999; Perfect *et al.*, 1998;

Williamson, 1997) have long been recognized as the major virulence factors. Urease activity has also been reported to play an important role in cryptococcal pathogenesis in studies in which three separate models of infection were used (Cox *et al.*, 2000). These studies, which included the murine intravenous and inhalation models as well as the rabbit meningitis model, revealed that virulence of the urease-negative strains was comparable to the wild-type strains in one model while being attenuated in others. Such contrasts in virulence further emphasize the importance of virulence genes with regard to the stages/sites of infection as well as the strains used. Although there have been case reports of urease-negative isolates of *C. neoformans* causing infection in

Abbreviations: 5-FC, 5-fluorocytosine; 5-FOA, 5-fluoroorotic acid.

Two supplementary figures are available with the online version of this paper.

humans (Bava *et al.*, 1993; Ruane *et al.*, 1988) as well as those isolated from the environment (Li & Wu, 1992; Li *et al.*, 1993), these are extremely rare. Urease activity is still considered one of the hallmarks of the genus *Cryptococcus* (Kwon-Chung *et al.*, 1987) and related genera among the Heterobasidiomycetes (Kwon-Chung & Bennett, 1992).

Urease is a metalloenzyme that facilitates the hydrolysis of urea to ammonia and carbamate, and under physiological conditions results in an increase in pH (Curtis *et al.*, 1994). The latter property has been regularly used as a diagnostic tool for the identification of *C. neoformans* in clinical laboratories (Canteros *et al.*, 1996; Zimmer & Roberts, 1979). Such pH increases caused by urease, in other organisms, have been associated with survival in animal tissues and the cause of tissue damage induced by the production of ammonium hydroxide (Mobley, 1996). Furthermore, urease has also been postulated to play a pivotal role in allowing *C. neoformans* to convert urea to a more readily usable form of nitrogen in its ecological niche. The exact role of urease in cryptococcal pathogenesis, however, is unclear. In bacterial pathogenesis, the enzyme appears to alter the host's immune function by increasing the microenvironmental pH at the infection site (Curtis *et al.*, 1994). While urease in bacteria that infect the stomach mucosa and urinary tract (Chen *et al.*, 1997; Mobley *et al.*, 1991) allows the microbes to survive at low pH, it appears to act differently in *C. neoformans* pathogenesis. It has been suggested that urease activity promotes sequestration of the organism in microcapillaries prior to dissemination into the brain and its inhibition correlates with a significant decrease in eliciting immune defence cells in the lungs of mice (Olszewski *et al.*, 2004).

In this study, we investigated the genetic basis of a urease-negative *C. neoformans* strain that was isolated from the spinal

fluid of an immunocompetent patient. A novel urease gene, *URE2*, was identified in this strain by complementation cloning and its roles in urea utilization and virulence were examined.

METHODS

C. neoformans strains and media. *C. neoformans* strains and plasmids used in the study are listed in Table 1. A clinical urease-negative strain, B-4587, was isolated from the spinal fluid of an immunocompetent patient in China who had suffered from severe headaches for 2 months. The strain was determined to be of serotype A according to the test results with the Iatron Crypto Check kit (Iatron Laboratories). Strain B-4587FOA is a spontaneous *ura5* mutant obtained by plating strain B-4587 on 5-fluoroorotic acid (5-FOA) agar and subsequent plating on media containing orotidine or orotic acid (Kwon-Chung *et al.*, 1992). B3501A is a serotype D reference strain that was sequenced at Stanford University (<http://www-sequence.stanford.edu/group/C.neoformans/>). The TAV strains are genetically manipulated strains either of B-4587 or H99 as stated in Table 1. All strains were maintained on slants of YPD agar (per litre: 10 g yeast extract, 20 g peptone, 20 g glucose, 20 g Bacto-agar) and stored at 4 °C until use. For each experiment, 24-h-old yeast cells grown on YPD at 30 °C were used. Min agar (YNB) contained 6.7 g yeast nitrogen base without amino acids, 20 g glucose and 20 g Bacto-agar per litre. Urea agar was composed of 1.7 g l⁻¹ yeast nitrogen base without amino acids and ammonium sulfate (Difco), 20 g l⁻¹ glucose and supplemented with 3 g l⁻¹ urea (Sigma) as the sole nitrogen source. This medium was used to isolate urease-positive transformants. Christiansen's urea agar was also used to confirm their urease activity (Kwon-Chung & Bennett, 1992). Transformants resistant to geneticin (G418, Gibco) were screened on YPD plates supplemented with 100 µg G418 ml⁻¹.

Transformation methods. The urease-negative clinical strain B-4587 was transformed by the electroporative method (Edman & Kwon-Chung, 1990) with either plasmid pPM8 (vector only), the plasmids harbouring the H99 genomic DNA library (Mondon *et al.*, 2000), the plasmid containing the truncated/intact *URE2* gene sequence (this study), or the plasmid containing the intact *URE1* gene sequence (Cox *et al.*, 2000), a gift from Gary Cox, Department

