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First report of urease activity in the novel systemic fungal pathogen *Emergomyces africanus*: a comparison with the neurotrope *Cryptococcus neoformans*

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Running title: Urease of *Cryptococcus neoformans* and *Emergomyces africanus*

ABSTRACT

Cryptococcus neoformans is an opportunistic pathogen responsible for the AIDS-defining illness, cryptococcal meningitis. During the disease process, entry of

cryptococcal cells into the brain is facilitated by virulence factors that include urease enzyme activity. A novel species of an *Emmonsia*-like fungus, recently named *Emergomyces africanus*, was identified as a cause of disseminated mycosis in HIV-infected persons in South Africa. However, in contrast to *C. neoformans*, the enzymes produced by this fungus, some of which may be involved in pathogenesis, have not been described. Using a clinical isolate of *C. neoformans* as a reference, the study aim was to confirm, characterize and quantify urease activity in *E. africanus* clinical isolates. Urease activity was tested using Christensen's urea agar, after which the presence of a urease gene in the genome of *E. africanus* was confirmed using gene sequence analysis. Subsequent evaluation of colorimetric enzyme assay data, using Michaelis-Menten enzyme kinetics, revealed similarities between the substrate affinity of the urease enzyme produced by *E. africanus* (K_m ca. 26.0 mM) and that of *C. neoformans* (K_m ca. 20.6 mM). However, the addition of 2.5 g/l urea to the culture medium stimulated urease activity of *E. africanus*, whereas nutrient limitation notably increased cryptococcal urease activity.
Key words: *Cryptococcus; Emergomyces;* enzyme kinetics; HIV; urease; virulence factor

INTRODUCTION

Recently, novel *Emmonsia*-like fungi have emerged as a cause of human disease across the globe (Kenyon *et al*. 2013; Schwartz *et al*. 2015b). Over the past five years, the number of human cases has escalated drastically with the majority of cases being South African HIV-infected individuals (Schwartz *et al*. 2015b). The *Emmonsia*-like fungi that primarily cause AIDS-related mycoses are characterized by small yeast-like cells *in vivo* (Schwartz *et al.* 2015b) and have recently been placed in a novel genus, *Emergomyces* (Dukik *et al*. 2017). The predominant species that causes disease in South Africa has been named *Emergomyces africanus* Dukik, Kenyon, Govender *et* de Hoog (Dukik *et al*. 2017) and is associated with mortality rates of around 48% (Schwartz *et al*. 2015b).

Members of the novel genus *Emergomyces* (including *E. africanus* and *Emergomyces pasteurianus,* previously called *Emmonsia pasteuriana*) (Dukik *et al*. 2017) are classified as thermally dimorphic due to their ability to undergo a

morphological transition from a mycelial phase (Fig. 1a, b, c and d) at 26 ºC to a yeast-like phase (Fig. 1e and f) at 37 ºC (Kenyon *et al*. 2013). Although the primary environmental source of *E. africanus* is unknown, its initial portal of entry is presumed to be inhalational and the main clinical manifestation of disease is the presence of widespread skin lesions (Schwartz *et al*. 2015a). Early clinical data, however, suggests that virtually all organ systems can be affected (Kenyon *et al*. 2013; Schwartz *et al*. 2015a; Schwartz *et al*. 2015b).

Clinical studies have established that *Emergomyces africanus* is an opportunistic pathogen that primarily causes an AIDS-related mycosis, as all patients diagnosed to date have been profoundly immunocompromised (Kenyon *et al.* 2013; Schwartz *et al*. 2015a). This is similar to the neurotropic basidiomycetous yeast *Cryptococcus neoformans*, which is known for its ability to cause severe disseminated disease in immunocompromised individuals, particularly HIV-infected patients (Meyohas *et al*. 1995; Wang, Aisen and Casadevall 1995; Kambugu *et al*. 2008; Park *et al*. 2009).

Cryptococcus neoformans, syn. *Cryptococcus neoformans* var. *grubii* (Hagen *et al*. 2015), is the primary causative agent of cryptococcosis in persons with advanced HIV infection (Meyer *et al*. 2011; Cogliati 2013; Kassi *et al*. 2016). Cryptococcal infection commences in the lungs, following the inhalation of basidiospores or yeast cells into the alveolar spaces of the human host (Giles *et al*. 2009; Velagapudi *et al*. 2009; Nielsen and Kwon-Chung 2011). From there, *C. neoformans* can disseminate to the central nervous system (CNS) (Chang *et al*. 2004; Dromer and Levitz 2011). More specifically, cryptococcal cells are ingested by blood phagocytes, which act as a vehicle for fungal dissemination to the CNS (Santangelo *et al*. 2004; Charlier *et al*. 2009; Sorrell *et al*. 2016; Santiago-Tirado *et al*. 2017). The AIDS-defining illness, cryptococcal meningitis (Fries and Cox 2011) is one of the leading causes of mortality amongst HIV-positive individuals, particularly in the developing world (Lawn *et al*. 2008; Jarvis *et al*. 2014).

Upon entry into the CNS, *C. neoformans* may employ multiple virulence factors to assist in its migration across the blood-brain barrier (BBB; Dromer and Levitz 2011; Liu, Perlin and Xue 2012). Virulence factors that have been linked to *C. neoformans* brain invasion include the presence of mating types with varying levels of virulence (Nielsen *et al*. 2005), capsule formation (Charlier *et al*. 2005), phospholipase B

(Maruvada *et al*. 2012) and laccase secretion (Qiu *et al*. 2012), as well as the production of urease (Shi *et al*. 2010).

