INFECTION AND IMMUNITY, Feb. 2000, p. 443–448 Vol. 68, No. 2
0019-9567/00/$04.00+0

Urease as a Virulence Factor in Experimental Cryptococcosis

GARY M. COX,[1]* JEAN MUKHERJEE,[2] GARRY T. COLE,[3] ARTURO CASADEVALL,[2] AND JOHN R. PERFECT[1]

Division of Infectious Disease, Department of Medicine, Duke University Medical Center, Durham, North Carolina 27710[1]; Division of Infectious Diseases, Department of Medicine, and Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, New York 10461[2]; and Department of Microbiology and Immunology, Medical College of Ohio, Toledo, Ohio 43614[3]

Received 18 June 1999/Returned for modification 6 August 1999/Accepted 8 November 1999

Urease catalyzes the hydrolysis of urea to ammonia and carbamate and has been found to be an important pathogenic factor for certain bacteria. *Cryptococcus neoformans* is a significant human pathogenic fungus that produces large amounts of urease; thus we wanted to investigate the importance of urease in the pathogenesis of cryptococcosis. We cloned and sequenced the genomic locus containing the single-copy *C. neoformans* urease gene (*URE1*) and used this to disrupt the native *URE1* in the serotype A strain H99. The *ure1* mutant strains were found to have in vitro growth characteristics, phenoloxidase activity, and capsule size similar to those of the wild type. Comparison of a *ure1* mutant with H99 after intracisternal inoculation into corticosteroid-treated rabbits revealed no significant differences in colony counts recovered from the cerebrospinal fluid. However, when these two strains were compared in both the murine intravenous and inhalational infection models, there were significant differences in survival. Mice infected with a *ure1* strain lived longer than mice infected with H99 in both models. The *ure1* strain was restored to urease positivity by complementation with *URE1*, and two resulting transformants were significantly more pathogenic than the *ure1* strain. Our results suggest that urease activity is involved in the pathogenesis of cryptococcosis but that the importance may be species and/or infection site specific.

Urease is a metalloenzyme that catalyzes the hydrolysis of urea to ammonia and carbamate. Under physiological conditions, this reaction can result in an increase in pH. Urease activity has been found in several bacteria, fungi, and plants and has been shown to be an important pathogenic factor for the bacteria *Helicobacter pylori* and *Proteus mirabilis* (12, 14, 33). *H. pylori* is the major cause of peptic ulcer disease in humans, and urease is essential for pathogenesis in experimental models. Urease-negative strains of *H. pylori* constructed using a variety of molecular and genetic techniques were unable to infect gastric mucosa (12, 33). It has been postulated that the hydrolysis of urea and the resulting increase in pH allow *H. pylori* to survive within the gastric mucosa. It has also been postulated that much of the tissue damage induced by this organism is a result of ammonium hydroxide produced through the actions of urease (22, 23), and there are data that the actions of urease may alter the function of white blood cells (18–20). Because of the importance of this enzyme in the pathogenesis of peptic ulcer disease, urease inhibitors and urease vaccines are currently being developed for clinical use.

Many fungi pathogenic to humans have urease activity, among which are *Cryptococcus neoformans*, *Coccidioides immitis*, *Histoplasma capsulatum*, *Sporothrix schenckii*, and species of *Trichosporon* and *Aspergillus*. The first urease gene cloned from a human pathogenic fungus was that of *C. immitis* (35). The coccidioidal urease gene has been shown to be expressed in vivo, and there are suggestions that it plays a role in both sporulation and pathogenesis (7). Therefore, we wanted to further investigate the role of urease in fungal infections by using *C. neoformans* as a model pathogen.

C. neoformans is a heterothallic yeast with several known virulence factors, including a polysaccharide capsule, melanin production, and the ability to grow at 37°C (21). Although there have been some rare case reports of urease-negative *C. neoformans* strains causing human infection (2, 29), the vast majority of clinical isolates produce large amounts of urease. In fact, the rapid detection of urease activity is one means of tentatively identifying *C. neoformans* from clinical specimens (3, 36). It is likely that the primary role of this enzyme for *C. neoformans* is to convert urea to a usable nitrogen source in its ecological niche. However, by analogy to the findings with some bacteria, this enzyme may be important for the ability of this yeast to survive within mammalian hosts.

In this report, we followed a classical molecular pathogenesis approach by identifying the *C. neoformans* urease gene and then constructing urease-negative mutants through targeted gene disruption. The virulence of one of these urease-negative mutants was compared to that of its wild-type parent in three animal models of cryptococcosis. The results demonstrate that urease activity is a factor in the pathogenesis of cryptococcosis but that the importance may be species and/or infection site specific.

MATERIALS AND METHODS

Strains and media. *C. neoformans* strains H99 (serotype A, mating type α) and M001 (an *ade2* auxotroph [32] derived from H99 using UV mutagenesis) were recovered from 15% glycerol stocks stored at −80°C prior to use in this study. Urease-negative transformants were selected on adenine dropout media (32). The strains transformed back to urease positivity were maintained on yeast extract-peptone-dextrose (YPD) agar containing 200 U of hygromycin B/ml (9). Strains were tested for urease activity using Christensen's urea agar and broth (Becton Dickinson, Cockeysville, Md.). Minimal medium plates were made using 0.67% yeast nitrogen base without amino acids (Difco, Detroit, Mich.), 2% glucose, and 1.6% agar. Prior to use in the mouse studies, yeast strains were grown for 18 to 20 h at 30°C with shaking in YPD broth (1% yeast extract, 2% peptone, 2% dextrose), harvested, washed three times with sterile phosphate-buffered saline (PBS), and counted with a hemacytometer to determine cell number. Prior to use in the rabbit experiments, the strains were streaked to confluence on YPD and grown at 30°C for 48 h. Yeasts were then harvested from the plates with a cotton swab, suspended in PBS with vortexing, and counted with

* Corresponding author. Mailing address: Box 3353, Duke Medical Center, Durham, NC 27710. Phone: (919) 681-5055. Fax: (919) 684-8902. E-mail: cox00005@mc.duke.edu.