Table 1. *C. neoformans* strains and plasmids

Strain or plasmid	Comments	References
<i>C. neoformans</i>		
H99	Serotype A, clinical isolate	Perfect <i>et al.</i> (1998)
LP1	<i>ade2 ura5</i> auxotroph of JEC-21, serotype D isolate	Lee <i>et al.</i> (2005)
B-4587	Serotype A, urease-negative clinical strain from China	This study
B-4587FOA	<i>ura5</i> auxotroph of B-4587 isolated on 5-FOA medium	This study
TAV-756, TAV-758, TAV-759, TAV-760	Urease-positive electroporative transformants of B-4587 obtained by complementation with genomic library of H99	This study
TAV-4587V	Stable, urease-negative, ectopic, vector-only (pPM8) transformant of B-4587	This study
TAV-4587C	Stable, urease-positive, ectopic, <i>URE2</i> transformant of B-4587	This study
TAV-H99SD(1,2)	Urease-negative <i>ure2::G418</i> disruption strain of H99	This study
Plasmids		
pPM8	Stable episomal plasmid with <i>URA5</i> gene used to construct genomic H99 library	Mondon <i>et al.</i> (2000)
pAV-756, pAV-758, pAV-759, pAV-760	Episomal plasmids recovered from urease-positive transformants of B-4587 complemented with genomic library of H99 containing <i>URE2</i> gene	This study
pAV-768	Plasmid containing the truncated construct of <i>URE2</i>	This study
pAV-797	Plasmid containing the <i>ure2::G418</i> disruption construct	This study
pAV-772	Plasmid containing the <i>ure2::ADE2</i> disruption construct	This study

of Medicine, Duke University Medical Center, Durham, NC, USA. Plasmids containing the gene conferring resistance to G418, plasmids harbouring the *URE2::G418* disruption constructs and plasmids containing the *URE2::ADE2* disruption constructs were used in biolistic transformations (Toffaletti *et al.*, 1993) to delete the native *URE2* gene from either the LP1 or the H99 strain.

Cloning and sequencing of the *URE2* gene. A genomic DNA library of the serotype A strain H99 of *C. neoformans*, constructed in the *URA5*-containing plasmid pPM8 (Mondon *et al.*, 2000), was linearized and used to complement the urease-negative *ura5* strain B-4587FOA by electroporative transformation. Genomic DNA was extracted from eight independent urease-positive transformants grown on urea agar (Varma & Kwon-Chung, 2000). Genomic DNAs from these urease-positive clones were separately used to transform the urease-negative clinical isolate B-4587. The genomic DNA from these transformants was electrophoresed undigested on 10 g l⁻¹ agarose gels and analysed via Southern blot hybridizations to detect the presence of episomes in each. The episomal plasmids were recovered from four of the urease-positive transformants (TAV-756, TAV-758, TAV-759, TAV-760). Genomic DNA from each of the urease-positive transformants was digested with *NotI* to remove telomeric sequences. The DNA was then ligated with DNA polymerase to recircularize the plasmids prior to amplification in *Escherichia coli*. The episomal plasmids (pAV-756, pAV-758, pAV-759 and pAV-760) recovered from each of the urease-positive transformants were also used to electrophoretically transform strain B-4587, and the DNA inserts harboured by the plasmids were subsequently sequenced using the standard dideoxy method.

Functional analysis of the *URE2* gene. To determine whether expression of an intact and functional *URE2* gene was required for urease activity in the B-4587 background, two approaches were taken. In the first approach, a truncated version of the *URE2* gene was generated by digestion with *SnaBI* that resulted in a deletion of 1395 bp across the conserved coding region of the gene. In parallel experiments, plasmids containing either the deletion construct or the intact *URE2* gene were linearized with *NotI* prior to being used for transformations of the strain B-4587FOA by electroporation. The transformants were then tested for urease activity by growth on urea agar. In the second approach, a disruption construct of the *URE2* gene was designed in which a significant portion of the internal coding sequence of the gene was replaced by either the *ADE2* or the *G418* gene. Genomic DNA sequences extending 1 kb proximal to the 5' and 3' regions of the *URE2* coding region were amplified by PCR from the H99 or the LP1 strain. Primer sequences were used from the respective flanking regions as well as those that created overlaps with the 5' and 3' ends of the *G418* gene sequence or restriction enzyme matches with the *ADE2* gene for restriction digestion and ligation. A DNA fragment containing the gene conferring resistance to G418 was amplified by PCR using specific primers with homology to the overlapping template DNA sequences between the *URE2* and *G418* genes. This enabled the generation of an overlapping DNA sequence by PCR that contained the *G418* gene sequence flanked by DNA sequences specific to the 5' and 3' ends of the *URE2* gene from H99. The biolistic method was used to transform the PCR fragment containing the *ure2::G418* disruption construct into the G418-sensitive strain H99. The putative *ure2*-disruption strains were grown on YPD agar supplemented with 100 µg G418 ml⁻¹. Disruption of the native *URE2* gene was confirmed by PCR and by Southern blot analysis. Specific oligonucleotides were designed to generate a PCR product that initiated from within the inserted *G418* gene sequence and extended to the flanking DNA sequences derived from the genomic sequence of the *URE2* gene. A similar strategy was employed to obtain a disruption of the *URE2* gene in the serotype D strain LP1. In this case, however, the *URE2* gene was disrupted by insertion of the *ADE2* gene in its coding