The nickel-requiring enzyme urease is responsible for the enzymatic hydrolysis of urea into ammonia and carbonic acid (Mobley, Island and Hausinger 1995). This enzyme, recently described as a “niche factor”, is produced by numerous commensal microorganisms of the human body (Mora and Arioli 2014). However, increasing evidence exists for its association with the disease progression of many microbial pathogens, in which case it is referred to as a virulence factor (Rutherford 2014). Urease was first implicated in the pathogenesis of *C. neoformans* during a study conducted on experimental cryptococcosis in murine models (Cox *et al*. 2000). It was found that mice infected with a urease-negative *C. neoformans* mutant showed increased survival compared to mice infected with the wild-type strain. Subsequent research revealed that urease activity enhances microvascular sequestration of *C. neoformans* cells in the CNS, thus facilitating CNS invasion (Olszewski *et al*. 2004). Additionally, a more recent study found that urease aids in the transmigration of cryptococcal cells across the BBB (Shi *et al*. 2010). The inoculation of mice with a *ureI* mutant strain of *C. neoformans* resulted in a 3-fold decrease in the number of transmigration sites into the brain parenchyma. A further two studies demonstrated that *ureI* mutants of *C. neoformans* were unable to cross the BBB as effectively as wild-type strains (Singh *et al*. 2013; Santiago-Tirado *et al*. 2017). In addition to its role at the BBB, cryptococcal urease was found to stimulate immature dendritic cell accumulation and an ineffective T2 immune response in the lungs of infected mice (Osterholzer *et al*. 2009). Thus, urease expression by *C. neoformans* resulted in an impaired clearance of pulmonary cryptococcal cells, leading to an increased fungal burden at the primary site of infection.

The abovementioned studies therefore highlight the important role of cryptococcal urease in host colonization. However, the specific mechanism by which the urease of *C. neoformans* facilitates this process is still unknown. It was suggested that within the CNS, urease-derived ammonia may result in endothelial cell toxicity, which compromises the barrier function of the BBB endothelial cells (Olszewski *et al*. 2004). Interestingly, a study conducted on a closely related species, i.e. *Cryptococcus gattii*, revealed that the Ure1 protein contributes to virulence via ureolytic-independent urease activity (Feder *et al*. 2015). The authors therefore

highlighted that in addition to the ureolytic activity of urease, the inhibition of urea-independent mechanism/s of urease toxicity should be studied so that potential therapeutic tools can be identified. It is interesting to note that the use of urease as a therapeutic agent to prevent infection was studied for an unrelated urease-producing pathogenic fungus, *Coccidioides immitis* (Li *et al*. 2001). Recombinant urease and urease DNA of *C. immitis* were evaluated as possible vaccine candidates for the treatment of coccidioidomycosis. The administration of recombinant urease or urease DNA in mice, prior to inoculation with a lethal dose of *C. immitis*, was found to elicit an immunoprotective response.

To date, the ability of *E. africanus* to produce urease has not been determined. Using a clinical isolate of *C. neoformans* as a urease-positive reference strain, the aim of this study was therefore to confirm, characterize and quantify urease activity in *E. africanus*. To achieve this, we first screened clinical isolates of *E. africanus* for urease production using a conventional plate assay. Thereafter, polymerase chain reaction (PCR) amplification was used to confirm the presence of the urease gene within the genome of *E. africanus*. The substrate affinity of urease within crude protein extracts of a representative strain was then determined using Michaelis-Menten enzyme kinetics. In addition, we investigated the effect of different nutrient conditions on the urease activity of crude protein extracts.

MATERIALS AND METHODS

Strains and maintenance

The clinical strains, and their origins, used in this study are listed in Table 1. Both *E. africanus* isolates were maintained in a mycelial phase (Fig. 1a, b and c) by periodic transfer to brain heart infusion (BHI) broth (pH 7.4; Merck, Darmstadt, Germany) supplemented with 2 % (w/v) bacteriological agar (Merck) and incubated at 26 ºC. The yeasts *Candida albicans* and *C. neoformans* were maintained at 26 ºC by periodic transfer to yeast extract-malt extract (YM) broth (pH 5; Yarrow 1998).

Selection of urease-positive strains

Strains listed in Table 1 were screened for urease activity on Christensen's urea agar according to the method described by Kurtzman *et al*. (2011). *Candida albicans* CAB 397 was included as a negative control. For both *E. africanus* strains, inocula

were obtained from two-week-old cultures grown on BHI agar plates at 26 ºC. Inocula of *C. neoformans* CAB 1055 and *C. albicans* CAB 397 were obtained from 5-day-old cultures grown at 26 ºC on YM agar plates. Inoculated agar plates were incubated at 26 ºC with daily inspection for five days. Urease activity was evidenced by the presence of a deep pink colour, which was compared to the reaction obtained on control plates without urea.

Identification of urease genes and primer design

Predicted gene and protein sequences of a strain belonging to *E. africanus* were obtained from the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands (Dukik *et al*. 2017). The predicted proteins were then compared and aligned to protein sequences of known fungal ureases, obtained from the UniProt database (http://www.uniprot.org/), using BLAST+ (v2.2.31) with a minimum E-value of 1.0e-8 (Altschul *et al*. 1990). One of the predicted protein sequences had the greatest sequence identity (86 %) with the urease of *Blastomyces dermatiditis* ATCC 26199 (UniProt accession number T5BDP5) and was thus retained as a putative urease of *E. africanus*. The CLC Main Workbench 7.6.4 (CLC Bio) was used to conduct a multiple sequence alignment where the putative urease amino acid sequence was aligned with other known urease protein sequences available on Genbank (http://www.ncbi.nlm.nih.gov/genbank). Thereafter, a phylogenetic tree was constructed using the maximum likelihood approach with the JTT matrix and 100 bootstrap replicates (Fig. S1 in the Supplementary Material). In addition, the same software was used to predict the open reading frame (ORF) within the gene encoding the putative protein. Thereafter, primers were designed to amplify the identified ORF within the genomes of the two *E. africanus* strains used in this study. The nucleotide sequences of the forward and reverse primers were 5'-CGATCGAATGAAATCATATGGATACCG-3' and 5'-CGTACATGTCGTTGCGGTTGTTCC-3', respectively. These primers were produced by Inqaba Biotechnical Industries (Pretoria, Gauteng, RSA).