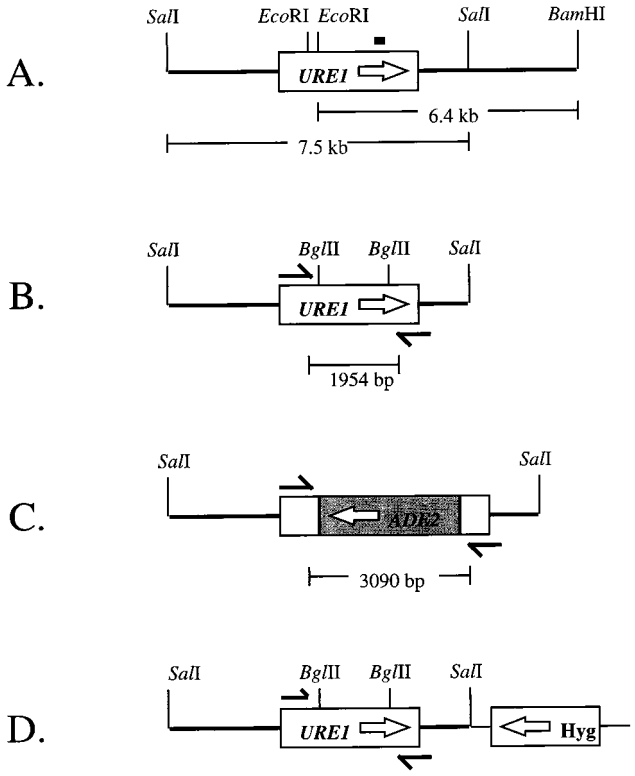

FIG. 1. (A) Restriction map of the genomic fragment containing the urease gene (*URE1*). Pertinent restriction enzyme sites are shown. The small bar above *URE1* denotes the area that was originally amplified using primers derived from conserved areas of bacterial urease coding sequences. (B) The *Sal*I genomic fragment containing *URE1* that was subcloned to a plasmid and used to create the disruption construct. This fragment was digested with *Bgl*II, and the deleted fragment containing most of *URE1* was replaced with *ADE2*. The resulting construct (C) was used in the gene disruption studies. The solid arrows flanking the *Bgl*II sites designate the sites of the PCR primers that were used to screen for disruption of the native gene, and the expected sizes of the amplicons with the native and disrupted genes as templates are noted (B and C, respectively). The *ADE2* fragment that was used to replace the deleted portion of *URE1* is shaded in panel C. (D) Construct used to complement *ure1* strain to urease positivity. A hygromycin B resistance cassette (Hyg) was ligated into the plasmid containing the *Sal*I fragment and used as a selectable marker.

a hemacytometer. Inoculum sizes for both the mouse and rabbit experiments were confirmed by plating dilutions on YPD agar plates.

Isolation of the cryptococcal urease gene. A 294-bp fragment of the cryptococcal urease gene (*URE1*) was amplified from *C. neoformans* strain H99 genomic DNA by PCR using primers complementary to conserved areas of bacterial urease structural genes (BU5′-ATGGTTTGCCACCACCT and BU3′-AGCTGGGTTGATTGTGTATT) (G. M. Cox, G. T. Cole, and J. R. Perfect, Program Abstr. 3rd Int. Conf. Cryptococcus Cryptococcosis, poster 1.6, p. 150). The amplified DNA fragment was ligated into a plasmid for sequencing to confirm its identity as a urease-encoding sequence and then used to probe a *C. neoformans* strain H99 genomic library (8). Two hybridizing fragments of 7.5 and 6.4 kb were recovered from one of the phage isolates obtained from the library by digestion with *Sal*I and *Bam*HI/*Eco*RI, respectively (Fig. 1). The fragments were subcloned into a plasmid (pBluescript II; Stratagene, La Jolla, Calif.), and the *Sal*I-digested fragment was sequenced in both directions. Putative introns in the coding region were identified by both comparing the putative amino acid sequence with those of the sequenced fungal urease genes from *C. immitis* (35) and *Schizosaccharomyces pombe* (31) and by identifying the 5′ and 3′ splice sites of GTNNGY and YAG, respectively.

Disruption of the native urease gene. The 7.5-kb *Sal*I genomic fragment containing the urease gene was digested with *Bgl*II to release an internal 1,745-bp fragment from the urease gene (Fig. 1). The *Bgl*II sites of the plasmid were filled in using Klenow fragment, and the deleted fragment was replaced by a 2,891-bp blunt ended genomic fragment containing the phosphoribosylaminoimidazole carboxylase gene (*ADE2*) (32). The resulting construct was linearized with *Bam*HI, and dideoxy ATP was added to the ends of the linearized construct using terminal deoxynucleotidyl transferase as described previously (17). The construct was then used to transform strain M001 with a biolistic device as described previously (32). Transformants were initially selected on adenine dropout plates and then tested for urease activity by suspending single colonies in 1 ml of Christensen's broth in 12- by 75-mm polystyrene tubes. The tubes containing the suspensions of the transformants in Christensen's medium were incubated with agitation overnight at 30°C. Christensen's medium contains 300 mM urea and phenol red as a pH indicator. In Christensen's medium, urease activity converts urea to ammonia and increases the pH, which is reflected by a color change of the medium from yellow to bright pink. Transformants that appeared urease negative on visual inspection were screened again on Christensen's agar at 30°C for 48 h. The urease-negative transformants were then screened for disruption of the native urease gene using PCR (primers Bgl5, GACTCTTGTCCTATGGC TA, and Bgl3, GGATCGAAGCGTTCGCAT) and Southern blotting. Genomic DNA from each transformant was double digested with both *Bam*HI and *Eco*RI, separated on agarose gels, and transferred to nylon membranes. The Southern blots were probed with [^{32}P]dCTP-labeled DNA made with a random primer labeling kit (Gibco BRL, Gaithersburg, Md.) and the 6.4-kb *Bam*HI-*Eco*RI genomic fragment containing part of *URE1* as a template (Fig. 1). Transformants that demonstrated displacement of the native gene by Southern analysis and disruption of the native gene by PCR were compared to strain H99 in terms of growth rates in YPD broth at 37°C, melanin production on dopamine agar (28), and capsule size by microscopic measurement of India ink preparations after growth on YPD at 30°C in 5% CO_2 (13). Total RNA was isolated from selected strains and used in Northern blots probed with the *Sal*I fragment containing *URE1*. The amount of RNA loaded onto the gels was controlled for by probing duplicate blots with the actin gene (8). All Northern blots were exposed to film for 4 days before processing.

