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O papel da urease e proteínas acessórias na virulência de *Cryptococcus gattii*

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A Deus: O Cara que sempre me surpreende com novos dias, novas oportunidades e novos desafios.

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Para Sofia, que me ensina tanto todos os dias. Sabedoria no nome e na alma... Obrigada por ter me escolhido como mãe!

"A ciência de maneira nenhuma nega a existência de Deus. Quando considero quantas e quão maravilhosas coisas o homem compreende, pesquisa e consegue realizar, então reconheço claramente que o espírito humano é obra de Deus, e a mais notável."

Galileu Galilei

SUMÁRIO

RESUMO

Ureases (EC 3.5.1.5) são metaloenzimas dependentes de Ni^{2+}que hidrolisam ureia para produzir amônia e CO_2. Estas enzimas são encontradas em fungos, bactérias e plantas, compartilhando estruturas similares. Nosso grupo vem demonstrando que ureases possuem propriedades biológicas independentes da atividade ureolítica que potencialmente contribuem para a patogenicidade de micro-organismoss produtores de urease. A presença de urease em bactérias patogênicas (p.e. *Helicobacter pylori*, *Proteus mirabilis*) está correlacionada com a patogênese em algumas doenças humanas. Alguns fungos de importância médica também são produtores de urease entre eles *Cryptococcus neoformans*, *Coccidioides immitis*, *Histoplasma capsulatum*, *Sporothrix schenckii*. *Cryptococcus gattii* é um dos agentes etiológicos da criptococose em humanos e animais e acomete mais frequentemente indivíduos imunocompetentes. A maioria dos isolados produzem urease e vários autores sugerem que a liberação de amônia pela atividade da urease de *Cryptococcus* tem papel importante na patogenia da doença favorecendo uma maior permeabilidade que proporciona a transmigração das leveduras para o sistema nervoso central (SNC). No presente trabalho, para analisar o potencial de virulência da urease de *C. gattii* foram construídos mutantes com inativação do gene estrutural *URE (ure1)* e dos genes que codificam as proteínas acessórias (*URE*D, *URE*F – *ure4* e *ure6* respectivamente). Assim como já descrito para *H. pylori*, a urease de *C. gattii* desempenha papel importante na virulência independente da atividade enzimática. Esta função ocorre anterior a invasão do SNC diminuindo a multiplicação da levedura em macrófagos, aumentando a carga infecciosa no sangue e atenuando a mortalidade tanto no mutante *ure1*Δ como no mutante *ure6*Δ em camundongos infectados por via intranasal.

Palavras-chave: *Cryptococcus gattii* R265, urease, proteínas acessórias, mutantes, fator de virulência.

ABSTRACT

Ureases (EC 3.5.1.5) are metalloenzymes Ni^{2+} dependents that hydrolyze urea to produce ammonia and CO_2. These enzymes are found in fungi, bacteria and plants, show very similar structures. Our group has shown that plant and bacterial ureases display biological properties independent of their ureolytic activity that may contribute to the pathogenesis of urease-producing microrganisms. The presence of urease in pathogenic bacteria (e.g. *Helicobacter pylori*, *Proteus mirabilis*) strongly correlates with pathogenesis in some human diseases. Many medically important fungi also produce urease, among which are *Cryptococcus neoformans*, *Coccidioides immitis*, *Histoplasma capsulatum*, *Sporothrix schenckii*. *Cryptococcus gattii* is one of the etiologic agent that causes criptococcosis in human and animals, which often affects immunocompromised patients. The majority of clinical isolates produce large amounts of urease, and several authors suggest that the ammonia realease from urease activity might introduce a local damage of the endothelium, thus increasing permeability which provides yeast transmigration to central nervous system (CNS). To analyse virulence potential of *C. gattii* urease, mutants inactivating structural *URE* (*ure1*) gene and coding genes for accessory proteins required to assemble the Ni^{2+}-containing active site (*URE*D, *URE*F – *ure4* and *ure6* respectively). As already described to *H. pylori* urease, the *C. gattii* urease play important roles in virulence independent of ureolytic activity before CNS invasion, reducing yeast multiplication in macrophage, decreasing blood burden and also attenuating mortality either *ure1*Δ and accessory *ure*6Δ mutant in mice intranasal infection.

Keywords: *Cryptococcus gattii* R265, urease, accessory proteins, mutants, virulence factor.

LISTA DE ABREVIATURAS

ρ-HMB	ρ-hidroximercuriobenzoato
°C	graus Celsius
A	Absorbância
AIDS	Síndrome da Imuno Deficiência Adiquirida
bp	base pairs (pares de base)
CGU	*Cryptococcus gattii* Urease
CNTX	canatoxina
DNA	ácido desoxirribonucléico
HIV+	Vírus da Imunodeficiência Humana Positivo
HPU	*Helicobacter pylori* urease
IM	Intramuscular
IP	Intraperitoneal
IV	Intravenoso
JBU	Jack Bean Urease (Urease de *C. ensiformis*)
kDa	Quilo daltons (1.000 daltons)
L-DOPA	L-3,4-dihidroxifenilalanina
MM	marcador molecular
mM	milimolar
nm	nanometros
OD	Densidade Ótica
SNC	Sistema Nervoso Central

INTRODUÇÃO

1 *Cryptococcus sp*. E A CRIPTOCOCOSE

A criptococose é uma micose sistêmica que acomete o homem e vários outros mamíferos sendo uma das infecções fúngicas mais prevalentes afetando o sistema nervoso central (SNC). Há alguns anos, quando se pensava em criptococose, logo se associava sua ocorrência à espécie *Cryptococcus neoformans*, considerando que menos de 1% dos casos dessa doença era ocasionado por *Cryptococcus gattii*. Porém, esse panorama foi modificado após a epidemia causada por *C. gattii* nas ilhas Vancouver, Canadá (Kidd *et al.*, 2004) e desde 1999 essa espécie que era pouca estudada passou a ser amplamente citada na literatura e objeto de vários estudos incluindo ainda sua dispersão e ocupação de novos nichos ecológicos (Nicol *et al*., 2008; Byrnes III & Heitman 2009). Entre 2002 e 2006, a média anual de incidência de criptococose foi de 6,5 casos por milhão em British Columbia e 27,9 casos por milhão nas Ilhas Vancouver (BC Centre for Disease Control, 2007). O número de casos notificados por ano atingiu um patamar na Ilha de Vancouver em 2002, mas aumentou no continente desde 2005 e não foi observada variação na incidência de criptococose de acordo com os meses ou estações do ano (Galanis & MacDougal, 2010).

Anualmente, *C. gattii* e *C. neoformans* causam doença em aproximadamente um milhão de indivíduos contabilizando cerca de 620.000 óbitos sendo 1/3 de todas as mortes associadas a pacientes com AIDS. Esses óbitos ultrapassam a casuística de tuberculose na África (Byrnes III *et al.*, 2011). Em 1894, *C. neoformans* foi reconhecido como patógeno de humanos e animais, além de uma levedura normalmente encontrada no meio ambiente. Por muito tempo, este fungo não foi alvo de mais pesquisas pela baixa casuística da criptococose (Idnurm *et al.*, 2005). Com o aumento dos tratamentos com altas doses de corticosteróides (pacientes transplantados ou com desordens linfoproliferativas), o número de pacientes imunossuprimidos aumentou consideravelmente, acrescentado ainda do número de pacientes HIV-positivos. Desde então, a criptococose tem sido uma das três doenças oportunistas mais comuns em pacientes com AIDS. Nos demais grupos citados acima, que são terapeuticamente imunossuprimidos, estima-se 6% de risco de desenvolverem criptococose clínica (Chayakulkeeree & Perfect, 2006). Dados

da Organização Mundial da Saúde (OMS) mostram que aproximadamente três milhões de pacientes infectados pelo HIV morrem por ano no mundo. As complicações neurológicas atingem entre 70-90% dos pacientes infectados, sendo que as com complicações associadas à criptococose possuem maior incidência em pacientes HIV+(Lin & Heitman, 2006). O aumento dos casos de criptococose em proporção direta com o aumento do número de indivíduos com AIDS fez ressurgir o interesse por estudos nessa área.