DNA extraction and PCR amplification

Emergomyces africanus JX398291 and *E. africanus* JX398293 were cultured for two weeks on BHI agar plates at 26 ºC. Thereafter, genomic DNA was extracted using the ZR Fungal/Bacterial DNA MiniPrep Kit (Zymo Research, Orange County, CA,

USA) according to the manufacturer's protocol. PCR amplification of the urease gene was then performed using the abovementioned primer set. The reaction components consisted of 1 µl genomic DNA, 1 µl of each primer (10 µM), 10 µl 2x KAPA Taq ReadyMix (KAPA biosystems, Massachusetts, USA) and milliQ water to give a volume of 20 µl. The amplification process was carried out in an Applied Biosystems 2720 thermal cycler with an initial denaturation step at 95 °C for 5 min, 30 cycles of denaturation at 95 °C for 30 sec, annealing at 58 °C for 30 sec, extension at 72 °C for 45 sec and a final extension step at 72 °C for 7 min. Thereafter, urease nucleotide sequences were obtained using the Applied Biosystems ABI3130xl genetic analyser and compared to known nucleotide sequences available on GenBank using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast).

Inoculum preparation for urease assays

Inoculum preparation differed depending on the fungal species. Inocula of *C. neoformans* CAB 1055 were obtained from five-day-old yeast cultures on YM agar plates incubated at 26 ºC, while yeast-like growth of *E. africanus* JX398293 was first obtained by repeated transfers to fresh BHI agar plates at 37 ºC (Fig. 1e). In the latter case, a loop-full of the yeast-like growth was then aseptically transferred to a test tube containing 5 ml of BHI broth. The inoculated tube was subsequently incubated at 37 ºC on a TC-7 tissue culture roller drum (60 rpm; New Brunswick Scientific Co. Inc.) and the yeast-like phase (Fig. 1f) was maintained by repeatedly transferring cells to test tubes containing fresh BHI broth. This yeast-like growth of *E. africanus* JX398293, obtained after three days of incubation at 37 ºC in BHI broth, served as inoculum for subsequent experiments.

Protein extract preparation for urease assays

A suspension of *E. africanus* JX398293 yeast-like cells was used as inoculum for two conical flasks (1 L) each containing 100 ml BHI broth. Following inoculation, flasks were incubated at 37 ºC for three days on an orbital shaker (Model G53, New Brunswick Scientific Co. Inc., Edison, NJ, USA) set at 200 revolutions per minute (rpm). For *C. neoformans* CAB 1055, four conical flasks (1 L) were each inoculated with a loop-full of cells obtained from cultures grown on YM agar plates. All four flasks were subsequently incubated with shaking (200 rpm) at 37 ºC for 16 hours.

After the respective incubation periods, crude protein preparation was conducted according to a method described by Moller *et al*. (2016) with modifications. Cell suspensions were centrifuged at 10,000 g for 10 min at 20 ºC and resulting pellets were washed three times with distilled water (dH_2O). Pellets were then washed a final time (7000 g, 10 min, 4 ºC) in 400 µl chilled lysis buffer consisting of 40 mM Tris-HCl (pH 8), 1 mM EDTA (pH 8), 10 % glycerol and 1 mM phenylmethylsulfonyl fluoride (PMSF). Subsequently, each pellet was re-suspended in 400 µl chilled lysis buffer and transferred to a 2 ml screw-cap microcentrifuge tube containing 0.5 ml acid-washed glass beads (400-600 µm; Sigma-Aldrich, St. Louis, MO, USA). Cells were then disrupted by vigorous mixing on a Vortex Genie 2 (Scientific Industries, Bohemia, NY, USA) for 30 sec, followed by 1 min cooling on ice, for a total of seven cycles, before the removal of cell debris using centrifugation (13,000 g, 15 min, 4ºC). The protein content of the resulting supernatant was then measured using the Bradford method conducted with the BioRad protein assay kit II (BioRad Laboratories, Hercules, CA, USA). Following quantification, protein extract was assayed immediately for urease activity.

Urease activity assay

Protein extract was assayed for urease activity using urea concentrations ranging from 0.625 to 30 g/l. In brief, an aliquot of protein extract, containing 100 µg protein, was added to a spectrophotometer tube containing a specific concentration of urea (Sigma-Aldrich), 50 mM HEPES buffer (pH 7.4; Sigma-Aldrich) and dH_2O to a final volume of 1 ml. Thereafter, tubes were incubated at 37 ºC for 60 min on a G53 orbital shaker (200 rpm). Ammonia production within the reaction mixture was measured after 0, 10, 20, 30, 40 and 60 min using the phenol-hypochlorite assay of Weatherburn (1967) with slight modifications. At the respective time interval, 50 µl of reaction mixture was added to a spectrophotometer tube containing 500 µl of reagent A (10 g/l phenol [Merck] and 50 mg/l sodium nitroprusside [Merck]). An equal volume (500 µl) of reagent B (5 g/l sodium hydroxide [Merck] and 8.4 ml/l sodium hypochlorite [commercial bleach]) was subsequently added and the reaction mixture was mixed well before incubation at 37 ºC for 30 min. During this time, any ammonia present within the sample was allowed to react with both reagents, producing a blue colour. Absorbance at 625 nm was then measured using a SmartSpec Plus spectrophotometer (BioRad Laboratories Ltd., Johannesburg, RSA).

Reaction mixture without urea served as the blank and reaction mixture lacking crude extract was used as a control. Absorbance values were converted to nanomoles (nmol) of ammonia (Table S1 and S2 in the Supplementary Material) using an ammonium chloride standard curve consisting of concentrations up to 1000 µM. Thereafter, graphs of ammonia concentration [NH_3], versus time (t), for each urea concentration [S], were plotted on the same axis (data not shown). A linear regression analysis was then conducted to determine the initial reaction velocity (V_i) for each substrate concentration from the slopes of the straight lines that best fitted the plotted data points.