Restoration of urease activity. A hygromycin B resistance cassette (9) was ligated into the *Eco*RV site of the plasmid containing the intact *Sal*I genomic fragment. A *ure1* strain was then restored to urease positivity by biolistic transformation with this construct. Transformants were selected on YPD-hygromycin B media, and twenty were randomly chosen for testing on Christensen's agar. Eighteen of the 20 hygromycin B-resistant colonies were found to have urease activity, and two transformants, the *ure1*+*URE1*-1 and *ure1*+*URE1*-2 strains, were selected for further study.

In vivo testing. A total of 10^8 cryptococci of either strain were inoculated into the cisternae magnae of 10 (3 with the *ure1* strain, 7 with H99) corticosteroid-treated rabbits as described previously (26). Cerebrospinal fluid (CSF) was sampled 2, 5, 8, and 12 days after infection, and dilutions were plated onto YPD for colony counts. Cryptococci were also used to infect 4- to 6-week-old female BALB/c mice (NCI/Charles River Labs, Raleigh, N.C.) using lateral tail vein injections and 4- to 6-week-old female A/Jcr mice (NCI/Charles River Labs) using nasal inhalation. Fifteen mice were infected with 10^7 yeasts of the *ure1*, H99 (*URE1*), and *ure1*+*URE1*-1 strains in a volume of 100 μl via lateral tail vein injections. Ten mice were infected with 10^5 yeasts of the *ure1*, *URE1*, *ure1*+*URE1*-1, and *ure1*+*URE1*-2 strains via nasal inhalation. The nasal inhalations were performed by first anesthetizing the mice with phenobarbital via intraperitoneal injections and then suspending the mice via the incisors on a silk thread so that the necks were fully extended. The yeast inocula were made up in 50-μl volumes and slowly pipetted directly into the nares. The mice were suspended for 10 min after infection. The mice were fed ad libitum and monitored with twice-daily inspections. Mice that appeared moribund or in pain were sacrificed using CO_2 inhalation. Survival data from the mouse experiments were analyzed by a Wilcoxon test for censored data using True Epistat (standard version; Epistat Services). Student's *t* test was used in evaluating the colony counts from the rabbit experiments. Comparisons with P values <0.05 were considered to be significant.

Nucleotide sequence accession number. The *C. neoformans URE1* sequence has been submitted to GenBank and is available under accession no. AF006062.

RESULTS

We were able to clone the *C. neoformans* strain H99 urease gene (*URE1*) by using PCR and primers derived from bacterial urease gene sequences to amplify a conserved area of the gene. The amplified fragment was used to probe Southern blots of strain H99 genomic DNA samples individually digested with *Bam*HI, *Eco*RV, *Hin*dIII, and *Sal*I. Only single hybridizing bands were seen with each digested DNA sample, suggesting that the urease gene exists in a single copy in the haploid form of this organism (data not shown). A *Sal*I genomic fragment obtained from the library was found to contain the entire urease gene, which was 3,032 bp and contained 10 introns. The putative amino acid sequence of *URE1* contains 832 residues and has 59 and 57% identity to the sequences of the urease genes from *C. immitis* and *S. pombe*, respectively. The original PCR amplicon used to probe the genomic library was subsequently found to correspond to base pairs 2168 to 2455 of *URE1* (Fig. 1) and amino acids 583 to 682. The sequence also showed conservation of all of the histidine residues thought to

```
                    * *                 *
C. neoformans  ...GRLDVHVHYISPQL........VQINLHSDTLNES...
C. immitis     ...GGIDTHVHFICPQQ........IQCLIHTDTLNES...
S. pombe       ...GGMDSHVHFICPQQ........VQCLIHTDTLNES...
H. pylori      ...GGIDTHIHFISPQQ........VQVAIHTDTLNEA...
```

FIG. 2. Comparison of portions of the putative urease amino acid sequences from *C. neoformans*, *C. immitis*, *S. pombe*, and *H. pylori*. The sequences correspond to amino acids 395 to 408 and 507 to 519 of the *C. neoformans* urease. Conserved histidine residues thought to be important in binding to the nickel metallocenter (amino acid 400), binding to the substrate (amino acid 402), and catalysis (amino acid 512) are in boldface and marked with a star.

be involved in binding to the nickel metallocenter, binding to the substrate, and catalysis (Fig. 2).