Este fungo foi inicialmente identificado pela sua característica fenotípica de produzir o pigmento melanina e uma cápsula polissacarídica. Uma combinação de estudos moleculares (Meyer *et al*., 1999; Boekhout *et al*., 2001; Meyer *et al*., 2003; Latouche *et al*., 2003) agruparam as linhagens obtidas mundialmente do Complexo Cryptococcus em oito tipos moleculares distintos: VNI e VNII (*C. neoformans* var. *grubii*, sorotipo A); VNIV (*C. neoformans* var. *neoformans*, sorotipo D); VNIII (híbrido, sorotipo AD); e VGI, VGII, VGIII e VGIV, todos correspondentes aos sorotipos B e C (*C. gattii*), indicando uma evolução de forma independente em paralelo. Um tipo molecular separado, VNB, foi proposto para a linhagem isolada em Botswana (Litvintseva *et al*., 2006). Essa classificação quanto ao tipo molecular pode ser observada esquematicamente na figura 1. Esses genótipos possuem distribuição geográfica e grau de virulência distinto. Os sorotipos A (*C. neoformans var grubii)* e D (*C. neoformans var neoformans)* geralmente causam a doença em pacientes imunocomprometidos. Já os sorotipos B e C (*C. gattii* descrito como uma espécie independente por (Kwon-Chung & Varma, 2006;Kwon-Chung *et al.*, 2002) causam a doença preferencialmente em pacientes imunocompetentes (Idnurm *et al.*, 2005).

Há uma distinta distribuição geográfica conforme a classificação genotípica detectada (Figura 2): o genótipo VGI, é considerado endêmico na Austrália e seu isolamento é descrito em diversas regiões geográficas (Chen *et al.*, 2000). O genótipo VGII foi responsável por aproximadamente 95% das infecções causadas no surto de criptococose no Canadá e nos Estados Unidos e também foi isolado no Brasil (Kidd *et al.*, 2004;Byrnes *et al.*, 2010). O surgimento de *C. gattii* nos Estados Unidos surpreende devido a alteração de clima preferencial desta linhagem (Byrnes III *et al.*, 2011). Os genótipos VGIII foram encontrados em regiões Iberoamericanas e na Índia, e VGIV na África do Sul e nos Estados

Unidos, sendo estes dois últimos tipos mais raros (Chen *et al.* 2000). As epidemias na América do Norte estão variavelmente ligadas ao subgenótipos VGII onde se encontra classificada a linhagem R265, especificamente VGIIa (D'Souza *et al.*, 2011).

Também foram relatadas evidências de isolados de aves e fezes de psitacídeos de *C. neoformans var grubii* na Colômbia (Caicedo *et al.*, 1999) e no Brasil (Raso *et al.*, 2004). Este e outros achados são sugestivos de um padrão de transmissão zoonótica para pacientes imunocompetentes, tendo estes animais como importantes reservatórios do patógeno e aumentando a atenção a constante emergência da criptococose no mundo (Lagrou *et al.*, 2005).

A mudança na epidemiologia de *C. gattii* é provavelmente consequência de alterações na ecologia e biologia do fungo e ilustra seu potencial em causar doenças sérias ao homem. Estudando relações filogenéticas entre as espécies do Complexo *Cryptococcus*, Ngamskulrungroj e colaboradores (2009) constataram que o genótipo VGII, responsável pela epidemia em Vancouver, foi o único que apresentou clara evidência de recombinação em todos os testes realizados. Eventos frequentes de recombinação na população global desse genótipo poderiam explicar sua capacidade de adaptação a novas condições ambientais expandindo seu nicho ecológico, como no caso da epidemia em Vancouver (Ngamskulrungroj *et al*., 2009). Kidd e colaboradores (2004) sugeriram que o clima temperado de Vancouver pode proporcionar um nicho favorável à sobrevivência e dispersão de *C. gattii*, assim como também, o aquecimento global poderia favorecer sua colonização em novas regiões geográficas.

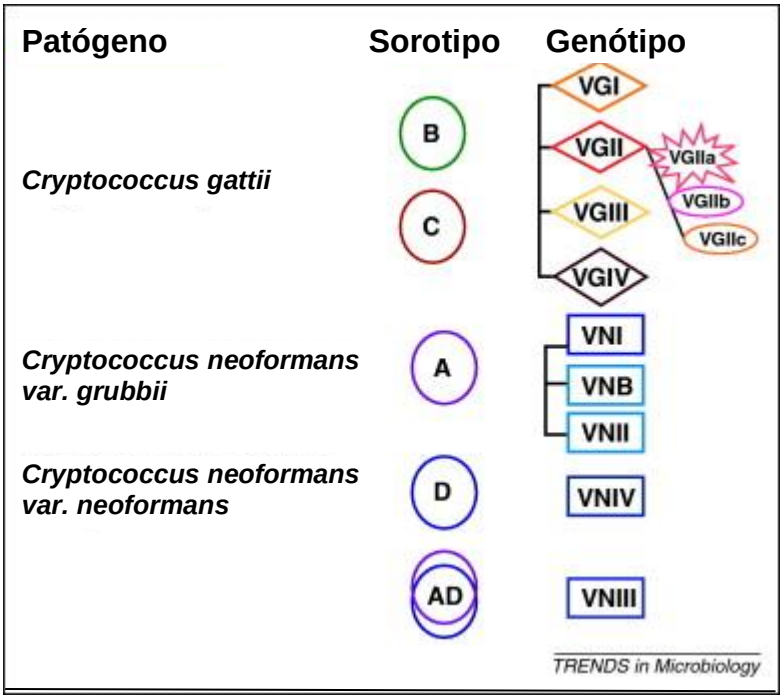

Figura 1: Sorotipos e genótipos de *Cryptococcus neoformans* e *Cryptococcus gattii.* Linhagens de *C. gattii* pertencem ao sorotipo B ou C; *C. neoformans* var grubii são sorotipo A, enquanto que linhagens de *C. neoformans* var *neoformans* são sorotipo D. A categorização taxonômica das linhagens pertencentes ao sorotipo AD não é clara. Genótipos de *C. gattii* e de *C. neoformans* baseiam-se em tipagem de sequência multilocus de fragmentos de diferentes genes (MLST). Os surtos de criptococose por *C. gattii* na América do Norte estão associados ao genótipo VGII. A linhagem R265, pertence ao subgenótipo VGIIa. Extraído de Chaturvedi & Chaturvedi, 2011.