region that was then used as a selection marker for transformation. Oligonucleotide primers used to generate overlapping PCR fragments for insertion were as follows. For the *ADE2* gene: 5'-GTACTAGT-CATCTTCAGACCCCAATCTCACAC-3' and 5'-CAGGATCCCGCA-CAGTTTATCACGACCACG-3'; 5'-CAGAATCCGTCAAACAGCAA-CTTCTTCTGGG-3' and 5'-GTATCGATCAATGCGAAAAGCGAAA-TGGAG-3'; for the *G418* gene: 5'-GCTCTCCAGCTCACATCTCC-CAACTCCCTCAACTCTTCATCCG-3' and 5'-GCATGATGAACAA-AGTAAGG-3'; 5'-TCGTCCTTCCTGCAGATAGGAGGG-3' and 5'-GCTAGTTTCTACATCTCTCCGTGCGATAAAGGTGATTGAAAAG-G-3'. For Southern analysis, genomic DNA from each of the transformants was digested with different restriction enzymes and screened with the *URE2/G418* gene sequences to identify hybridization patterns consistent with disruption of the native *URE2* gene. This allowed discrimination of the *ure2* disruption strain from the untransformed strains. The resulting *ure2* disruption strains were then tested for urease activity by culturing them on urea agar medium.

To determine if the urease-negative phenotype of strain B-4587 was the result of anomalies within its *URE2* gene sequence, the latter was sequenced and compared with that of the wild-type strain H99. Since the *URE2* gene is quite large, specific DNA primers were designed to generate overlapping DNA fragments from the *URE2* gene of strain B-4587. The use of short overlapping PCR fragments for sequencing not only minimized potential polymerase-generated sequence errors but also allowed for multiple sequencing reactions of each fragment to confirm the gene sequence. The sequences of these PCR fragments were then compiled to generate the intact *URE2* gene sequence from strain B-4587. This sequence was then compared with the *URE2* genomic sequence in the *C. neoformans* database of strain H99 (<http://cneo.genetics.duke.edu/>).

Analysis of *URE1* and *URE2* transcriptional levels by RT-PCR. Cells of the urease-positive strain H99, the urease-negative *ure2* disruption strain, the urease-negative clinical strain B-4587 and the urease-positive *URE2*-complemented transformant B-4587C were grown overnight in YPD broth at 30 °C. The cells were harvested by centrifugation and washed twice with sterile water. Total RNA was extracted from each strain in the exponential growth phase by using the FastRNA kit-Red (Qbiogene). The total RNAs extracted from each strain were treated with DNase I, and RT-PCR was performed for each sample using the Qiagen One-Step RT-PCR kit (B-D Sciences). Oligonucleotide primers used for RT-PCR were specifically designed to generate DNA fragments from RNA products of the *URE2* gene (5'-ACCTTGATGGCGTCTTTGGAC-3'; 5'-TCCTGTTGATGAGGGTCTGTCTG-3'), the *URE1* gene (5'-CGTT-CAAGTTGGCTCCCACTAC-3'; 5'-TGTCCTGTAAAAACATCTTCG-GCTG-3') and the constitutively expressed product of the *GPD* gene (5'-TGCTATTGGTCTGAATTGTTTC-3'; 5'-TGGTGCAAGAAGCGT-TGGACA-3').

Virulence studies in mice. Virulence in mice was determined for the urease-negative strains B-4587, TAV-4587V (ectopically integrated, stable, vector-only transformant of B-4587) and TAV-H99SD (*ure2::G418* disruption strain of H99) as well as the urease-positive wild-type strain H99 and B-4587C (ectopically integrated, stable, urease-positive clone of B-4587 transformed with a wild-type copy of the *URE2* gene). Animal studies were performed using 7-week-old female BALB/c mice and their mortality was monitored following two different routes of infection – 1 × 10⁶ cells per mouse for intravenous injection via the tail vein and 2 × 10⁷ cells per mouse for intranasal inhalation – using sets of 10 mice for each strain. Overnight cultures of each strain in the exponential growth phase were harvested by centrifugation, washed and suspended in 9 g l⁻¹ saline solution. Appropriate dilutions of each cell suspension were made in saline to yield the desired number of cells in the required volumes (200 µl for tail vein intravenous and 20 µl for intranasal

Fig. 1. Urease-positive transformants grown on YNB agar containing urea as the sole nitrogen source. Satellite colonies proximal to the transformants reflect feeding from the transformants that degraded urea. (a) Growth of colonies after 3 days; (b) magnification of inset in (a); (c) growth of colonies after 6 days.

inhalation) needed to administer per mouse. The inoculum sizes were confirmed by quantifications of expected colony-forming units on agar media.

RESULTS

Characterization of the urease-negative strain from CNS infection

The urease-negative isolate (B-4587) of *C. neoformans* was isolated from the cerebrospinal fluid (CSF) of a 46-year-old male patient who had neither immunological abnormalities nor an underlying illness but had suffered from headaches for more than 2 months before seeking medical attention. The first CSF sample drawn upon his hospitalization yielded a serotype A, MAT α strain of *C. neoformans* that was urease-negative. The patient was treated with ketoconazole plus 5-fluorocytosine (5-FC) and his CSF became culture-negative after 5 months of treatment. Strain B-4587 is highly encapsulated, strongly melanin positive on birdseed agar and grows well at 37 °C. However, it failed to grow on agar with urea as the sole source of nitrogen and was also negative on Christiansen's urease testing medium, which is routinely employed by clinical microbiology laboratories for the diagnosis of urease-positive organisms.