The *Sal*I fragment of *URE1* was used to create a gene disruption construct (Fig. 1). *ADE2* was used to disrupt *URE1*, and the resulting construct was used to transform strain M001. A total of 140 Ade2$^+$ transformants were screened for urease activity, and 54 (39%) were found to be persistently urease negative. In order to confirm disruption of the native *URE1* in the urease-negative transformants, we used PCR, Southern blot, and Northern blot analyses. Twenty of the urease-negative transformants were randomly selected, and displacement of the native gene in all 20 was demonstrated by PCR. Figure 3a shows a representative agarose gel used to evaluate disruption of the native *URE1*. The PCR product with either M001 or H99 genomic DNA as the template was 1,954 bp, whereas the PCR products with DNA from each of the transformants as the template were 3,090 bp. The 1,136-bp displacement of the

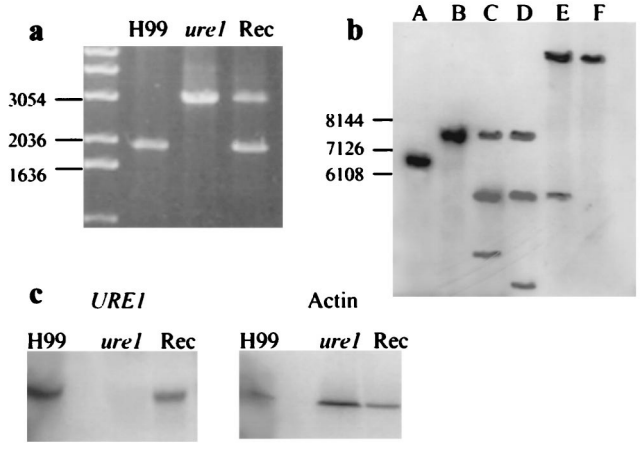

FIG. 3. (a) Agarose gel of PCR products using primers Bgl5 and Bgl3 (Fig. 1) and genomic DNA from strain H99 and the *ure1* and *ure1*+*URE1*-1 (Rec) strains as the template. The amplicon of the native *URE1* fragment from H99 is 1,954 bp, whereas the amplified fragment from the *ure1* strain is 3,090 bp. The displacement of the *ure1* band suggests that the native *URE1* was disrupted. The amplicons from the *ure1*+*URE1*-1 strain demonstrate the disrupted native locus as well as a band corresponding to the wild-type copy of *URE1* used to reconstitute the strain to urease positivity. Size standards in base pairs are indicated to the left of the gel. (b) Southern blot of *Bam*HI/*Eco*RI-digested genomic DNA from H99 (A), the *ure1* strain (B), the *ure1*+*URE1*-1 strain (C), and the *ure1*+*URE1*-2 strain (D) and of *Bam*HI-digested genomic DNA from the *ure1*+*URE1*-1 strain (E) and the *ure1*+*URE1*-2 strain (F). The blot was probed with a labeled *Eco*RI-*Bam*HI genomic fragment containing a portion of *URE1* (Fig. 1A). The hybridizing band seen in the *ure1* strain is displaced compared to that of H99 suggesting replacement of the native urease gene in the *ure1* strain with the disruption construct. The hybridization patterns in the two reconstituted strains demonstrate the presence of the disrupted urease gene as well as copies of the reconstitution construct that have integrated into the genome at different locations. Size standards in base pairs are indicated to the left of the gel. (c) Northern blots of total RNA obtained from H99, the *ure1* strain, and the *ure1*+*URE1*-1 strain (Rec) probed with *URE1* (left) and the actin gene (right).

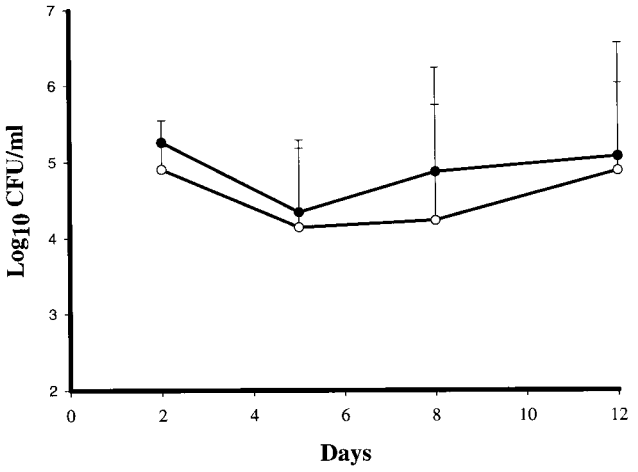

FIG. 4. Log_{10} of the CFU per milliliter (with standard deviations) recovered from the spinal fluid of rabbits infected with H99 (solid circles) and the *ure1* strain (open circles) plotted against time.

PCR products is accounted for by *ADE2* used in the disruption construct being larger than the genomic fragment that it replaced. Southern blots demonstrated displacement of the native urease fragment from the 6.5-kb fragment seen in H99 to the expected 7.6-kb fragment in all 20 of the urease-negative transformants that were checked (example shown in Fig. 3b), and for 2 of the transformants there was evidence for a single integration event within the genome. Northern blots confirmed the absence of *URE1* transcripts in the urease-negative transformants (example shown in Fig. 3c). The twenty urease-negative transformants were found to be grossly identical to strain H99 in terms of the cryptococcal virulence properties of growth rate at 37°C on YPD, growth on minimal media, capsule size after growth in elevated partial CO_2 pressure, and melanization on dopamine agar (data not shown). Thus, urease does not appear to influence these known cryptococcal virulence phenotypes. One of the transformants that appeared to have a single integrative event on the Southern blot was chosen for further study and was designated the *ure1* strain. This strain was reconstituted back to urease positivity using random ectopic integration of the entire *URE1* locus. Two reconstituted strains (the *ure1*+*URE1*-1 and *ure1*+*URE1*-2 strains) demonstrated possession of the intact *URE1* on PCR analysis (Fig. 3a) and Southern blotting (Fig. 3b) and *URE1* transcripts on Northern analysis (Fig. 3c). The hybridization patterns on Southern blotting the two reconstituted strains were different, suggesting that the ectopic integrations were in different locations. The growth rates in YPD broth at 37°C of the two reconstituted strains showed that the colony counts after 24 h of growth were approximately 1/2 log unit lower than the colony counts for H99.