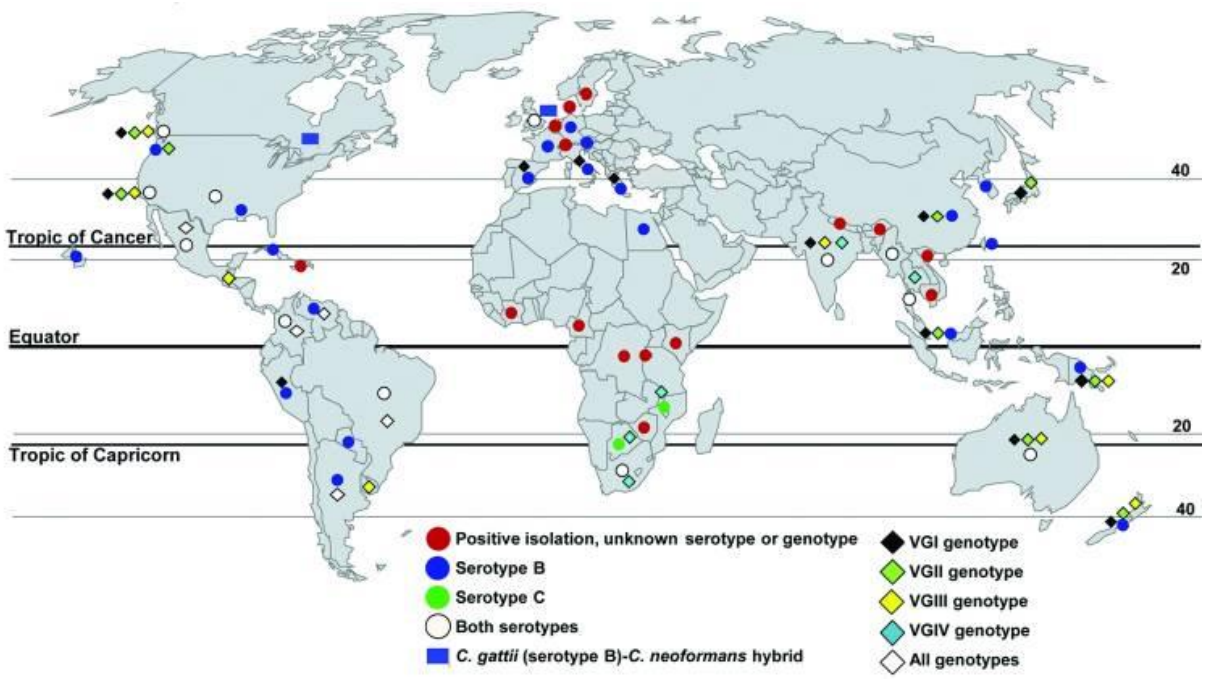

Figura 2. Distribuição de isolados de *Cryptococcus gattii* de origem clínica humana, veterinária e ambiental. Os círculos indicam os sorotipos, os losangos indicam os genótipos e os retângulos indicam os híbridos entre *C. gattii* e *C. neoformans*. Extraído de Springer & Chaturvedi, 2010.

1.1 Ciclo reprodutivo e infeccioso de *C. gattii*

C. neoformans e *C. gattii* são frequentemente isolados de pacientes e do ambiente na forma leveduriforme. No entanto, pode haver uma transição dimórfica com formação de hifas (forma filamentosa) por duas rotas distintas: *mating* (ciclo sexuado) e frutificação monocariótica (ciclo assexuado) (Fraser *et al*., 2005; Idnurm *et al*., 2005; Heitman, 2006) (Figura 3). O ciclo sexual de *Cryptococcus sp.* envolve a fusão de células haplóides *mating type* opostos (**a** e **α)** que estimuladas pela limitação de nutrientes liberam feromônios e quando se encontram sofrem fusão para produzir filamentos dicarióticos (Fraser *et al.,* 2003; Idnurm *et al.,* 2005; Heitman, 2006). Na formação do basídio, os núcleos se fusionam e ocorre a meiose, formando quatro produtos meióticos, os quais passam por mitose e brotam na superfície. As três variedades de *Cryptococcus* mostram diferenças na combinação entre os tipos **a** e **α**. Particularmente, na epidemia de Vancouver *C. gattii* foi caracterizada como uma levedura geralmente haplóide que predominantemente se reproduz assexuadamente,

por brotamento. Baseado nisso, Wickes e colaboradores (1996) demonstraram que *C. neoformans*, na ausência de um parceiro de *mating type* oposto, passa por um evento de frutificação monocariótica, com a consequente produção de basidiósporos de mesmo *mating type*. Essa habilidade também foi comprovada em *C. gattii*, nos isolados provenientes do surto na Ilha Vancouver (Fraser *et al.,* 2005).

Ambos os *mating type* de *C. gattii* já foram encontrados em diversas regiões, porém a maior frequencia é de isolados apresentando MATα (Campbell & Carter 2006). O evento de frutificação monocariótica ocorre principalmente em isolados MATα o que explicaria a alta frequencia de linhagens MATα, comparado com os isolados MATa, tanto para *C. neoformans* como para *C. gattii* (Wickes *et al.,* 1996; Fraser *et al.,* 2005; Idnurm *et al.,* 2005).

O ciclo de infecção é muito similar entre *C. gattii* e *C. neoformans*. A principal forma de infecção por *Cryptococcus* ocorre por exposição ambiental (Chayakulkeeree & Perfect, 2006; Chaturvedi & Chaturvedi (2011). O fungo está no ambiente, sendo encontrado no solo, árvores e fezes de aves, sobrevivendo em animais silvestres. A porta de entrada principal para o início da criptococose se dá pela inalação do patógeno na forma de levedura dessecada, ou como esporo. A partir daí a forma inalada se deposita nos alvéolos, podendo atravessar o endotélio de microcapilares até ultrapassar a barreira hemato-encefálica, e estabelecer infecção do SNC (Idnurm, 2005). Essa infecção pode ocorrer em mamíferos terrestres e marinhos, ressaltando a natureza ubíqua desse patógeno (D'Souza *et al*., 2011; Chaturvedi & Chaturvedi 2011) (Figura 4). Ocasionalmente também pode ocorrer contaminação por via digestiva, inoculação direta por traumatismo ou por implante de tecidos e órgãos contaminados (Delgado *et al*., 2005). Após entrada no hospedeiro suscetível, o fungo pode produzir um quadro latente ou agudo (Chayakulkeeree & Perfect, 2006)

As infecções pulmonares são mais brandas, pois neste ambiente o fungo pode ser eficientemente fagocitado e destruído pelas células de defesa do sistema imune, ou sobreviver e se multiplicar no interior de macrófagos. Quando atinge o SNC, inicia a fase crônica e mais grave da doença, vindo a desenvolver quadros de meningoencefalite ou meningite (Lin & Heitman 2006).