Michaelis-Menten constant (K_m) determination

A non-linear regression analysis, based on the principle of least squares, was used to fit the Michaelis-Menten function to constructed data plots of initial reaction velocities against corresponding urea concentrations. This analysis was performed on the Solver supplement of Microsoft Office Excel, which was used to generate estimates of the Michaelis-Menten enzyme kinetic parameters, V_{max} and K_m, by minimizing the sum of squares of the non-linear function below:

$$V_i = \frac{V_{max}[S]}{K_m + [S]}$$

Effect of different nutrient conditions on urease activity

The urease activity assay described above was conducted on protein extracts obtained from *C. neoformans* CAB 1055 and *E. africanus* JX398293 grown in BHI broth with and without supplemented urea. In addition, the effect of nutrient starvation in the presence and absence of urea was investigated by conducting the same assay on protein extracts obtained from cells that were transferred from BHI broth to a nutrient-limited medium. All experiments were conducted in triplicate. If an insufficient amount of protein extract was obtained for a biological repeat, multiple flasks were inoculated and the resulting lysate pooled.

For *C. neoformans* CAB 1055, a loop-full of growth from a YM agar plate served to inoculate each of two conical flasks (1 L), each containing 100 ml BHI broth, one of which was supplemented with 2.5 g/l urea. Following inoculation, both flasks were incubated with shaking (200 rpm) at 37 ºC for 16 hours. Similarly, a suspension of *E. africanus* JX398293 yeast-like cells was used to inoculate an additional two flasks, containing the same two media as described above. Flasks inoculated with *E. africanus* JX398293 were subsequently incubated with shaking (200 rpm) at 37 ºC for three days. Once the respective incubation periods of each organism had elapsed, the resulting log-phase cultures were harvested by centrifugation (10,000 g, 10 min, 20 ºC) and washed three times in dH_2O. Thereafter, crude protein was extracted and quantified according to the method described above under the heading “Protein extract preparation”. Urease activity of 50 µg crude protein was assayed immediately using the protocol described under the heading “Urease activity assay”, but with a fixed urea concentration of 20 g/l. An ammonium chloride standard curve was used to calculate nmol ammonia formed in a reaction volume of 1 ml at each time interval. Graphs of nmol ammonia against time were constructed and linear regression analysis was used to determine the initial reaction velocities (nmol ammonia/min).

Using similar techniques as above, the effect of nutrient limitation in the presence and absence of urea was investigated for both *C. neoformans* CAB 1055 and *E. africanus* JX398293. Prepared inoculum of each strain was added to two conical flasks (1 L), each containing 100 ml BHI broth. Flasks inoculated with *C. neoformans* CAB 1055 were incubated with shaking (200 rpm) at 37 ºC for 16 hours, whereas inoculated flasks containing *E. africanus* JX398293 were incubated with shaking (200 rpm) at 37 ºC for three days. After the respective incubation periods, resulting growth was washed via centrifugation at 10,000 g for 10 min at 20 ºC. For each organism, two cell pellets, originating from growth in the two respective flasks each containing 100 ml BHI broth, were individually resuspended in 500 µl dH_2O and transferred to separate 1 L conical flasks, each containing 100 ml of a nutrient-limited medium (pH 6.8; 0.1 % [w/v] glucose, 0.91 % [w/v] monopotassium phosphate and 0.95 % [w/v] dipotassium phosphate), one of which was supplemented with 2.5 g/l urea. Thereafter, the four inoculated flasks were incubated under shaking conditions (200 rpm) at 37 ºC for three hours. Following incubation,

cells from the respective flasks were pelleted by centrifugation (10,000 g; 10 min; 20 ºC) and washed three times in dH_2O, followed by a final wash step (7000 g, 10 min, 4 ºC) in 400 µl chilled lysis buffer. Washed pellets were resuspended in another 400 µl chilled lysis buffer; where after crude protein was extracted and quantified. Urease activity of 50 µg protein was assayed in the same manner as above, again using a urea concentration of 20 g/l. As described previously, the initial reaction velocities (nmol ammonia/min) were calculated using an ammonium chloride standard curve and linear regression analysis.

Lastly, the effect of added nitrogen on *C. neoformans* urease activity was tested using the abovementioned protocol with the following modifications. Yeast cells grown for 16 hours in 100 ml BHI broth were transferred to a 1 L conical flask containing 100 ml of the nutrient-limited medium, supplemented with 4.086 g/l ammonium chloride (Kimix, Cape Town, Western Cape, SA). The resulting nitrogen concentration was similar to that found in yeast nitrogen base without amino acids (Difco, Michigan, USA). The inoculated flask was subsequently incubated for three hours at 37 ºC under shaking conditions (200 rpm). Thereafter, crude protein was extracted, quantified and assayed for urease activity using methods described above.

Statistical analyses

All data are expressed as mean ± 1 standard error of the mean. One-way ANOVA was used for the analysis of variance in data sets obtained from the characterization of urease activity in *C. neoformans* CAB 1055 and *E. africanus* JX398293. Furthermore, mean values were compared using Fisher's LSD post hoc test for multiple comparisons with a set significance level of $p < 0.05$. Statistical analyses were performed using the Statistica software package (Version 13, Dell, Round Rock, TX, USA).