The virulence of the *ure1* strain was compared to that of H99 (*URE1*) in three animal models. The first model was the rabbit meningitis model (26). Equal numbers of *ure1* and *URE1* strain cells were injected into the cisternae magnae of corticosteroid-treated rabbits, and the CSF was serially sampled. Figure 4 is a graph showing the $\log_{10}$ CFU per milliliter of CSF from the infected rabbits plotted against time. The colony counts of the two strains were not significantly different ($P > 0.8$) at any of the observed time points. This suggested that the virulence levels of the two strains were approximately equal in this model over a 12-day period. Fifty colonies of *ure1* strain cells recovered from the rabbits on day 12 were individually tested for

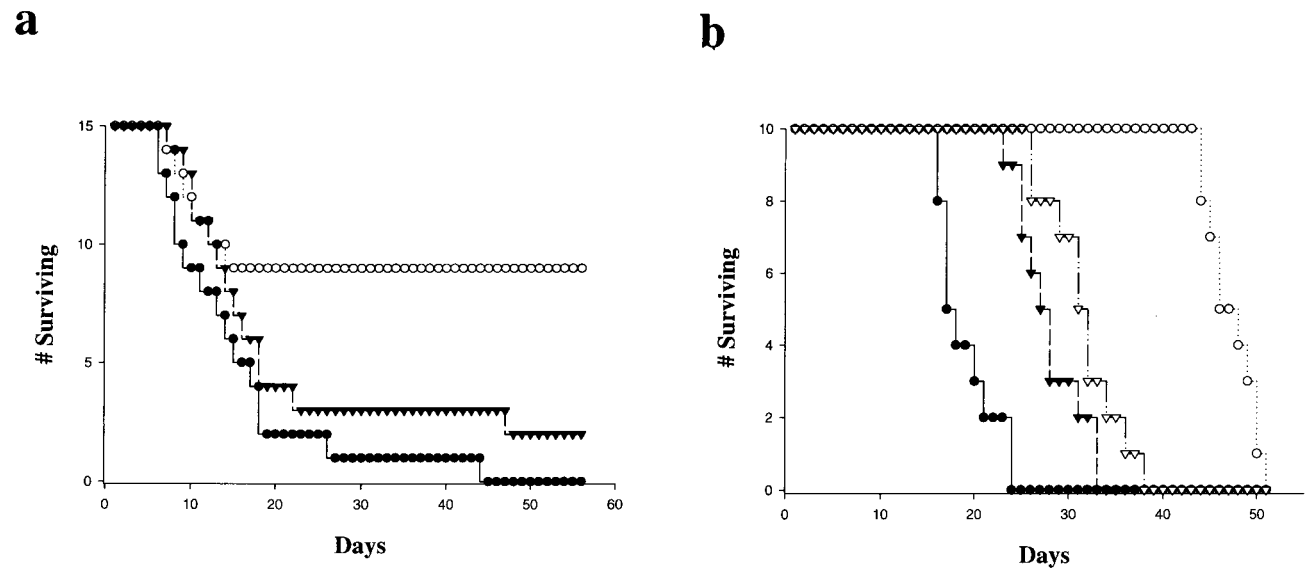

FIG. 5. (a) Number of surviving mice that were infected via intravenous injection with the *ure1* strain (open circles), H99 (solid circles), and the *ure1*+*URE1*-1 strain (inverted triangles) plotted against time. (b) Number of surviving mice that were infected via the inhalational route with the *ure1* strain (open circles), H99 (solid circles), the *ure1*+*URE1*-1 strain (solid inverted triangles), and the *ure1*+*URE1*-2 strain (open inverted triangles) plotted against time.

urease activity on Christensen's agar, and all remained urease negative.

The other models used for virulence assessment were the murine intravenous and inhalational models of cryptococcosis, where the requirements for pathogenicity are likely to be different from those for the rabbit model. In addition to the *ure1* and *URE1* strains, the *ure1* strains complemented back to urease positivity (*ure1*+*URE1*-1 and *ure1*+*URE1*-2 strains) were used to infect mice. In both murine models, the *ure1* strain-infected mice lived significantly longer than the *URE1* strain-infected mice (Fig. 5). In the murine intravenous infection model, the mean survival of the *URE1* strain-infected mice was 15.7 days, compared to 37.9 days for the *ure1* strain-infected mice ($P < 0.003$; Fig. 5a). The *ure1*+*URE1*-1 strain was found to have virulence intermediate to the other two strains when tested in the intravenous model. Mice infected with this strain had a mean survival of 22.4 days, which was still significantly shorter than survival of the *ure1* strain-infected mice ($P = 0.04$). All surviving mice were euthanized after 8 weeks of infection, and the brains were harvested for individual culture. All of the samples grew *C. neoformans*, and there were no significant differences in the quantitative counts between the limited numbers of surviving animals in the two groups. Twenty colonies from each animal were tested for urease activity in Christensen's broth, and every recovered isolate that was tested had the expected urease phenotype.

In the murine inhalational model, the mean survival of the *URE1* strain-infected mice was significantly shorter than that for the *ure1* strain-infected mice (19 versus 47.3 days; $P < 0.001$; Fig. 5b). Two reconstituted strains (*ure1*+*URE1*-1 and *ure1*+*URE1*-2 strains) were also tested in this particular model, and both were associated with significantly decreased survival compared to the *ure1* strain ($P < 0.02$; Fig. 5b). On day 38 of infection, every mouse infected with a urease-positive strain had died, whereas all of the *ure1* strain-infected mice were still alive. There were differences in the appearance of the mice infected via the inhalational route at time of death. The mice infected with the urease-positive strains appeared to succumb with pulmonary distress and had no clinical evidence of hydrocephalus. The *ure1* strain-infected mice displayed no pulmonary distress, but all of these mice ultimately did develop hydrocephalus and wasting that were similar to what was observed in mice infected with *C. neoformans* via the intravenous inoculation route.

DISCUSSION

Because there is precedence in bacteria for urease as an important pathogenic factor, this study explored the contribution of urease to virulence in an experimental fungal infection. We were able to clone and sequence the single-copy *C. neoformans* urease gene and found that the predicted amino acid sequence of the cryptococcal urease gene was highly conserved with those of urease genes from other organisms.