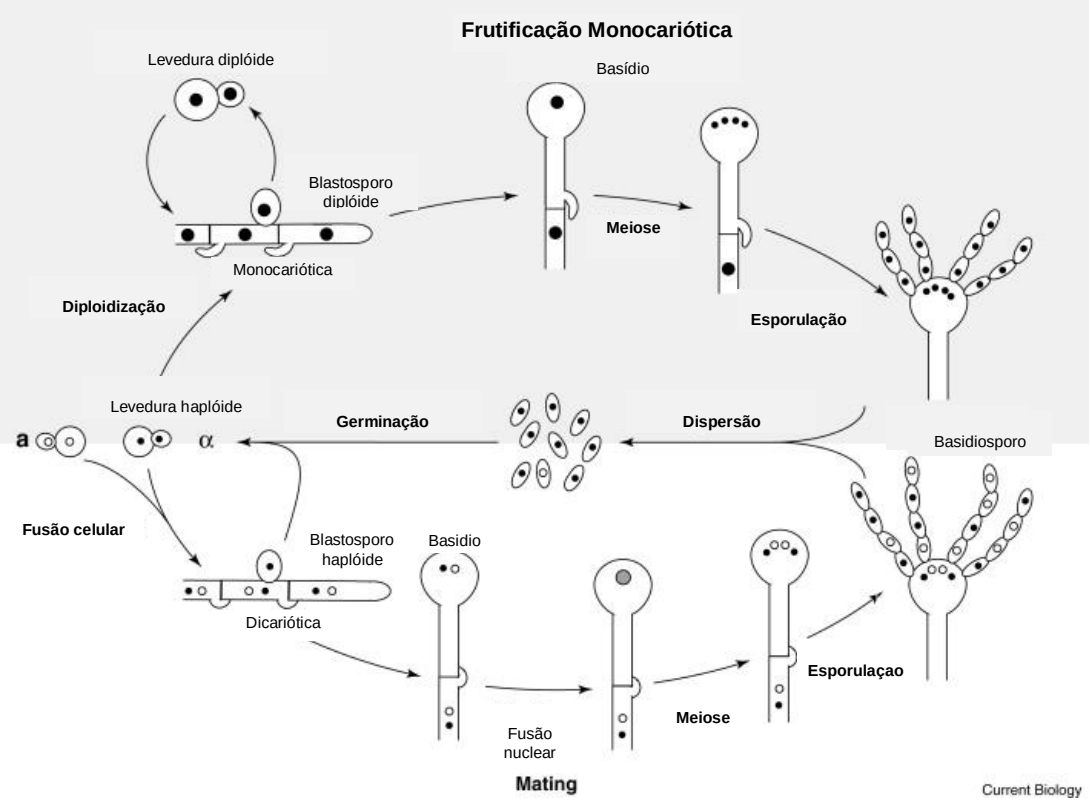

Figura 3: Modelo de Ciclo reprodutivo de *Cryptococcus neoformans*: o painel inferior descreve a reprodução sexual tradicional por *mating* a partir da fusão de duas células de *mating type* opostos, MATa e MATα. Em resposta a limitação de nutrientes, as células do tipo **a** e α (*mating type*) secretam hormônios peptídicos que promovem a fusão celular. Após a fusão, ocorre a formação de uma hifa dicariótica, que passa por um processo de divisão celular (meiose), resultando na produção de basidiósporos de ambos os *mating type*. Os núcleos migram pelos filamentosos gerando células separadas. O painel superior mostra a forma modificada de reprodução sexuada, que ocorre entre os (*mating type*) do mesmo tipo durante a frutificação monocariótica. Na frutificação monocariótica, células do mesmo *mating type* (geralmente MATα), sofrem diploidização formando uma hifa monocariótica diplóide. Esta, por sua vez, sofre meiose, produzindo basidiósporos de mesmo *mating type*. Ambos os processos liberam basidiósporos para o ambiente, os quais em condições favoráveis germinam, dando origem às células leveduriformes. Extraído de Heitman, 2006.

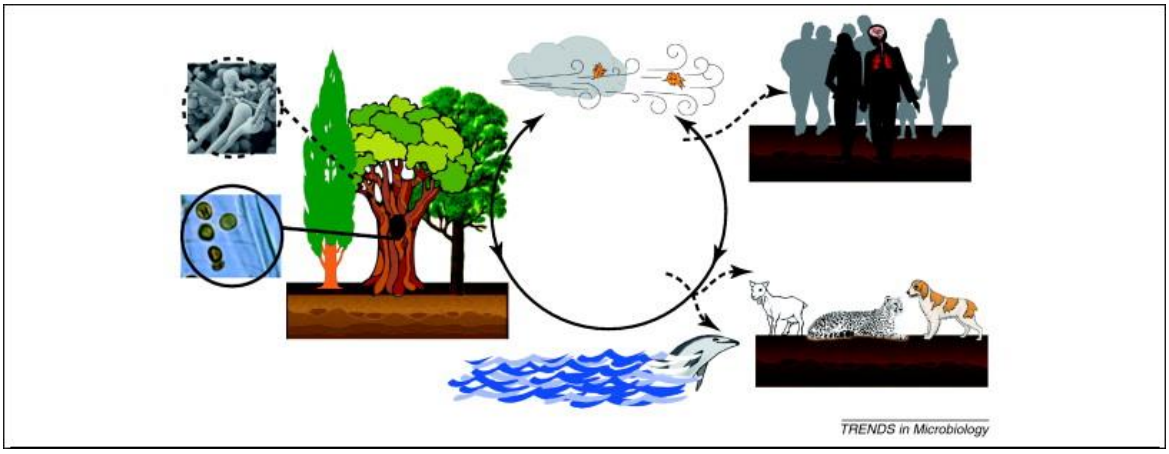

Figura 4: Ciclo natural do *Cryptococcus gattii* e eventos relacionados a criptococose. O patógeno pode ser encontrado no solo, ar e água. As leveduras de ambos *mating types* foram isoladas a partir de árvores em troncos ocos (círculo sólido); ainda não está claro se *C. gattii* completa seu ciclo sexuado no ambiente como é demonstrado em laboratório (círculo tracejado). O hospedeiro (humano ou animal) entra em contato com a célula leveduriforme ou formas esporuladas do ciclo sexuado permanecendo assintomático (linha tracejada com setas), mas um pequeno número de humanos ou animais infectados desenvolvem infecção pulmonar e cerebral grave. Figura extraída de Chaturvedi & Chaturvedi, 2011.

1.2 Cryptococcus gattii Linhagem R265

Antes de 1999 não havia evidências de infecções por *C. gattii* na Ilha de Vancouver – Canadá e se estendendo até o norte dos Estados Unidos. Atualmente, esta epidemia abrange uma ampla escala geográfica com níveis de infecção tão elevados ou superiores do que qualquer outra epidemia global; atualmente, as Ilhas de Vancouver apresentam uma casuística de 25 casos/milhão de habitantes (Byrnes III, *et al*., 2011). Este patógeno era normalmente encontrado em nichos de árvores (Chayakulkeeree & Perfect, 2006) e restringia-se a regiões de clima tropical e subtropical (Austrália, Sudeste da Ásia, África Central e regiões tropicais e subtropicais das Américas). Estudos revelaram que 44% dos pacientes com criptococose na Austrália causada por *C. gattii* eram aparentemente imunocompetentes (Chen *et al*., 2000). A súbita emergência de vários isolados em Vancouver mostrou que a ecologia e distribuição de *C. gattii* estava mudando. Estudos do ambiente da ilha apontaram uma surpreendente alteração no nicho ambiental desta

espécie, com habitat em uma variedade de árvores de clima temperado (Kidd *et al*., 2004).

A epidemia de Vancouver de 1999 a 2003 foi documentada como a maior do mundo, com casuística 10 a 40 vezes mais alta do que a da Austrália, onde *C. gattii* é considerada endêmica (Kidd *et al*., 2004). Como já descrito, o *mating type* é um fator de virulência reconhecido (Fraser *et al*., 2003). O *mating* no mundo microbiano justifica a diversidade genética e produz linhagens que podem colonizar novos nichos ambientais. No parasito *Toxoplasma gondii*, a meiose pode produzir isolados mais patogênicos pela recombinação de duas linhagens menos virulentas. No patógeno de plantas *Ustilago maydis*, o *mating* é essencial para a infecção. Nesta ocorrência em Vancouver, verificou-se que todos os isolados foram classificados como *mating type* α e análises por PCR, *fingerprinting* e AFLP, revelaram a presença de 2 genótipos. A grande maioria dos isolados clínicos e ambientais pertencia ao tipo molecular hipervirulento VGII/AFLP6. Posteriormente, a análise genotípica mostrou que as linhagens da epidemia eram clones e descendiam de dois ancestrais do mesmo *mating type* (α) – denominada *same-sex mating*. Estes isolados também foram muito mais virulentos quando testados em modelos animais. Uma minoria de isolados significativamente menos virulentos compartilhava um genótipo idêntico de isolados férteis, de uma população recombinante da Austrália. Este mesmo estudo também constatou a elevada virulência da principal linhagem isolada de Vancouver (R265) em ensaios com infecção intranasal em cobaias, produzindo maior mortalidade comparada a outras linhagens, principalmente em relação ao isolado avirulento (R272) (Fraser *et al.*, 2006).