Cloning of the *URE2* gene

In order to functionally complement the urease-negative phenotype, B-4587FOA was used as the host strain for cloning the *URE2* gene from an H99 genomic library (Mondon *et al.*, 2000). Strain B-4587FOA was electroporatively transformed using the library and the transformed cells were plated on YNB medium that contained urea as the sole source of nitrogen. Several transformants grew on urea medium within 3 days, indicating either complementation or reversion of the urease-negative phenotype. When these transformation plates were incubated for several more days, satellite colonies appeared around the original transformants that suggested the feeding from the urease-positive clones by secretion of urease (Fig. 1). Genomic DNA from each of these urease-positive transformants successfully complemented the urease-negative phenotype of the clinical strain B-4587 (Fig. 2). Southern blot analysis revealed the presence of an episomal plasmid in each of the transformants tested (data not shown). The recovered episomal plasmid from each of the urease-positive transformants was

able to successfully complement the urease-negative phenotype of strain B-4587. This indicated that the urease-positive phenotype of the transformants was the result of gene complementation and not reversion. Besides, the urease-negative phenotype of the strain B-4587 was so stable that revertants had never been observed. Four of the episome-harboured urease-positive transformants were analysed further. Sequence analysis of the 6 kb genomic insert in the plasmid harboured by each of the transformants revealed the presence of a 4.5 kb gene that exhibited no sequence similarity to the previously reported *C. neoformans* urease gene *URE1*. This gene was named *URE2* since it functionally complemented the urease-negative phenotype, although it showed extensive homology, especially at the N- and C-termini, with the protein encoded by the *sudA* gene of *Aspergillus nidulans* (see Fig. S1, available as supplementary data with the online version of this paper). Transformation with a plasmid containing the urease-encoding gene *URE1* failed to complement the urease-negative phenotype of the strain B-4587 (data not shown). An analysis of the genomic DNA sequence of the *URE2* gene with its predicted cDNA sequence (TIGR, <http://www.tigr.org>) revealed the presence of fourteen introns within the coding region of the gene. A C-T-rich stretch was observed in the putative promoter

Fig. 2. Growth on YNB urea agar: B-3501A (urease-positive wild-type isolate, serotype D); H99 (urease-positive patient isolate, serotype A); B-4587 (urease-negative clinical isolate, serotype A); B-4587/vector (B-4587 transformed with vector alone); B-4587/*URE2*-2 and B-4587/*URE2*-9 (*URE2* transformants of B-4587).

Fig. 3. Identification of transformants containing a disruption of the native *URE2* gene by PCR and Southern analysis. (a) *URE2* gene map. S, *SpeI*; E, *Eco47III*; N, *NcoI*; B, *BamHI*; R, *EcoRI*; BE, *BstEII*; C, *ClaI*. (b) PCR analysis generated DNA fragments using primers that extended from within the sequence of the *G418* gene to the flanking DNA sequences adjacent to the insertion site within the *URE2* gene. Lane 1, size marker; lane 2, primer only; lanes 3–4, primer + template. (c) Southern blot analysis of two *ure2::G418* transformants of strain H99. Genomic DNAs were either used uncut (lanes 1–3) or digested with *NotI* (lanes 4–9). Lanes 1–6, probed with PCR fragment containing the *G418* gene; lanes 7–9, probed with PCR fragment containing *URE2* gene sequence. Lanes 1, 4 and 7, strain H99; lanes 2, 5 and 8, strain H99SD1; lanes 3, 6 and 9, strain H99SD2.

region upstream of the 5' end of the gene which also contained a TAATA box upstream from the initiating ATG. No other possible ORF was detected proximal to the 5' and 3' ends of the gene. The *URE2* gene of *C. neoformans* encodes a putative protein composed of 1243 amino acids. A search of the *C. neoformans* cDNA sequence database revealed homology of the *URE2* genomic DNA sequence to an ORF annotated to encode a hypothetical protein.

Functional characterization of the *URE2* gene

Transformants of B-4587 containing the *URE2* gene were able to grow on urea agar (Fig. 2) and turned Christiansen's urease test medium colour to magenta within 24 h. This indicated that the *URE2* gene product was involved in restoring the ability of B-4587 to utilize urea as the sole nitrogen source. To determine if this was indeed the case, two approaches were taken. First, 1395 bp of the *URE2* gene's internal coding region were deleted. The resulting truncated gene construct was used in parallel with a plasmid containing an intact *URE2* gene to transform the urease-negative strain B-4587. The transformants with the truncated version of the *URE2* gene were not able to grow on medium with urea as the sole nitrogen source while those with the intact version did (data not shown). Second, the coding region of the native *URE2* gene in the wild-type strain H99 was disrupted by insertion with the DNA

sequence of the gene conferring resistance to G418. Disruption of the native *URE2* gene was confirmed by Southern blot analysis as well as by PCR analysis (Fig. 3). The *ure2::G418* disruption transformant of strain H99 (H99SD) failed to grow on agar medium supplemented with urea as the sole nitrogen source (Fig. 2). Retransformation of strain H99SD with a plasmid containing the *URE2* gene restored the strain's ability to grow on urea agar medium. A similar disruption of the *URE2* gene in the serotype D strain LP1, by insertion of the *ADE2* gene in the coding region, also led to failure of growth on urea agar (data not shown).