We were able to make urease-negative mutants using targeted gene disruption and found that urease activity does not appear to have an effect on the in vitro assays of the known cryptococcal virulence properties of growth at 37°C, capsule size, and melanin production. We then compared the virulence of a *ure1* mutant with that of wild-type strain H99. The choice of H99 as the comparative strain was based on precedence in the serotype A literature (1, 11, 16, 24) and previous experiments demonstrating that reconstitution of the *ade2* auxotrophic strain M001 to prototrophy with the same genomic locus as that used in this study restores virulence to wild-type levels in both the rabbit (27) and murine models (personal observations). Thus, M001 does not appear to have any unspecified mutations that affect virulence in the two animal models used here.

A comparison of the *ure1* and *URE1* (H99) strains in the corticosteroid-treated rabbit meningitis model revealed no differences in virulence. These results suggest that urease activity is not necessary for *C. neoformans* to survive in the CSF of immunosuppressed rabbits after direct inoculation into this site. The fact that the *ure1* strain was able to survive in the harsh environment of the CSF as well as the wild-type control is further support for the lack of any undefined mutations in the urease-negative mutant that may affect virulence. When the *URE1* and *ure1* strains were tested in two complementary murine models of disseminated infection, there were marked increases in the survival of mice infected with the urease-

negative strain relative to that of mice infected with the wild type. In order to show that these differences in virulence between the two strains were clearly related to the urease gene disruption and not to some unspecified mutation resulting from the transformation process, the urease-negative mutant was reconstituted to urease positivity by complementation with the wild-type gene. The virulence of two independent urease-positive, reconstituted mutants was significantly greater than that of the urease-negative mutant. Both of the reconstituted strains were generated by random, ectopic insertion of the wild-type gene, as has been done in other studies (1, 6, 24). If there is any effect on virulence, a random insertion is much more likely to attenuate the ability to cause disease (34). The fact that virulence was restored to the *ure1* strain by complementation in two independent strains is compelling evidence in support of the claim that the attenuation in virulence seen in the *ure1* strain was due to the lack of urease activity rather than some unspecified mutation in the M001 strain or some mutation that resulted from the gene disruption process. Therefore, it appears that urease activity is a virulence property in the murine model of cryptococcosis. The virulence of the two complemented strains was not restored to the level of H99, and this is most likely explained by the effects of the ectopic insertions that occurred during reconstitution and the fact that these strains had a subtle defect in in vitro growth rates at 37°C.

The finding of the urease-negative strain having virulence comparable to that of the wild-type strain in one model while having attenuation of virulence in another model emphasizes an important concept about virulence studies: virulence genes may only be important at certain stages or sites of infection and may only be important in certain animal models. Contrasting the differences between the two models may give some insight into the virulence defect of the *ure1* strain in the murine models. The rabbit model of meningitis is relevant because it measures the ability of yeast to survive at the most clinically important site of infection. It has been used to quantitate differences in virulence attributed to several cryptococcal genes, including genes for *n*-myristoyl transferase (16), calcineurin A (24), and the G protein gamma subunit (*GPA1*) (1) and *ADE2* (27). On the other hand, the intravenous murine model measures the ability of yeast to disseminate throughout the body after intravenous injection, while the murine inhalational model measures the ability of yeast to systemically disseminate from the lungs. The murine intravenous injection model has been used in *C. neoformans* virulence studies involving genes important in capsule synthesis (4–6), *URA5* (34), the laccase gene (30), and *FKBP12* (11). The rabbit model of meningitis predominantly measures extracellular survival of yeast in the CSF after direct inoculation into this relatively immunologically sequestered site in animals immunocompromised as a result of corticosteroid treatment, and there are no demands made on the yeast to invade the central nervous system (CNS) from the bloodstream. The murine models used in these studies differ from the rabbit model in several important areas: the basal body temperature of the mouse is the same as that of humans (as opposed to the 39°C basal body temperature of rabbits), the inoculated yeast are exposed to an intact host immune system, involvement of the CNS results from hematogenous dissemination, and intracellular parasitism is common. Another difference between the murine and rabbit models concerns the end points for the measurement of virulence. The rabbit model measures survival of the organism at the site of infection, while the murine model, as we used it, measures survival of the host. Therefore, since each model tested for different properties of the pathogen, they can be viewed as complementary. We certainly do not advocate the routine use of multiple animal models in future virulence studies with this fungus, but it is clear that careful attention needs to be paid to the choice of model and to the interpretation of results.

The role of urease in the pathogenesis of cryptococcosis is unknown. In bacterial pathogenesis studies, urease appears to have a role in the alteration of host immune function and also increases microenvironmental pH at the site of infection. *H. pylori* mutants lacking urease activity are phagocytosed more efficiently than the parental strain (20), and urease-containing fractions of *H. pylori* have been shown to activate monocytes (19), to cause the secretion of inflammatory cytokines (10), and to act as chemotactic factors for leukocytes (10, 18). If we assume that the phagocyte-urease interaction is important in the pathogenesis of cryptococcosis, the differences in the virulence of the *ure1* strain in the animal models can be explained by the fact that the CNS rabbit model has a profound CSF leukopenia and likely relies less on phagocytic killing than the murine intravenous model. We also observed a significant reduction in the numbers of elicited immune defense cells in the lungs of the mice infected with the urease-negative strain (personal observations). Studies examining the immune response to the urease-negative *C. neoformans* mutants by in vivo quantitation of cytokines and infecting immunodeficient mice may help to determine the mechanisms through which urease contributes to pathogenesis, and we plan to perform these studies in the future. The ability of urease to increase microenvironmental pH, such as that within a phagolysosome, probably does not play a significant role in the pathogenesis of cryptococcosis. A recent study using human phagocytes in vitro demonstrated that the phagosomal pH remained relatively constant after ingestion of cryptococci (15).