RESULTS

Identification of urease-positive strains

All tested strains, except the negative control *C. albicans* CAB 397, produced positive urease reactions on Christensen’s urea agar after five days of incubation (Fig. S2 in the Supplementary Material). In addition, the urease gene of *E. africanus*

was partially amplified from the genomic DNA of the two representative *E. africanus* strains (Genbank accession numbers KY241790 and KY241791). In both cases, the ca. 700 bp amplified region of the urease gene was found to share 86 % sequence identity with the partial urease messenger RNA (mRNA) of *Blastomyces dermatitidis* SLH14081 (Genbank accession number XM_002623809.1). Furthermore, a BLASTx analysis of the amplified region revealed high sequence identities with the N-terminal domain of the structural alpha-subunit of other fungal ureases (Fig. S3 in the Supplementary Material).

Michaelis-Menten enzyme kinetics

A typical hyperbolic curve was obtained when the Michaelis-Menten function was fitted to constructed plots of initial reaction velocities against corresponding urea concentrations (Fig. 2). Furthermore, calculated K_m values of *C. neoformans* CAB 1055 and *E. africanus* JX398293 were ca. 20.6 mM and ca. 26.0 mM, respectively.

Urease activity in BHI broth with and without supplemented urea

Protein extracts of *C. neoformans* CAB 1055 cells cultured in BHI broth had a urease activity of 6.22 ± 0.16 nmol ammonia/min, which increased slightly but insignificantly with urea supplementation (Fig. 3a). In contrast, urease activity in protein extracts of *E. africanus* JX398293 cells increased significantly with the addition of urea from 6.24 ± 0.34 to 7.38 ± 0.37 nmol ammonia/min (p value = 0.02) (Fig. 3b).

Urease activity in a nutrient-limited medium with and without supplemented urea

Similar to the findings obtained when *C. neoformans* CAB 1055 was cultured in BHI broth, urea supplementation of the nutrient-limited medium was found to have no significant effect on cryptococcal urease activity (Fig. 3a). Likewise, the results obtained for the *E. africanus* strain with regard to urea supplementation in the nutrient-limited medium was similar to that obtained with BHI broth. Urease activity in cellular protein extracts of *E. africanus* JX398293 increased significantly with the addition of urea, from 6.19 ± 0.18 to 7.34 ± 0.19 nmol ammonia/min (p value = 0.02) (Fig. 3b).

It must be noted, however, that in contrast to the results obtained with *E. africanus* JX398293 (Fig. 3b), cryptococcal urease activity was found to be significantly higher in cells originating from the nutrient-limited medium compared to those from the BHI broth (Fig. 3a). An activity of 17.6 ± 1.97 nmol ammonia/min was obtained for cryptococcal cells originating from the nutrient-limited medium, which is more than double the enzyme activity of cellular protein extracts from *C. neoformans* CAB 1055 cultured in BHI broth alone (p value < 0.01).

Effect of ammonia addition on *C. neoformans* urease activity in a nutrient-limited medium

The supplementation of a nutrient-limited medium with ammonia did not affect the urease activity of cryptococcal protein extracts (results not shown). An enzyme activity of 18.0 ± 0.63 nmol ammonia/min was obtained, which did not differ significantly from the resulting activities when cryptococcal cells were transferred to an ammonia-free nutrient-limited medium, both in the presence and absence of urea.

DISCUSSION

This study provides strong evidence for the existence of a urease enzyme in *E. africanus*. In addition to the positive urease reaction on Christensen's urea agar, we were able to confirm the presence of a urease gene within the genome of strains representing *E. africanus*. By performing subsequent enzyme assays and applying Michaelis-Menten enzyme kinetics, the substrate affinity of the urease produced by *E. africanus* JX398293 (K_m of ca. 26.0 mM) was found to be similar to that of *C. neoformans* CAB 1055 (K_m of ca. 20.6 mM). The K_m of other fungal ureases is scarcely reported in the literature and the few that are available differ greatly from the values obtained in this study. For example, in a study conducted in 2002, the urease of the pathogenic fungus *Coccidioides immitis* was found to have a K_m of 4.1 mM (Mirbod, Schaller and Cole 2002). In another study, a K_m of 1.03 mM was reported for the urease of *Schizosaccharomyces pombe* (Lubbers *et al*. 1996). Thus, indications are that the ureases of both *C. immitis* and *S. pombe* have a higher affinity for the substrate urea than that of *C. neoformans* and *E. africanus*. In contrast, a vast amount of literature is available on the K_m values of bacterial ureases (Jones and Mobley 1987; Mobley *et al*. 1988; Gatermann, John and Marre 1989; Dunn *et al*. 1990; Clemens, Lee and Horwitz 1995), which have been found to

range from as low as 0.3 mM for *Mycobacterium tuberculosis* (Clemens, Lee and Horwitz 1995) to as high as 60 mM for *Proteus mirabilis* (Jones and Mobley 1987). Therefore, it is evident that the K_m values we obtained in this study are within the range detected for bacterial ureases.

Further experimentation revealed significant changes in the urease activity of *C. neoformans* CAB 1055 and *E. africanus* JX398293 when the cells were subjected to different nutrient conditions. Unlike *C. neoformans* CAB 1055, urea supplementation (2.5 g/l) of both the BHI broth and the nutrient-limited medium was found to significantly increase urease activity in protein extracts of *E. africanus*. Similarly, the presence of urea (ca. 1 g/l) was previously found to induce urease activity of the pathogenic bacterial species *Proteus mirabilis* (Jones and Mobley 1988) and *Providencia stuartii* (Mulrooney *et al*. 1988). As concluded for the abovementioned bacteria, indications are that the urease of *E. africanus* JX398293 is regulated by the presence of urea in different environments.

Microorganisms exposed to high urea concentrations, such as within the urinary tract of animals and the soil environment, commonly possess ureases with large K_m values ranging from 13 to 130 mM (Mobley, Island and Hausinger 1995). Thus, the abovementioned urease K_m value of *E. africanus*, points to a natural habitat associated with either soil or animals. *Emergomyces africanus* is a member of the ascomycetous fungal order Onygenales (Dukik *et al*. 2017) containing many soil dwelling keratinolytic species, the latter indicating association with animals (de Hoog *et al*. 2000). In addition, it is well known that many of the dimorphic Onygenales able to infect animals show urease activity, a rare trait among ascomycetous fungi (Summerbell, Kane and Pincus 1990; Paré *et al*. 1997; Bagagli *et al*. 1998; Roilides *et al*. 1999; de Hoog *et al*. 2000; Brandt *et al*. 2005; Mirbod-Donovan *et al*. 2006; Marin-Felix *et al*. 2015; Muñoz *et al*. 2015).