Transcriptional analysis of *URE2* and *URE1*

Transformation of the isolate B-4587 with the *URE1* gene did not complement the urease-negative phenotype. This indicated that the inability of the strain to utilize urea was not due to a malfunction of the *URE1* gene. A subsequent RT-PCR analysis of the isolate did indeed reveal the presence of the predicted *URE1* gene transcript (see Fig. S2, available as supplementary data with the online version of this paper). Disrupting the internal coding sequence of the native *URE2* gene in a wild-type strain predictably resulted in a loss of the *URE2* gene transcript. This also resulted in the strain becoming urease-negative despite the presence of the *URE1* gene transcript. These observations suggested that an intact

Fig. 4. Virulence tests. Virulence was recorded as mortality of BALB/c mice that were each infected with different strains, either intravenously with 1×10^6 cells or intranasally with 2×10^7 cells. (a) Virulence comparison by intravenous infection between the wild-type strain H99, the urease-negative strain B-4587, the urease-negative vector-only transformant B-4587V, the complemented urease-positive *URE2* transformant B-4587C and the urease-negative *ure2::G418* disruptant of H99 (H99SD). (b) Virulence comparison by inhalation between the wild-type strain H99 and the urease-negative strain B-4587. (c) Virulence comparison by inhalation between the urease-negative vector-only transformant B-4587V, the complemented urease-positive *URE2* transformant B-4587C and the urease-negative *ure2::G418* disruptant of H99 (H99SD).

URE2 gene was apparently required for the *C. neoformans* strains to express the urease-positive phenotype. RT-PCR analysis, however, did show the presence of the *URE2* gene transcript in the clinical isolate B-4587 despite the strain being urease-negative.

The DNA sequence of the *URE2* gene in the urease-negative strain B-4587 was compared with the *URE2* gene of strain H99. The sequence of the *URE2* gene was remarkably well conserved between H99 and B-4587. The comparison revealed only three single-nucleotide substitutions in the DNA sequence of the *URE2* gene between strains B-4587 and H99. Two of these (C to T, and A to G) were observed within the 2nd and 11th exons, respectively. The third (C to T), however, was observed 15 bp downstream of the coding sequence of the gene.

Role of *URE2* in murine cryptococcosis

Virulence in mice was studied in sets of 7-week-old female BALB/c mice that were infected by two separate modes. Each mouse was infected either by intravenous tail vein injections

with 1×10^6 organisms or via inhalation with 2×10^7 organisms. No apparent difference in virulence was observed between the strains when administered via the intravenous route except that mortality in strain H99SD appeared to be slightly delayed ($P=0.03$, log-rank test) (Fig. 4a). All mice infected via this route died of cryptococcal infection within 7–13 days regardless of the urease phenotype of the infecting organism (Fig. 4a). Although mortality among mice infected via the inhalation route was generally delayed by 20–30 days compared to the intravenous route, mice infected with H99 and the urease-negative B-4587 strain succumbed to cryptococcosis at the same rate (Fig. 4b). Furthermore, the urease-negative vector transformant (B-4587V) and the urease-positive *URE2* transformant of B-4587 (B-4587C) were equally virulent (Fig. 4c). Virulence with the *ure2::G418* disruption isolate of H99 (H99SD), however, was significantly reduced compared to both B-4587 ($P=0.02$, log-rank test) and its *URE2* complemented transformant (B-4587C), where it was found to be comparable (Fig. 4c). This indicated that the importance of the urease phenotype in pathogenicity of

C. neoformans is strain dependent, and the route of infection emphasizes this importance.

DISCUSSION

In this study, we have identified a novel gene by complementational cloning in a urease-negative strain of *C. neoformans*. Urease-negative isolates of *C. neoformans*, although rare, have previously been reported from two clinical cases (Bava *et al.*, 1993; Ruane *et al.*, 1988) and two from pigeon droppings (Li & Wu, 1992; Li *et al.*, 1993). Unlike our urease-negative strain, however, the two urease-negative clinical isolates reported had caused disseminated infection in AIDS patients. Our case, therefore, is believed to be the first report of a urease-negative *C. neoformans* infection in an immunocompetent patient who was apparently cured with a regimen of 5-FC and ketoconazole. The combination of ketoconazole and 5-FC is rarely used as the drug treatment of choice for cryptococcosis of the central nervous system (Kwon-Chung & Bennett, 1992), and its therapeutic success in this case is likely due to the patient's normal immune status. The circumstances under which the patient might have been exposed to *C. neoformans* have not been determined. This encapsulated, melanin-positive and urease-negative strain became urease-positive, as evidenced by growth on urea agar, when transformed with a genomic plasmid library from the serotype A reference strain H99. The appearance of satellite colonies proximal to the transformants suggested secretion of urease by the transformants that degraded the urea in the agar into ammonia and carbamate, thereby allowing growth of urease-negative untransformed clones. Involvement of the *URE2* gene product in the utilization of urea for growth in *C. neoformans* was further evidenced by the observation that strains containing the truncated versions of the *URE2* gene failed to grow on medium with urea as the sole nitrogen source. A similar failure of the *ure2* disruption strains of H99 or LP1 to grow on urea agar, coupled with restoration of this ability upon retransformation with the *URE2* gene, indicated a prominent role of the *URE2* gene product in the organism's ability to utilize urea as the sole nitrogen source. A search of the *C. neoformans* genomic database, with the DNA sequence of the gene contained within the insert, found the putative protein encoded by the ORF to be annotated as a hypothetical protein. It is not uncommon to find genomic databases littered with ORFs annotated to encode hypothetical proteins. We find this especially to be true when screening the genomic database of *C. neoformans*. The use of complementation cloning, as we have done to identify the gene with an apparent association with urease metabolism, can be a useful tool to assign function to such genes. Although a homology-based search of the NCBI database using the sequence of our gene revealed the ORF to have significant similarity to the *sudA* gene of *A. nidulans*, the gene was named *URE2* due to its role in urease activity.