Em relação ao quadro clínico, a localização preferencial de *C. gattii* é no parênquima cerebral e não na meninge, resultando numa maior prevalência de criptococomas cerebrais, ou hidrocefalia, após instalação do quadro respiratório inicial. Os pacientes com envolvimento do parênquima cerebral acabam por desenvolver aumento da pressão intracraniana, neuropatia craniana e baixa resposta à terapia antifúngica (Kozel, 1995;Mitchell, 2006).

Estudos epidemiológicos com indivíduos infectados por *C. gattii* sugerem que os quadros são predominantemente pulmonares enquanto aqueles infectados por *C. neoformans* comumente manifestam meningoencefalite. Análises de infecção por inalação comparando as linhagens R265 de *C. gattii* e

H99 de *C. neoformans* em modelo murino revelaram que *C. neoformans* se dissemina mais rápido no cérebro, enquanto *C. gattii* apresenta um quadro infeccioso mais localizado nos pulmões. No entanto, quando a em infecção experimental é por via intravenosa os animais infectados por *C. gattii* R265 apresentaram quadros mais avançados de criptococose e meningoencefalite grave sugerindo que esta linhagem pode atravessar a barreira hemato-encefálica mais eficientemente do que *C. neoformans* H99 (Ngamskulrungroj *et al.*, 2012a)

1.3 Fatores de virulência

C. neoformans e *C. gattii* produzem fatores de virulência que os distinguem de *Saccharomyces cerevisiae* e outros fungos: uma cápsula polissacarídica, produção de melanina (Figura 5) e a habilidade de desenvolvimento a 37^{o}C (Perfect 2006).

1.3.1 Melanina

A melanina protege *C. neoformans* e *C. gattii* das radiações ultravioleta e outras agressões do meio ambiente como altas temperaturas, congelamento e descongelamento (Wang & Casadevall 1994). A enzima responsável pela melanogênese foi identificada em 1982 como uma fenol-oxidase ligada a membrana. A formação de melanina demonstrou ser essencial fator de virulência sendo especialmente produzida pela levedura no SNC do hospedeiro (Polacheck & Kwon-Chung, 1988). Células melanizadas (Figura 5) são mais resistentes a injúria por ação química oxidativa, luz UV, anfotericina B, macrófagos e micróglia. A melanina também reduz a fagocitose mediada por anticorpos em macrófagos de murinos. Estas observações sugerem que este pigmento seja um fator que acentue a virulência, protegendo *Cryptococcus* contra mecanismos de defesa do hospedeiro e fatores ambientais mais agressivos. A melanina deposita-se na parede celular, acreditando tratar-se de um polímero (Wang *et al.*, 1996).

Durante o desenvolvimento da doença, a melanina pode se formar pela oxidação de catecolaminas no cérebro do hospedeiro, protegendo o patógeno de radicais livres tóxicos produzidos pelo sistema imune do hospedeiro (Casadevall *et al.*, 2000). Lacase é a enzima chave necessária para a síntese de melanina. Estudos moleculares da síntese de melanina, identificaram 2 genes essenciais para sua biossíntese, os genes *LAC1* e *LAC2* (Zhu & Williamson 2004; Missall *et al.,* 2005). Outros genes essenciais foram descritos, tais como *VPH1, CLC1, CCC2* e *ATX1*, embora o mecanismo de ação não esteja totalmente elucidado (Erickson *et al.* 2001; Zhu & Williamson 2003; Walton *et al.,* 2005). O gene mais bem caracterizado é *LAC1*, mutantes para esse gene apresentam fenótipo albino na presença de compostos fenólicos e virulência atenuada em modelos animais. Mutantes nulos de apresentam inabilidade de escapar dos pulmões, sem afectar o desenvolvimento tanto no sangue ou no cérebro, indicando que a lacase tem um papel na disseminação de *Cryptococcus* na corrente sanguínea linhagens mutantes (Liu *et al.*, 2012). A melanina foi conceituada como um *scavenger* de radicais livres e tem função na proteção ao estresse oxidativo. A lacase também faz o seqüestro de ferro durante a infecção, levando a um rompimento oxidativo de fagócitos (Brown *et al.*, 2007). Quando em ação conjunta com fosfolipase B, a lacase promove a saída do patógeno do pulmão facilitando a penetração no SNC (Idnurm *et al*., 2005). A prevalência de substratos para lacase no SNC foi proposta como uma possível justificativa para o neurotropismo do fungo (Steen *et al.*, 2003).

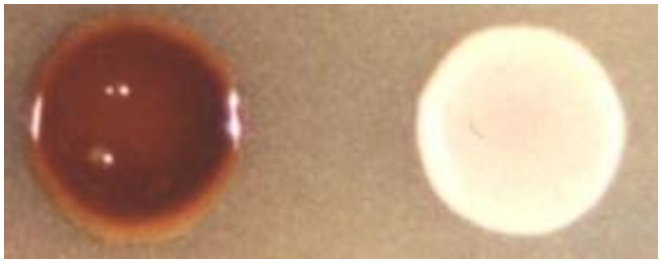

Figura 5: Fatores de virulência de *C. gattii:* produção de melanina. Colônia de *C. gattii* melanizada apresentando coloração castanha (esquerda) em comparação a uma colônia não formadora de melanina (direita). Imagem extraída de Ma & May, 2009.

1.3.2 Cápsula polissacarídica

A cápsula polissacarídica é um importante fator de virulência das espécies do complexo *Cryptococcus* e é composta basicamente de 90 a 95% de glicuronoxilomanana (GXM), 5% de galactoxilomanana (GalXM) e aproximadamente 1% de manoproteínas (McFadden *et al.,* 2007). GXM é um polímero, que confere as características antigênicas das espécies do complexo, possibilitando a diferenciação dos sorotipos das mesmas. Estudos recentes mostraram características distintas nas espécies *C. gattii* e *C. neoformans*, onde *C. gattii* apresenta menor diâmetro e maior reatividade sorológica (Fonseca *et al.*, 2010). A cápsula está diretamente relacionada com a sobrevivência celular em condições de desidratação apresentando uma fina espessura, no hospedeiro, promove a evasão do sistema imune e consequente sobrevivência do patógeno. Em estudos de infecção experimental de camundongos, a composição antigênica da cápsula *in vivo* é distinta em diferentes órgãos infectados, sugerindo uma evolução da estrutura capsular durante a infecção (Charlier *et al.*, 2009). Mutantes acapsulados são avirulentos sendo facilmente fagocitados por macrófagos e neutrófilos (Kwon-Chung & Rhodes, 1986).

Uma família de genes controla a biossíntese de uma cápsula variável e complexa, capaz de interferir no processo de fagocitose por macrófagos (McFadden *et al.*, 2006). Análises no genoma de *C. neoformans* demonstraram a presença de mais de 30 genes que provavelmente estejam envolvidos na formação da cápsula (Loftus *et al.,* 2005). Alguns genes já foram testados e assumidos como essenciais para a síntese da cápsula, tais como: *CAP59, CAP64, CAP60* e *CAP10* (Chang & Kwon-Chung 1994; Chang *et al.,* 1996; Chang & Kwon-Chung 1998, 1999).