There were differences noted in the appearance of the inhalationally infected mice at the time of death that were related to the infecting strain. Mice infected with the wild-type and reconstituted strains displayed respiratory distress at the time of death, compared to the mice infected with the urease-negative strain, which succumbed with hydrocephalus and wasting. We assume that these differences are due to the ability of the urease-positive strains to cause an acute pneumonitis that is capable of killing the animal before significant brain involvement occurs. It appears as if the urease-negative strain was not able to cause such a significant pneumonitis as to take the life of the host, but the pathogen was able to disseminate to the brain and cause a meningoencephalitis that resulted in hydrocephalus and death. In order to support the assumption that the urease-negative strain has compromised virulence in the lung compared to the urease-positive strains, experiments defining the pulmonary immune response to these strains are under way.

An additional finding in this study was the increased efficiency of gene disruption. Almost 40% of the transformants that were screened in this study were urease negative. This rate is more than seven times higher than that which has been described in other *C. neoformans* gene disruption studies (1, 4–6, 11, 16, 24, 30). The use of a biolistic device for DNA delivery appears to result in higher rates of targeted gene disruption in cryptococci than the other standard transformation method using electroporation, and it is also likely that our use of a serotype D *ADE2* as a selectable marker in the serotype A H99 increases disruption frequency by decreasing homologous integration into this site. However, a possible refinement that was used in this study was to add dideoxynucleotides to the ends of the linearized plasmid constructs used for transformation. The dideoxynucleotides decrease the frequency of plasmid religation, and this appears to increase the rate of

homologous recombination. Blocking the ends of knockout constructs with dideoxynucleotides has been used in other systems to improve knockout efficiency (17), and it is possible that there is a similar effect in *C. neoformans*. Formal studies comparing the effects of dideoxynucleotide treatment of the knockout constructs on transformation efficiencies and disruption rates are in progress.

These data support further investigation into the role of urease in cryptococcosis and other fungal infections. We feel that it is very reasonable to examine the virulence of isogenic, urease-negative mutants of other fungi in order to explore the breadth of this phenotype in pathogenesis. It is unlikely that inhibition of urease activity will, in itself, cure cryptococcosis since the urease-negative mutants are still pathogenic in animal models and because disseminated infections in humans with urease-negative strains have been described (2, 29). Nevertheless, urease does appear to be a component of the composite cryptococcal virulence phenotype, and urease inhibitors or vaccines may be useful in the treatment or prevention of cryptococcosis.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants AI22774 (A.C.), AI13342 (A.C.), AI01334 (G.M.C.), AI44975 (J.R.P.), and AI28388 (J.R.P.) from the National Institute of Allergy and Infectious Diseases and as part of the Veterans Affairs Research Center on AIDS and HIV infection. A.C. was a recipient of the Burroughs Wellcome Fund Experimental Therapeutics Award.

We acknowledge John McCusker for his suggestions regarding the use of the dideoxynucleotides and Joseph Heitman for his support and helpful suggestions.