In *A. nidulans*, SudAp is involved in chromosome scaffolding that facilitates segregation during mitosis

(Holt & May, 1996). The product of this gene, which appears to be well conserved, belongs to a family of extragenic suppressor proteins known as the DA-box protein family (Peterson, 1994). These DA-proteins, by genetic and biochemical analysis, have been shown to contribute directly to the composition of chromosomes in order to facilitate organization and segregation fidelity of chromosomes during mitosis (Peterson, 1994). The interaction of scaffold proteins with histones in the nucleus is known to affect several functional aspects of cell physiology, from structural aspects involving chromosomal condensation during cell division to gene regulation via transcriptional control (Peterson, 1994; Saitoh *et al.*, 1994; Saka *et al.*, 1994). It is possible that Sud1p of *C. neoformans* is a structural component of the mechanism integral to urease activity and may function in the secretion of urease, as suggested by the appearance of satellite colonies around the transformants.

While the NCBI database identified significant overall homology to the *sudA* gene in *A. nidulans*, a 288 bp region of the gene exhibited homology to DNA sequences present in genes encoding urease accessory proteins in other organisms. Urease accessory proteins, such as UreD, UreE and UreF, have been reported in *Lycopersicon esculentum* (tomato), *Glycine max* (soybean), *Arabidopsis thaliana* (thale cress) and the fission yeast *Schizosaccharomyces pombe* (Bacanamwo *et al.*, 2002). Although the DNA sequences of the *UreD* and *UreF* genes appear to be well conserved among plants and some species of divergent bacteria, the homology of these genes appears to be intermediate when compared to the fungus *S. pombe* (Bacanamwo *et al.*, 2002). The accessory proteins UreD and UreF are apparently essential for urease activity and reportedly form a complex to which the chaperone protein UreE delivers nickel, thus activating the urease enzyme (Lee *et al.*, 1992; Soriano *et al.*, 2000). Synthesis of metal-containing enzymes, such as urease, which requires the binding of nickel for function (Bacanamwo *et al.*, 2002; Eitinger *et al.*, 2000), often elicits the participation of several accessory proteins. It is postulated that the *UreD* gene is in some way involved in the production or incorporation of a nickel co-factor essential for urease activity. In previous reports, supplementing the urea growth media with nickel salts with or without bicarbonates was shown in other organisms to augment or restore the urease activity in some urease mutant strains which were otherwise unable to bind nickel (Soriano & Hausinger, 1999). However, the *URE2* mutant strains of *C. neoformans*, B-4587 and the *ure2::G418* strain of H99, were not able to grow on urea agar media supplemented with different concentrations and/or combinations of nickel and bicarbonate salts (data not shown).

The inability of strain B-4587 to grow on urea agar is apparently not due to a lack of *URE1* transcription, as evidenced by the RT-PCR result and the failure of the *URE1* gene to complement the urease-negative phenotype. This is

not surprising since there have been reports of urease-negative strains in which the *URE1* gene is expressed (Tanaka *et al.*, 2003). Since the clinical isolate B-4587 was urease-negative, we tried to determine if the basis of this malfunction was the result of any mutation in the DNA sequence of the *SUD1* gene. Although three nucleotide substitutions were detected, no discernible characteristics of the regions housing these substitutions were apparent. While these substitutions may play a role, their influence on the function of the protein is as yet unknown.

Virulence was recorded as mortality of the mice after infection. Two models of infection were used: the intravenous and the intranasal routes. Although *URE2* disruption strains were constructed in serotype A as well as serotype D backgrounds, the disruptant in the serotype A background was chosen for virulence comparisons since both the reference wild-type strain H99 and the clinical isolate B-4587 were of serotype A. For mice infected intravenously, virulence was comparable between the urease-negative strain B-4587, its vector-only transformant (B-4587V) and the urease-positive strains H99 and the *URE2*-complemented transformant B-4587C. The urease phenotype apparently had no effect on the virulence of strains in the B-4587 background when a systemic model of infection was used. This is in contrast to the report of Cox *et al.* (2000), where a significant attenuation in virulence was observed for mice infected intravenously with the urease-negative H99 mutant *ure1*. However, virulence in the *ure2::G418* disruption isolate of H99 (H99SD) was slightly attenuated compared to the wild-type strain H99 ($P=0.03$, log-rank test). This suggested that the urease-negative phenotype of H99 derived from a disruption of the *URE2* gene did affect its virulence in mice. In the inhalation model, mortality in mice was generally delayed compared to mice infected intravenously. Intranasal infection of mice is probably not the ideal method of organism delivery due to the inherent lack of inoculum control, which may result in variations in reproducible mortality times. As in the intravenous model, mortality of mice infected intranasally was also similar between the urease-negative strain B-4587, the urease-positive transformants, and strain H99. This showed that in both infection models, urease activity in strain B-4587 and its derivatives was not important for virulence in mice. However, in mice infected with the *ure2* disruption strain of H99 (H99SD), virulence via the inhalation model was found to be significantly more attenuated ($P=0.02$, log-rank test) than in strain B-4587 and its derivatives compared to the intravenous model. While all the mice infected intranasally with strains H99, B-4587 and B-4587C succumbed to infection in 40 days, 60 % of the mice infected with the *ure2* disruption strain H99SD were still alive after 50 days. Our results suggest that virulence of *C. neoformans* in mice is not necessarily dependent on urease activity by itself but it is strongly influenced by other factors such as products of other related genes like *URE2* and/or the route of infection. It is also possible that the differences in virulence observed between