A cápsula bloqueia o recrutamento de células inflamatórias, além de agir na depleção do sistema complemento e supressão de sinalização de hipersensibilidade que influenciaria diretamente a produção de anticorpo durante o processo infeccioso (Steen *et al*., 2003).

A GalXM é um polímero de menor massa comparado a GXM, espalhada por toda a extensão da cápsula (McFadden *et al.,* 2006). Sabe-se que GXM e

GalXM dividem propriedades imunomodulatórias (Zaragoza *et al.,* 2009; Fonseca *et al*., 2010). As manoproteínas, quando manosiladas e glicosiladas, agem como antígenos criptocócicos, os quais estimulam respostas de células T por promover a maturação e ativação de células dendríticas (Mansour *et al.,* 2002; Levitz & Specht 2006; Specht *et al.,* 2007). A cápsula aumentada durante a infecção é essencial para instalação do patógeno no hospedeiro. Existe correlação linear entre o diâmetro de GXM e o diâmetro da cápsula sugerindo que a síntese de GXM de diâmetro maior é essencial para o aumento da cápsula (Fonseca *et al.*, 2010). I*n vitro*, o aumento de cápsula é induzido por soro (Figura 6) (McFadden *et al.*, 2006).

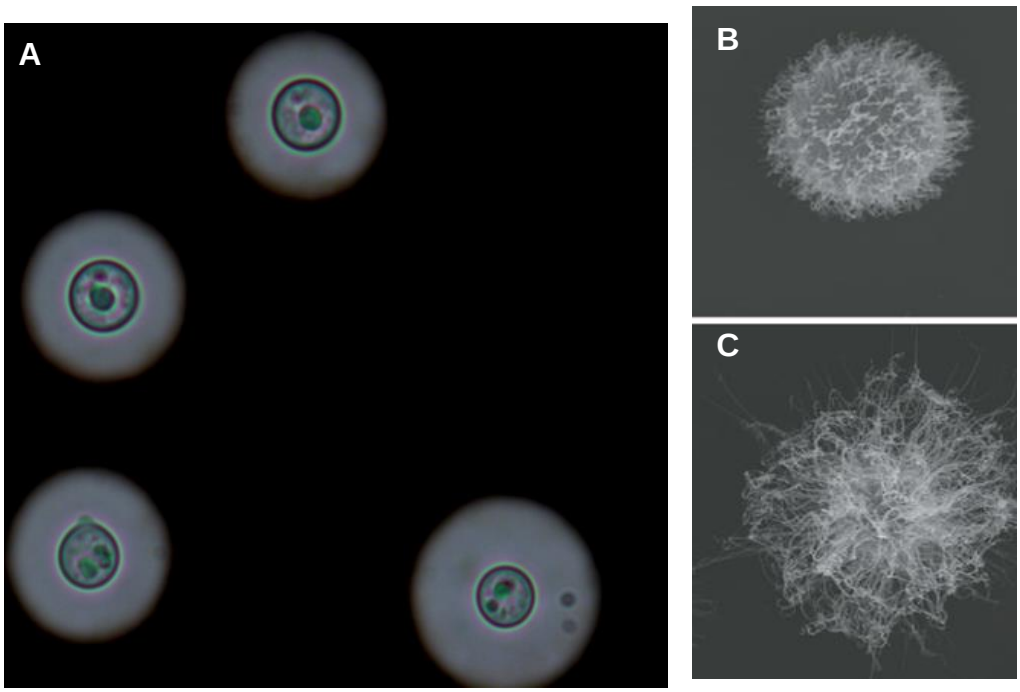

Figura 6: Fatores de virulência de *C. gattii* – cápsula polissacarídica. **A**; presença de cápsula de polissacarídeos (CPS) produzida em presença de desafios ambientais. Imagem produzida por Vanessa Feder. **B**; Imagem 3D em microscopia de varredura de cápsula menor e **C**; formação aumentada da CPS (adaptado de Mc Fadden *et al*., 2006).

1.3.3 Habilidade de desenvolvimento a 37°C

A capacidade do *C. neoformans* de crescimento na temperatura corporal de mamíferos (termotolerância) é uma característica fundamental para invasão do hospedeiro (Perfect, 2006). O desenvolvimento a 37ºC está associado a transição para forma de levedura em fungos dimórficos, sendo também

importante na virulência de *Histoplasma capsulatum*, *Paracoccidioides brasiliensis* e *Blastomyces dermatidis* (Kraus *et al.*, 2004). No gênero *Cryptococcus*, somente as espécies *C. neoformans* e *C. gattii* são capazes de crescer em temperatura superior a 30ºC. Este comportamento está relacionado a genes como *RAS1, CNB1, MPK1* e *CTS1* (codifica proteínas de ligação GTP) e ao gene *CNA1* (codifica a calcineurina), uma proteína necessária para resposta ao estresse e integridade celular em levedura.

A montagem de *contigs* de sequencias de cDNAs e análise comparativa com dados do GenBank levou a identificação de transcritos que podem ser críticos tanto para a interação patógeno-hospedeiro quanto em resposta a capacidade de crescimento em temperatura mais baixas ou mais elevadas. Estes produtos estão envolvidos na integridade da parede celular, resposta a stress, filamentação, metabolismo oxidativo e de ácido graxos foram induzidos a 37°C. Somado a estes dados, genes reguladores ao silenciamento de cromatina e transporte de fosfolipídeos são regulados positivamente a 25°C. Análises de RDA (Representational difference analyses) comparando condições saprofíticas e de temperatura no hospedeiro revelou a presença de novos genes com potencial envolvimento na virulência de *C. neoformans* (Rosa e Silva *et al.*, 2008)

1.3.4 Enzimas

Enzimas envolvidas no metabolismo do inositol demonstraram ser importantes na patogenicidade da criptococse. Fosforil inositol ceramida sintase (Ipc1) e sua proteína downstream App1 (proteína antifagocítica 1) são importantes na regulação da fagocitose de *Cryptococcus sp.* e sua potencial disseminação no hospedeiro. Enquanto isso, inositol fosfoesfingolipídio fosfolipase C1 (Isc1) desempenha função importante para a sobrevivência da levedura em macrófagos ativados. Mutantes com deleção de Isc1 gerou menor disseminação nos pulmões comparada com o tipo selvagem em modelo murino, além de ocorrer inabilidade de sobrevivência após fagocitose (Liu *et al.*, 2012).

As **fosfolipases** também são consideradas fatores de virulência e resultam na desestabilização de membranas e lise celular. Estudo com

mutantes no gene que regula a expressão de fosfolipase (*PLB1*) sugere sua importância na infecção inicial nos pulmões onde ocorre a secreção de fosfolipases. Outro papel possível na patogênese inclui a liberação de mensageiro secundário como inositol trifosfato, ácido araquidônico e lise de células fagocitárias (Cox *et al.*, 2001).