In contrast to the results obtained with *E. africanus* JX398293, cryptococcal urease activity increased significantly when the yeast was transferred from BHI broth to the nutrient-limited medium. This observed up-regulation was maintained despite supplementation with excess urea and ammonium chloride, respectively. The latter findings are in contrast to that of another study where the urease activity and gene expression of *C. neoformans* H99 were found to be regulated by the available

nitrogen source, including urea (Singh et al. 2013). It must be noted that compared to our study, different growth conditions were used to investigate urease activation in *C. neoformans* H99. Thus, unlike the generally accepted contention that urease expression is regulated by environmental changes in urea concentration, and/or the availability of nitrogen sources such as ammonia (Mobley, Island and Hausinger 1995), neither of these factors seem to impact the urease activity of *C. neoformans* CAB 1055 under the experimental conditions of our study. Additional work should, however, be conducted to determine the effect of a range of nitrogen concentrations on the urease activity of this cryptococcal strain incubated under different experimental conditions. For example, the effect of pH on the urease activity of *C. neoformans* and *E. africanus* was not tested in this study.

It is well-known that other microbial ureases, such as that of *Helicobacter pylori*, may be regulated in response to changes in environmental pH (Li, Chen and Burne 2000; Scott *et al*. 2000; Merrell *et al*. 2003). Typically, pH-regulation occurs under acidic conditions, where urease activity is induced by a low environmental pH. This leads to elevated levels of urease-derived ammonia, which ultimately results in the alkalinisation of the surrounding environment. It should be noted, however, that both the BHI broth and nutrient-limited medium used in our analyses had a pH of ca. 7. Thus, the abovementioned changes in urease activity that were observed for *C. neoformans* and *E. africanus*, under different nutrient conditions, seem not to have been the result of changes in the pH of the media.

In conclusion, this study is the first to confirm urease activity in a representative strain of *E. africanus*. In addition, our results provide evidence for the differential regulation of urease activity between *E. africanus* JX398293 and *C. neoformans* CAB 1055. Similar to that observed for pathogenic bacterial species, urease activity in *E. africanus* was enhanced by the presence of the substrate urea. Unlike *E. africanus* JX398293, urease activity of *C. neoformans* CAB 1055 was found to increase under conditions of nutrient limitation. Since the macrophage is believed to be a nutrient poor environment (Lucas and Lee 2000; Roetzer *et al*. 2010; Seider *et al.* 2014), it is possible that phagocytosis may enhance cryptococcal urease activity during infection of the human host. This, however, may not be the case for *E. africanus*, which has also been observed in blood phagocytes *(*Schwartz *et al*. 2015b). Future investigations should therefore include *in vitro* phagocytic studies in

combination with real-time quantitative PCR to analyse the transcription profile of fungal urease genes within the macrophage.

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Conflict of interest. None declared.

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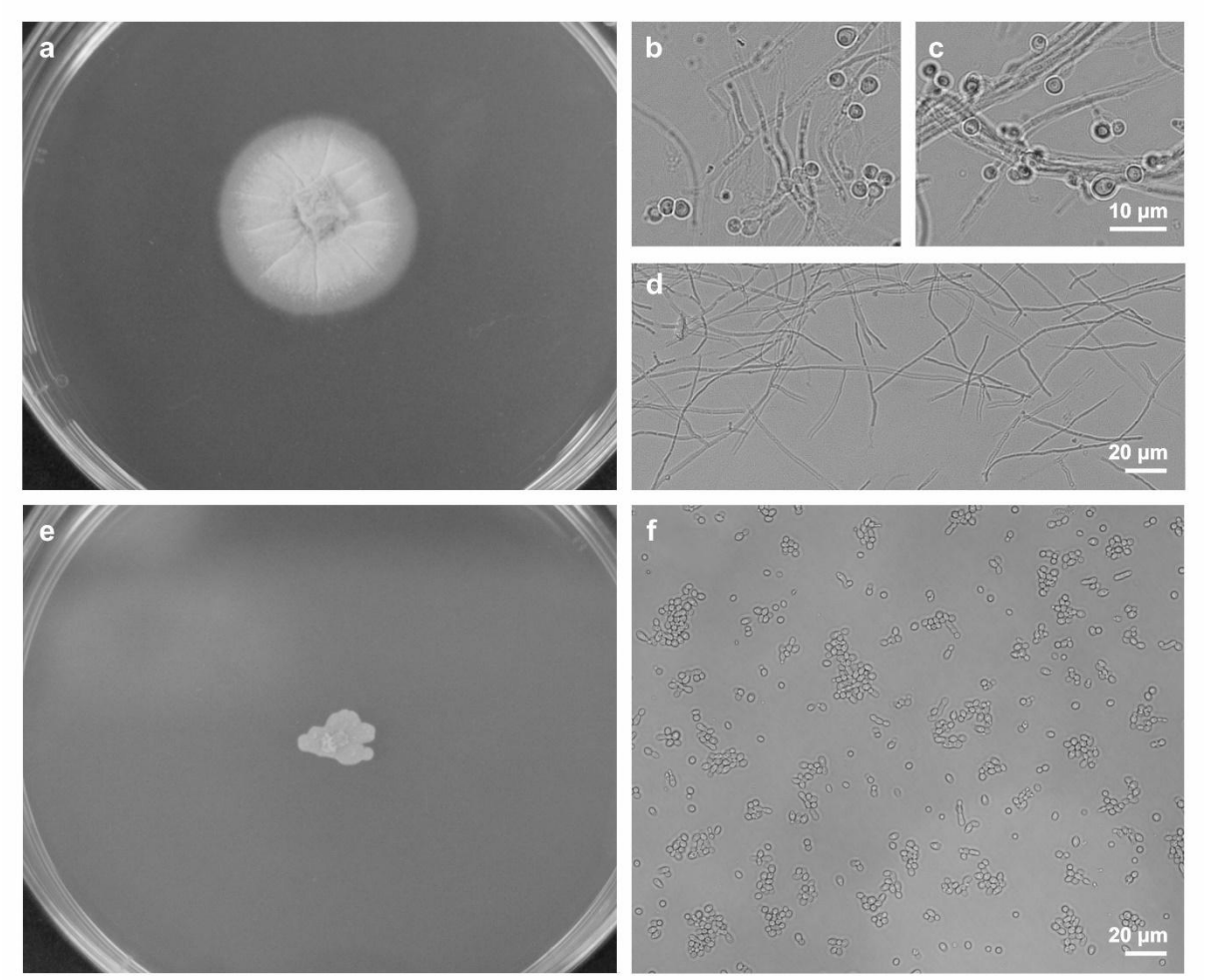