REFERENCES

1. **Alspaugh, J. A., J. R. Perfect, and J. Heitman.** 1997. *Cryptococcus neoformans* mating and virulence are regulated by the G-protein gamma subunit *GPA1* and cAMP. Genes Dev. **11:**3206–3217.
2. **Bava, A. J., R. Negroni, and M. Bianchi.** 1993. Cryptococcosis produced by a urease negative strain of *Cryptococcus neoformans*. J. Med. Vet. Mycol. **31:**87–89.
3. **Canteros, C. E., L. Rodero, M. C. Rivas, and G. Davel.** 1996. A rapid urease test for presumptive identification of *Cryptococcus neoformans*. Mycopathologia **136:**21–23.
4. **Chang, Y. C., and K. J. Kwon-Chung.** 1994. Complementation of a capsule deficiency mutation of *Cryptococcus neoformans* restores its virulence. Mol. Cell. Biol. **14:**4912–4919.
5. **Chang, Y. C., L. A. Penoyer, and K. J. Kwon-Chung.** 1996. The second capsule gene of *Cryptococcus neoformans*, Cap64, is essential for virulence. Infect. Immun. **64:**1977–1983.
6. **Chang, Y. C., and K. J. Kwon-Chung.** 1998. Isolation of the third capsule-associated gene, CAP60, required for virulence in *Cryptococcus neoformans*. Infect. Immun. **66:**2230–2236.
7. **Cole, G. T.** 1997. Ammonia production by *Coccidioides immitis* and its possible significance to the host-fungus interplay, p. 247–263. *In* H. van den Bossche, D. A. Stevens, and F. C. Odds (ed.), Host-fungus interplay. National Foundation for Infectious Diseases, Bethesda, Md.
8. **Cox, G. M., C. Dykstra, T. H. Rude, and J. R. Perfect.** 1995. *Cryptococcus neoformans* actin gene: characterization and its use as a phylogenetic marker. J. Med. Vet. Mycol. **33:**261–266.
9. **Cox, G. M., D. L. Toffaletti, and J. R. Perfect.** 1996. Dominant selection system for use in *Cryptococcus neoformans*. J. Med. Vet. Mycol. **34:**385–391.
10. **Craig, P. M., M. C. Territo, W. E. Karnes, and J. H. Walsh.** 1992. *Helicobacter pylori* secretes a chemotactic factor for monocytes and neutrophils. Gut **33:**1020–1023.
11. **Cruz, M. C., L. Cavallo, J. M. Gorlach, G. M. Cox, J. R. Perfect, M. E. Cardenas, and J. Heitman.** 1999. Rapamycin antifungal action is mediated via conserved complexes with FKBP12 and TOR kinase homologs in *Cryptococcus neoformans*. Mol. Cell. Biol. **19:**4101–4112.
12. **Eaton, K., C. Brooks, D. Morgan, and S. Krakowka.** 1991. Essential role of urease in pathogenesis of gastritis induced by *Helicobacter pylori* in gnotobiotic piglets. Infect. Immun. **59:**2470–2475.
13. **Granger, D. L., J. R. Perfect, and D. T. Durack.** 1985. Virulence of *Cryptococcus neoformans*: regulation of capsule synthesis by carbon dioxide. J. Clin. Investig. **76:**508–516.
14. **Jones, B., C. V. Lockatell, D. Johnson, J. Warren, and H. L. T. Mobley.** 1990. Construction of a urease-negative mutant of *Proteus mirabilis*: analysis of virulence in a mouse model of ascending urinary tract infection. Infect. Immun. **58:**1120–1123.
15. **Levitz, S. M., S. Nong, K. F. Seetoo, T. S. Harrison, R. A. Speizer, and E. R. Simons.** 1999. *Cryptococcus neoformans* resides in an acidic phagolysosome of human macrophages. Infect. Immun. **67:**885–890.
16. **Lodge, J. K., E. Jackson-Machelski, D. L. Toffaletti, J. R. Perfect, and J. I. Gordon.** 1994. Targeted gene replacement demonstrates that myristoyl CoA: protein N-myristoyl-transferase is essential for the viability of *Cryptococcus neoformans*. Proc. Natl. Acad. Sci. USA **91:**12008–12012.
17. **Mahoney, N. S., T. Hampton, R. Vidaver, and D. Ratner.** 1997. Blocking the ends of transforming DNA enhances gene targeting in *Dictyostelium*. Gene **203:**33–41.
18. **Mai, U. E. H., G. I. Perez-Perez, J. B. Allen, S. M. Wahl, M. J. Blaser, and P. D. Smith.** 1992. Surface proteins from *Helicobacter pylori* exhibit chemotactic activity for human leukocytes and are present in gastric mucosa. J. Exp. Med. **175:**517–525.
19. **Mai, U. E. H., G. I. Perez-Perez, L. M. Wahl, S. M. Wahl, M. J. Blaser, and P. D. Smith.** 1991. Soluble surface proteins from *Helicobacter pylori* activate monocytes/macrophages by lipopolysaccharide-independent mechanism. J. Clin. Investig. **87:**894–900.
20. **Makristathis, A., E. Rokita, A. Labigne, B. Willinger, M. L. Rotter, and A. M. Hirschl.** 1998. Highly significant role of *Helicobacter pylori* urease in phagocytosis and production of oxygen metabolites by human granulocytes. J. Infect. Dis. **177:**803–806.
21. **Mitchell, T. G., and J. R. Perfect.** 1995. Cryptococcosis in the era of AIDS 100 years after the discovery of *Cryptococcus neoformans*. Clin. Microbiol. Rev. **8:**515–548.
22. **Mobley, H. L. T.** 1996. The role of *Helicobacter pylori* urease in the pathogenesis of gastritis and peptic ulceration. Aliment. Pharmacol. Ther. **10**(Suppl. 1)**:**57–64.
23. **Murakami, M., K. Asagoe, H. Dekigai, S. Kusaka, H. Saita, and T. Kita.** 1995. Products of neutrophil metabolism increase ammonia-induced gastric mucosal damage. Dig. Dis. Sci. **40:**268–273.
24. **Odom, A., S. Muir, E. Lim, D. L. Toffaletti, J. R. Perfect, and J. Heitman.** 1997. Calcineurin is required for virulence of *Cryptococcus neoformans*. EMBO J. **16:**2576–2589.
25. **Park, I.-S., and R. P. Hausinger.** 1993. Site-directed mutagenesis of *Klebsiella aerogenes* urease: identification of histidine residues that appear to function in nickel ligation, substrate binding, and catalysis. Protein Sci. **2:**1034–1041.
26. **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.
27. **Perfect, J. R., D. L. Toffaletti, and T. H. Rude.** 1993. The gene encoding phosphoribosylaminoimidazole carboxylase (*ADE2*) is essential for growth of *Cryptococcus neoformans* in cerebrospinal fluid. Infect. Immun. **61:**4446–4451.
28. **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.
29. **Ruane, P. J., L. J. Walker, and W. L. George.** 1988. Disseminated infection caused by urease-negative *Cryptococcus neoformans*. J. Clin. Microbiol. **26:**2224–2226.
30. **Salas, S. D., J. E. Bennett, K. J. Kwon-Chung, J. R. Perfect, and P. R. Williamson.** 1996. Effect of the laccase gene, CNLAC1, on virulence of *Cryptococcus neoformans*. J. Exp. Med. **184:**377–386.
31. **Tange, Y., and Y. Niwa.** 1997. Identification of the ure1+ gene encoding urease in fission yeast. Curr. Genet. **32:**244–246.
32. **Toffaletti, D. L., T. H. Rude, S. A. Johnston, D. T. Durack, and J. R. Perfect.** 1993. Gene transfer in *Cryptococcus neoformans* by use of biolistic delivery of DNA. J. Bacteriol. **175:**1405–1411.
33. **Tsuda, M., M. Karita, M. G. Morsted, K. Okita, and T. Nakasaki.** 1994. A urease-negative mutant of *Helicobacter pylori* constructed by allelic exchange mutagenesis lacks the ability to colonize the nude mouse stomach. Infect. Immun. **62:**3586–3589.
34. **Varma, A., J. C. Edman, and K. J. Kwon-Chung.** 1992. Molecular and genetic analysis of *URA5* transformants of *Cryptococcus neoformans*. Infect. Immun. **60:**1101–1108.
35. **Yu, J.-J., S. L. Smithson, P. W. Thomas, T. N. Kirkland, and G. T. Cole.** 1997. Isolation and characterization of the urease gene (*URE*) from the pathogenic fungus *Coccidioides immitis*. Gene **198:**387–391.
36. **Zimmer, B. L., and G. D. Roberts.** 1979. Rapid selective urease test for presumptive identification of *Cryptococcus neoformans*. J. Clin. Microbiol. **10:**380–381.

Editor: T. R. Kozel