1.4 Urease e a Criptococose

Estudos publicados por diferentes grupos a partir de 2000 mostraram evidências de que a urease de *C. neoformans* estaria implicada como um fator de virulência na criptococose, particularmente na disseminação hematogênica do fungo e no estabelecimento da forma mais severa de criptococose, quando ocorre infecção meningocócica (Cox *et al*., 2000; Olszewski *et al*., 2004; Varma *et al*., 2006). Uma função da urease como fator de virulência através de um mecanismo não dependente da atividade ureolítica foi sugerido por (Olszewski *et al*., 2004; Varma *et al*., 2006). Esses resultados levaram alguns autores à proposição de que a urease de *Cryptococcus* poderia ser constituir um alvo terapêutico, com potencial de diminuir ou controlar a disseminação da infecção fúngica para o sistema nervoso central (Charlier *et al.* 2009; Shi *et al.* 2010) (Shi *et al.*, 2010;Casadevall, 2010).

Anteriormente, purificamos e caracterizamos a urease de *C. gattii* (CGU) linhagem R265 (Feder, 2008). A enzima foi purificada de extratos celulares através de duas cromatografias de troca iônica e uma cromatografia de exclusão molecular. Na caracterização da proteína purificada em solução, a CGU apresentou cinética típicas de outras ureases, com Km 2,0 mM de ureia e atividade específica 17807mU.mg^{-1}, pH ótimo 8,0. A CGU apresentou massa molecular de 90kDa em . Análise proteômica e sequenciamento (ESI-Q-TOF-MS/MS) da CGU resultou em 32% de cobertura e confirmação da sequencia predita a partir do genoma.

2 UREASE

Ureases (ureia amidohidrolases; EC 3.5.1.5) catalisam a hidrólise da ureia para formar 2 moléculas de amônia e uma de dióxido de carbono. A urease da semente de feijão-de-porco *Canavalia ensiformis* foi a primeira enzima a ser cristalizada em 1926, comprovando a natureza proteica das enzimas (Sumner, 1926). Também foi a primeira metalo-enzima dependente de Ni^{2+} a ser identificada. A urease catalisa a hidrólise da uréia para formar 2 moléculas de amônia e uma de dióxido de carbono (Dixon *et al.*, 1975).

As ureases são encontradas em inúmeros tecidos vegetais, sendo particularmente abundantes em sementes de leguminosas e curcubitáceas (Polacco & Holland, 1993), estando amplamente distribuídas em bactérias, fungos e plantas. Apesar da abundância de urease em alguns tecidos vegetais, sua função na planta é controversa. Postula-se que a principal função das ureases de plantas esteja relacionada à reciclagem de nitrogênio (Sirko & Brodzik, 2000) ainda que a uréia, que é seu principal substrato, não seja um metabólito importante em plantas (Polacco & Holland, 1993). Postula-se ainda, que em combinação com arginase, a urease pode ter função na utilização das reservas proteicas da semente durante a fase de germinação (Follmer, 2008). A urease de *C. ensiformis* ou *Jackbean Urease* (JBU), amplamente estudada e comumente adotada como modelo, constitui-se de uma cadeia polipeptídica com 840 aminoácidos e massa molecular de 90,77 kDa. A forma mínima da enzima ativa é trimérica, com 270 kDa, sendo que a forma nativa mais abundante é um hexâmero de 540 kDa (Zerner, 1991). Possui dois átomos de níquel no sítio ativo, cada um coordenado por dois resíduos de histidina (Follmer et al., 2001).

A canatoxina é uma proteína tóxica isolada por Carlini & Guimaraes, (1981) a partir de sementes de *Canavalia ensiformis* e posteriormente descrita como uma isoforma de urease, porém com apenas 30 – 40% da atividade enzimática sobre uréia, comparada com a urease da mesma semente (Follmer *et al.*, 2001). Oliveira e colaboradores (1999) descreveram preliminarmente a atividade fungicida da canatoxina sobre alguns fungos filamentosos Mais recentemente, demonstramos a ação antifúngica de ureases de diferentes fontes em concentrações sub-micromolares, obsevando-se inibição do

crescimento e germinação de fungos fitopatogênicos, mesmo com as ureases pré-tratadas com o inibidor irreversível ρ-hidroximercuribenzoato (ρ-HMB), portanto sem atividade ureolítica. (Becker-Ritt *et al.*, 2007); Oliveira et al., 1999). Somada à ação inseticida descrita para as ureases de *C. ensiformis* e da soja (Follmer et al., 2004), estes dados reforçam a proposição de um papel das ureases vegetais na proteção da planta contra insetos e fungos fitopatogênicos (Becker-Ritt & Carlini, 2012; Stanisçuaski & Carlini, 2012).

Comparativamente, verificou-se que a urease (JBU), além das propriedades inseticidas e fungitóxicas, possui outras atividades biológicas descritas para a canatoxina, como indução de exocitose resultando, por exemplo, em ativação plaquetária, e interação com gangliosídeos revisto em (Olivera-Severo *et al.*, 2006b). As atividades citadas também são independentes da atividade ureolítica já que persistem mesmo nas proteínas tratadas com ρ-HMB (Follmer et al., 2001). Esses dados indicam a existência, nestas proteínas, de pelo menos dois domínios protéicos distintos, responsáveis por atividades biológicas diferentes: um domínio com atividade hidrolítica sobre uréia, suscetível de inibição por agentes oxidantes; e pelo menos mais um segundo domínio, responsável pela toxicidade intraperitoneal (IP) da canatoxina e outras propriedades farmacológicas compartilhadas com a urease.

Na intenção de determinar se ureases de outros organismos compartilham com a JBU propriedades biológicas independentes da atividade ureolítica, a urease embrião específica de soja e a da bactéria de solo *Bacillus pausterii* foram testadas em plaquetas de coelho. Ambas ureases ativam plaquetas, induzindo secreção e agregação plaquetária, mesmo após tratamento com inibidor clássico ρ-HMB (Follmer *et al*., 2004; Olivera-Severo *et al.*, 2006ª; Olivera-Severo *et al*., 2006b). Posteriormente, verificou-se que a urease recombinante de *Helicobacter pylori*, uma bactéria causadora de gastrite e câncer gástrico em humanos, também desencadeia este processo em plaquetas (Wassermann *et al.*, 2010)

Em relação aos micro-organismoss, a atividade ureásica foi detectada em mais de 200 espécies de bactérias gram negativas e gram positivas. A urease sintetizada em bactérias de solo e em bactérias anaeróbicas, presentes

na flora digestiva de ruminantes, favorece a reciclagem de compostos nitrogenados (Mobley & Hausinger, 1989). As ureases bacterianas estão envolvidas na patogênese de muitas condições clínicas, como a ulceração péptica e a formação de urólitos e biofilmes de incrustação em catéter urinário, contribuindo para a patogênese de pielonefrites por infecções do trato urinário por *Proteus mirabilis*, encefalopatia causada por amônia, coma hepático, e em processos infecciosos crônicos que podem levar ao câncer gástrico característicos da infecção por *Helicobacter pylori* (Carlini & Polacco, 2008; Mobley *et al.*, 1995). Salienta-se que devido à atividade enzimática da urease, a espiroqueta *H. pylori* tem capacidade de adaptação ao ambiente gástrico, sobrevivendo em meios com pH variando entre 3,5 e 8,6 (Mobley *et al.*, 1991). No caso de infecção por *Proteus mirabilis,* a condição básica para a formação dos urólitos é a presença de cepas urease positivas, resultando em um crescimento excepcionalmente rápido destas pedras, de 4 a 6 semanas (Bichler *et al.*, 2002). Dois operons de urease (*URE*1 e *URE*2) foram descritos em *Brucella suis*. O operon *ure*1 é caracterizado como importante para a atividade ureolítica e sobrevivência em ambiente de pH baixo (Bandara *et al.*, 2007).