Figure 1. Mycelial and yeast-like phase of *E. africanus* JX398293 cultured at 26 and 37 °C, respectively. Mycelial growth on BHI agar plates incubated at 26 °C for 3 weeks (a). The surface of the fungal colony is cerebriform and powdery. Light microscopy images of hyphal fragments with conidia originating from a 3-week-old colony cultured on BHI agar plates at 26 °C (b and c). Scale bar represents 10 µm. Light microscopy image of hyphal fragments after growth in BHI broth at 26 °C for 5 days (d). Scale bar represents 20 µm. A 9-day-old yeast-like colony obtained by repetitive culturing on BHI agar plates at 37 °C (e). Light microscopy image of yeast-like cells cultured in BHI broth for 5 days (f). Scale bar represents 20 µm. A Nikon eclipse E400 microscope was used to capture all light microscopy images.

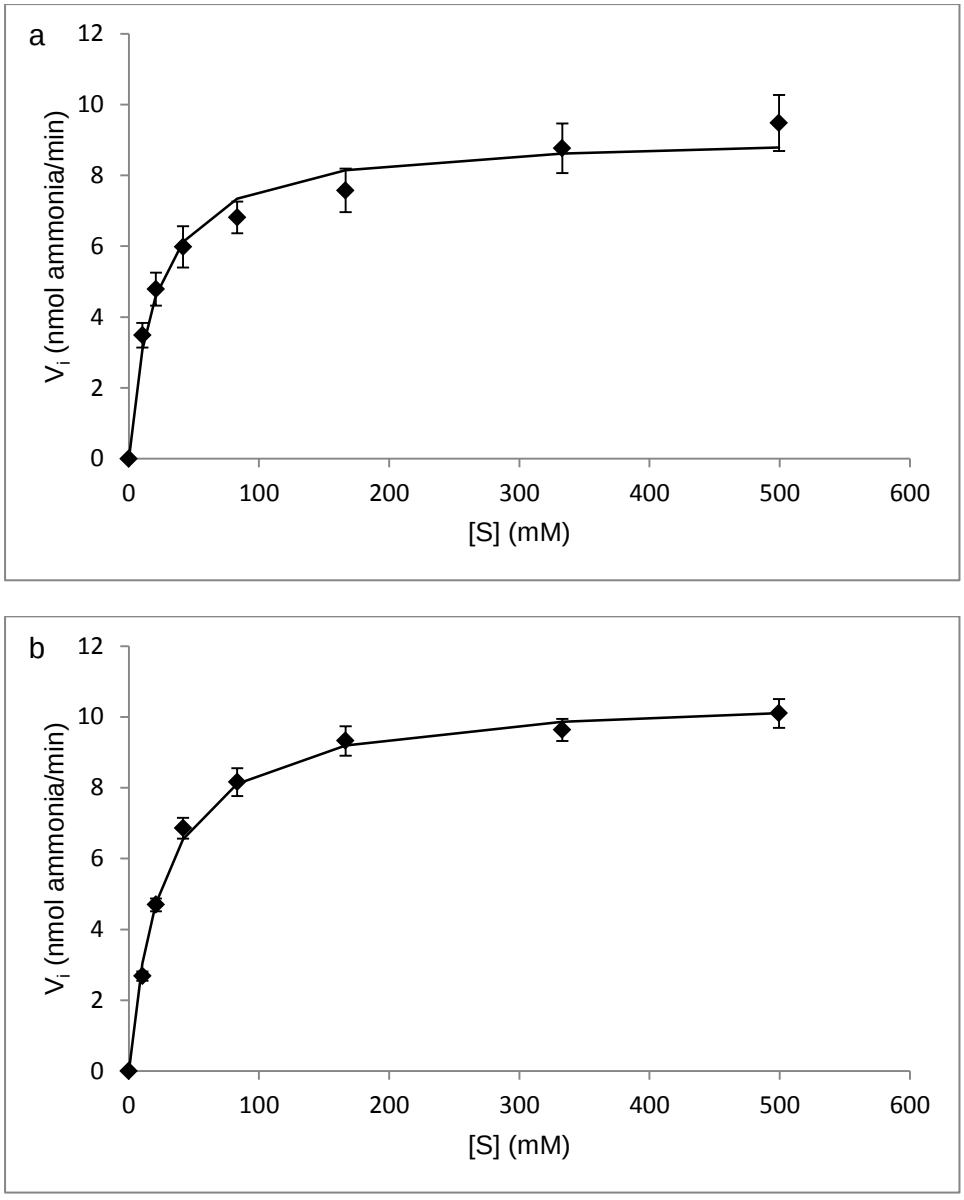

Figure 2. Plots of initial reaction velocity (V_i) versus substrate concentration [S] for *C. neoformans* CAB 1055 (a) and *E. africanus* JX398293 (b). Urease activity in 100 µg crude protein extract was assayed using a range of urea concentrations in a final reaction volume of 1 ml. The initial reaction velocity was calculated for each substrate concentration and expressed as nmol ammonia produced per min. Each data point represents the mean of four repetitions and whiskers indicate standard error.