our *URE2*-derived urease-negative strains and the *URE1*-derived urease-negative strains used by Cox and colleagues are in fact the result of the specific genes involved, the fungal strains used or the strains of mice (BALB/c or A/Jcr) rather than just urease activity. While the mechanism involving urease in pathogenicity is not known, genes affecting virulence may be important during certain stages or at specific sites of infection, and this may account for the difference in the two models. Urease is reported to be a major site-specific virulence factor in bacteria infecting the tissue lining of the stomach and urinary tract (Mobley *et al.*, 1991). It has also been reported that sequestration of the fungus within microcapillaries after inhalation is important prior to dissemination of *C. neoformans* to the brain (Olszewski *et al.*, 2004). The exact mechanism by which urease facilitates such sequestration is not known but the hydrolysis of urea proximal to endothelial cells may promote adhesion, or the release of ammonia could result in cell toxicity affecting astrocytes by weakening the cellular junctions of the blood–brain barrier. Since the *ure1* and *ure2* strains are indistinguishable in their urease activity, urease itself may not be important for the virulence in certain strains of *C. neoformans*. The relationship between urease activity and virulence of *C. neoformans* therefore warrants further study.

ACKNOWLEDGEMENTS

The authors would like to thank Dr Yun Chang for his critical reviews and suggestions. They would also like to thank Dr Gary Cox for the gift of the plasmid containing the *URE1* gene. This study was supported by funds from the intramural programme of the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA.

REFERENCES

- Bacanamwo, M., Witte, C. P., Lubbers, M. W. & Polacco, J. C. (2002). Activation of the urease of *Schizosaccharomyces pombe* by the UreF accessory protein from soybean. *Mol Genet Genomics* **268**, 525–534.
- Bava, A. J., Negroni, R. & Bianchi, M. (1993). Cryptococcosis produced by a urease negative strain of *Cryptococcus neoformans*. *J Med Vet Mycol* **31**, 87–89.
- Canteros, C. E., Rodero, L., Rivas, M. C. & Davel, G. (1996). A rapid urease test for presumptive identification of *Cryptococcus neoformans*. *Mycopathologia* **136**, 21–23.
- Chang, Y. C. & Kwon-Chung, K. J. (1994). Complementation of a capsule-deficient mutation of *Cryptococcus neoformans* restores its virulence. *Mol Cell Biol* **14**, 4912–4919.
- Chang, Y. C. & Kwon-Chung, K. J. (1998). Isolation of the third capsule-associated gene, *CAP60*, required for virulence in *Cryptococcus neoformans*. *Infect Immun* **66**, 2230–2236.
- Chang, Y. C. & Kwon-Chung, K. J. (1999). Isolation, characterization, and localization of a capsule-associated gene, *CAP10*, of *Cryptococcus neoformans*. *J Bacteriol* **181**, 5636–5643.
- Chang, Y. C., Penoyer, L. A. & Kwon-Chung, K. J. (1996). The second capsule gene of *Cryptococcus neoformans*, *CAP64*, is essential for virulence. *Infect Immun* **64**, 1977–1983.