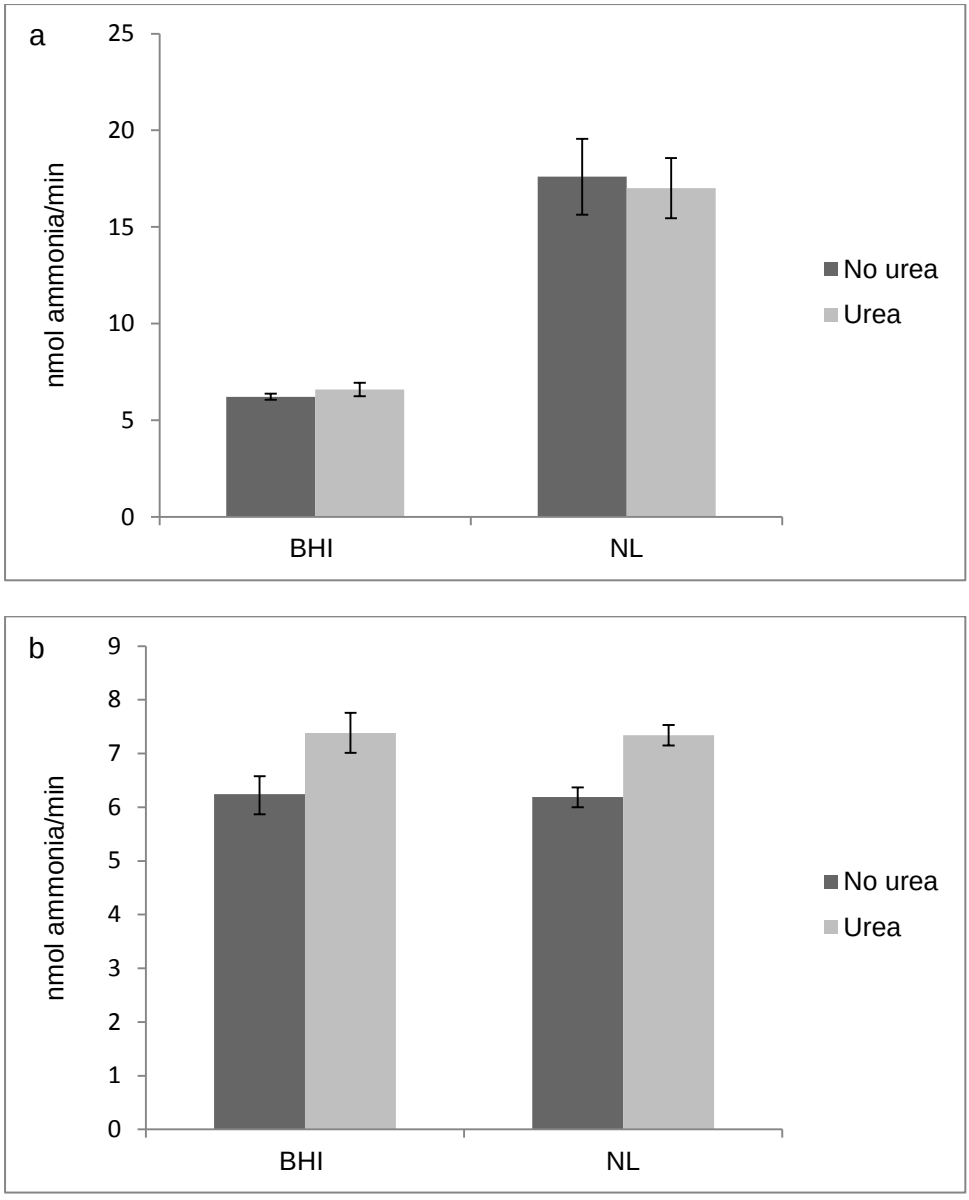

Figure 3. Urease activity of *C. neoformans* CAB 1055 (a) and *E. africanus* JX398293 (b) in BHI broth (BHI) and the nutrient-limited medium (NL), both in the presence and absence of 2.5 g/l urea. Enzyme activity in 50 µg crude protein extracts was represented as nmol ammonia produced per min (nmol ammonia/min) in a final reaction volume of 1 ml. Bars represent the mean of three repetitions and whiskers indicate standard error. Urease activity of *C. neoformans* CAB 1055 was significantly higher in the nutrient-limited medium ($p < 0.05$) and was not affected by urea supplementation. In contrast, nutrient limitation had no effect on the urease activity of *E. africanus* JX398293 and urea supplementation resulted in significantly higher urease activity levels in both BHI broth and the nutrient-limited medium ($p < 0.05$).

Table 1. Strains and their origins used in this study.

Species	Strain	Origin
Emergomyces africanus[a]	JX398291[*]	Clinical Isolate, NICD, RSA
Emergomyces africanus[a]	JX398293[*]	Clinical Isolate, NICD, RSA
Candida albicans[b]	CAB 397	Clinical Isolate, Tygerberg Hospital, RSA
Cryptococcus neoformans[b]	CAB 1055	Clinical Isolate, Tygerberg Hospital, RSA

*Identity of strains based on GenBank accession numbers for the sequences of the internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2 (Kenyon *et al*., 2013)

[a]Strains obtained from the culture collection of the National Institute for Communicable Diseases (NICD), RSA

[b]Strains obtained from the culture collection of the Department of Microbiology, University of Stellenbosch, RSA