- Chen, G., Fournier, R. L., Varanasi, S. & Mahama-Relue, P. A. (1997). *Helicobacter pylori* survival in gastric mucosa by generation of a pH gradient. *Biophys J* 73, 1081–1088.
- Cox, G. M., Mukherjee, J., Cole, G. T., Casadevall, A. & Perfect, J. R. (2000). Urease as a virulence factor in experimental cryptococcosis. *Infect Immun* 68, 443–448.
- Curtis, J. L., Huffnagle, G. B., Chen, G. H., Warnock, M. L., Gyetko, M. R., McDonald, R. A., Scott, P. J. & Toews, G. B. (1994). Experimental murine pulmonary cryptococcosis. Differences in pulmonary inflammation and lymphocyte recruitment induced by two encapsulated strains of *Cryptococcus neoformans*. *Lab Invest* 71, 113–126.
- Edman, J. C. & Kwon-Chung, K. J. (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.
- Eitinger, T., Degen, O., Bohnke, U. & Muller, M. (2000). Nic1p, a relative of bacterial transition metal permeases in *Schizosaccharomyces pombe*, provides nickel ion for urease biosynthesis. *J Biol Chem* 275, 33184.
- Holt, C. L. & May, G. S. (1996). An extragenic suppressor of the mitosis-defective *bimD6* mutation of *Aspergillus nidulans* codes for a chromosome scaffold protein. *Genetics* 142, 777–787.
- Kwon-Chung, K. J. & Bennett, J. E. (1992). *Medical Mycology*. Philadelphia: Lea & Febiger.
- Kwon-Chung, K. J., Polacheck, I. & Popkin, T. J. (1982). Melanin-lacking mutants of *Cryptococcus neoformans* and their virulence for mice. *J Bacteriol* 150, 1414–1421.
- Kwon-Chung, K. J., Wickes, B. L., Booth, J. L., Vishniac, H. S. & Bennett, J. E. (1987). Urease inhibition by EDTA in the two varieties of *Cryptococcus neoformans*. *Infect Immun* 55, 1751–1754.
- Kwon-Chung, K. J., Varma, A., Edman, J. C. & Bennett, J. E. (1992). Selection of *ura5* and *ura3* mutants from the two varieties of *Cryptococcus neoformans* on 5-fluoroorotic acid medium. *J Med Vet Mycol* 30, 61–69.
- Lee, M. H., Mulrooney, S. B., Renner, M. J., Markowicz, Y. & Hausinger, R. P. (1992). *Klebsiella aerogenes* urease gene cluster: sequence of *ureD* and demonstration that four accessory genes (*ureD*, *ureE*, *ureF*, and *ureG*) are involved in nickel metallocenter biosynthesis. *J Bacteriol* 174, 4324–4330.
- Lee, H., Chang, Y. C. & Kwon-Chung, K. J. (2005). *TUP1* disruption reveals biological differences between *MATa* and *MAT α* strains of *Cryptococcus neoformans*. *Mol Microbiol* 55, 1222–1232.
- Li, A. & Wu, S. (1992). An urease negative *Cryptococcus neoformans*. *Wei Sheng Wu Xue Bao* 32, 68–71.
- Li, A., Guo, N. & Wu, S. (1993). A strain of urease negative *Cryptococcus neoformans* isolated from the environment in China. *Chin Med Sci J* 8, 52–54.
- Liu, L., Wakamatsu, K., Ito, S. & Williamson, P. R. (1999). Catecholamine oxidative products, but not melanin, are produced by *Cryptococcus neoformans* during neuropathogenesis in mice. *Infect Immun* 67, 108–112.
- Mobley, H. L. (1996). The role of *Helicobacter pylori* urease in the pathogenesis of gastritis and peptic ulceration. *Aliment Pharmacol Ther* 10, 57–64.
- Mobley, H. L., Hu, L. T. & Foxal, P. A. (1991). *Helicobacter pylori* urease: properties and role in pathogenesis. *Scand J Gastroenterol* 187, 39–46.
- Mondon, P., Chang, Y. C., Varma, A. & Kwon-Chung, K. J. (2000). A novel episomal shuttle vector for transformation of *Cryptococcus neoformans* with the *ccdB* gene as a positive selection marker in bacteria. *FEMS Microbiol Lett* 187, 41–45.
- Olszewski, M. A., Noverr, M. C., Chen, G. H., Toews, G. B., Cox, G. M., Perfect, J. R. & Huffnagle, G. B. (2004). Urease expression by *Cryptococcus neoformans* promotes microvascular sequestration, thereby enhancing central nervous system invasion. *Am J Pathol* 164, 1761–1771.
- Perfect, J. R., Wong, B., Chang, Y. C., Kwon-Chung, K. J. & Williamson, P. R. (1998). *Cryptococcus neoformans*: virulence and host defences. *Med Mycol* 36, 79–86.
- Peterson, C. L. (1994). The SMC family: novel motor proteins for chromosome condensation? *Cell* 79, 389–392.
- Ruane, P. J., Walker, L. J. & George, W. L. (1988). Disseminated infection caused by urease-negative *Cryptococcus neoformans*. *J Clin Microbiol* 26, 2224–2225.
- Saitoh, N., Goldberg, I. G., Wood, E. R. & Earnshaw, W. C. (1994). ScII: an abundant chromosome scaffold protein is a member of a family of putative ATPases with an unusual predicted tertiary structure. *J Cell Biol* 127, 303–318.
- Saka, Y., Sutani, T., Yamashita, Y., Saitoh, S., Takeuchi, M., Nakaseko, Y. & Yanagida, M. (1994). Fission yeast *cut3* and *cut14*, members of a ubiquitous protein family, are required for chromosome condensation and segregation in mitosis. *EMBO J* 13, 4938–4952.
- Soriano, A. & Hausinger, R. P. (1999). GTP-dependent activation of urease apoprotein in complex with the UreD, UreF, and UreG accessory proteins. *Proc Natl Acad Sci U S A* 96, 11140–11144.
- Soriano, A., Colpas, G. J. & Hausinger, R. P. (2000). UreE stimulation of GTP-dependent urease activation in the UreD-UreF-UreG-urease apoprotein complex. *Biochemistry* 39, 12435–12440.
- Tanaka, R., Nishimura, K., Imanishi, Y., Takahashi, I., Hata, Y. & Miyaji, M. (2003). Analysis of serotype AD strains from F1 progenies between urease-positive- and negative-strains of *Cryptococcus neoformans*. *Nippon Ishinkin Gakkai Zasshi* 44, 293–297.
- Toffaletti, D. L., Rude, T. H., Johnston, S. A., Durack, D. T. & Perfect, J. R. (1993). Gene transfer in *Cryptococcus neoformans* by use of biolistic delivery of DNA. *J Bacteriol* 175, 1405–1411.
- Varma, A. & Kwon-Chung, K. J. (2000). Characterization of the *L41* gene in *Cryptococcus neoformans*: its application as a selectable transformation marker for cycloheximide resistance. *Yeast* 16, 1397–1403.
- Williamson, P. R. (1997). Laccase and melanin in the pathogenesis of *Cryptococcus neoformans*. *Front Biosci* 2, e99–e107.
- Zimmer, B. L. & Roberts, G. D. (1979). Rapid selective urease test for presumptive identification of *Cryptococcus neoformans*. *J Clin Microbiol* 10, 380–381.