A urease é sintetizada por vários gêneros de fungos de importância médica, como *Coccidioides, Candida, Aspergillus, Rhodotorula, Trichosporon e Cryptococcus* (Mirbod *et al.,* 2002), além de *Histoplasma capsulatum, Sporothrix schenkii* (Han *et al.,* 2002). Em 1997, o primeiro gene *URE* de fungo codificando uma urease foi isolado em *Coccidioides immitis* (Yu *et al., 1997)*. Alguns anos mais tarde, a urease de *C. immitis* foi purificada e caracterizada a sua forma nativa, apresentando massa molecular 450 kDa (Mirbod et al., 2002). Os autores sugeriram um papel dessa urease na patogenicidade do fungo, atuando na colonização no tecido hospedeiro, resultando na formação de abcessos com um microambiente alcalino diferenciado (Mirbod et al., 2002). Os genomas completos de *C. neoformans e de C. gattii*, depositados no Broad Institute (MIT e Harvard) e disponíveis em <http://broadinstitute.org>, mostraram a presença da urease e das proteínas acessórias UreD, UreF, UreG e ausência de UreE no complexo *Cryptococcus sp*. Há poucos estudos caracterizando estrutura e função de proteínas acessórias em leveduras. Entre esses poucos, (Bacanamwo *et al.*, 2002) descreveram a presença de UreD,

UreF e UreG no complexo de ativação da urease de *Schizossacaromyces pombe*.

Ureases apresentam homologia e mecanismos catalíticos similares, apesar de diferirem na estrutura quaternária. Enquanto que ureases vegetais e fúngicas comportam-se como trímeros ou hexâmeros de uma subunidade de 90 kDa, as ureases bacterianas são multímeros de complexos formados por duas ou três subunidades (Carlini & Polacco, 2008; Jones & Mobley, 1989). Algumas ureases apresentam uma forma nativa dimérica, como já descrito em certas planta (canatoxina, de *C. ensiformis*; a de folhas de amoreira *Morus alba*, entre outros) e certos fungos (*Aspergillus niger, Ustilago violacea* e *Schizossacharomyces pombe*) Particularmente, a urease de *C. gattii* foi caracterizada como um dímero de uma subunidade de 90 kDa (Feder, 2008). A figura 1 ilustra as diferentes organizações estruturais da urease em diferentes espécies.

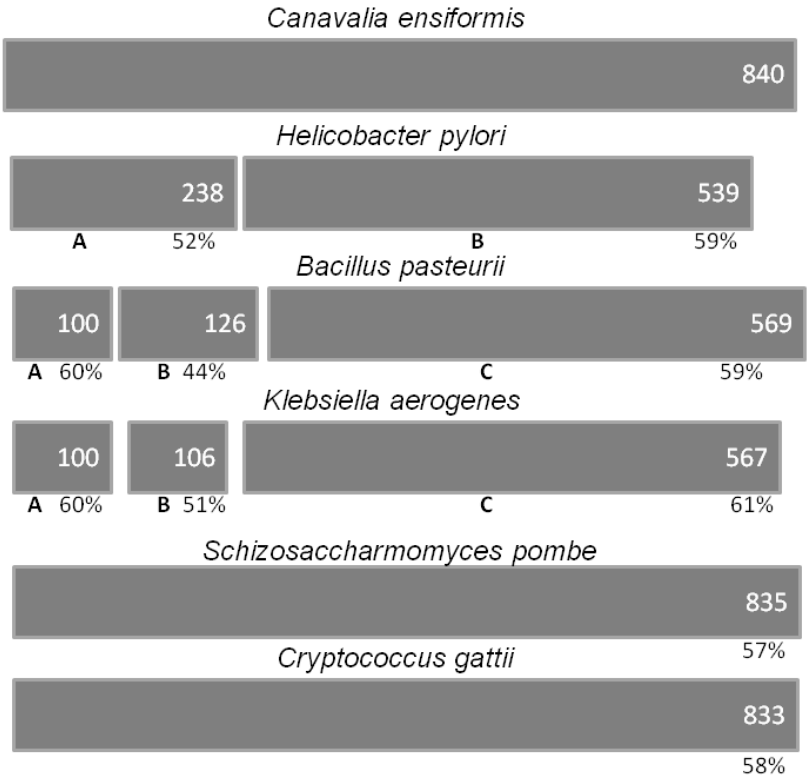

Figura 1: Comparação esquemática das subunidades estruturais das ureases de diferentes organismos. O número de aminoácidos de cada unidade está indicado dentro das caixas com letras indicando as respectivas subunidades. Os valores percentuais, abaixo de cada bloco, indicam a porcentagem de identidade com a urease de *C. ensiformis,* na mesma região. Referência a urease de *C. gattii* por Feder (2008). Imagem adaptada de Olivera-Severo *et. al.*, 2006.

2.1Urease e proteínas acessórias

Um processo bem regulado envolve a participação de uma série de proteínas acessórias que conferem estabilização da apoenzima e inserção do metal para a montagem correta de seus sítios ativos (Carter *et al.*, 2009;Real-Guerra *et al.*, 2012). Em todos modelos propostos, o sítio ativo da proteína encontra-se internalizado na molécula, sendo um metalocentro dinuclear de níquel, coordenado por histidinas e ligado a um resíduo de lisina carbamilado. A síntese do metalocentro de urease é um processo altamento complexo que requer, além do Ni^{2+}, dióxido de carbono para a carbamilação de lisina, várias proteínas acessórias e GTP. Estudos com o sistema recombinante de urease de *Klebsiella aerogenes* mostrou que o conjunto que forma a urease (UreABC) forma complexos com as proteínas acessórias UreD, UreF, UreG, UreE, nessa ordem, e a deleção de alguma destas proteínas resulta em atividade enzimática indetectável (Chang *et al.*, 2004). A urease e proteínas acessórias em fungos recebem nomenclatura específica, sendo a apoenzima denominada ure1 e as proteínas acessórias UreD, UreE, UreF e UreG respectivamente *URE4, URE5, URE6 E URE7*.

2.1.1 Proteína acessória URE D (*URE 4*)

Pouco ainda se sabe sobre propriedades estruturais e função da **UreD** em razão de sua insolubilidade quando isolada em condições nativas**.** Aparentemente é a primeira proteína a ligar-se a apo-urease. O conjunto de informações a respeito desta proteína deriva de sua insolubilidade quando isolada em condições nativas. Uma versão recombinante da Ure D fusionada a uma proteína ligadora de maltose permitiu mostrar sua capacidade de ligar Ni^{2+} e Zn^{2+}. Estudos propõem que ela possa ter uma relação direta na entrega de Ni^{2+} ao sítio ativo da enzima. No entanto, manipulações no gene da *ureD* mostraram uma ativação parcial da urease, mostrando que esta proteína pode ser parcialmente funcional (Carter & Hausinger, 2010; Ligabue-Braun *et al.*, 2012).

2.1.2 Proteína Acessória URE E (*URE 5*)

A estrutura da proteína acessória UreE já foi resolvida em *K. aerogenes* (Song *et al., 2001;*Colpas *et al.,* 1999)*, Bacillus pasteurii* (Ciurli *et al.,* 2002) e *H. pylori* (Bellucci *et al*., 2009), e todas as já descritas apresentam-se com estruturas altamente conservadas. A interação de UreE com seu mais provável parceiro UreG foi analisada em *H.pylori* e *K. aerogenes* e em ambos os casos, a interação entre essas moléculas parece ser intermediada por íons Zn^{2+} e Ni^{2+}. No entanto, várias outros organismos não incluem UreE no seu conjunto de proteínas acessórias, como em *Arabidopsis thaliana* (Witte *et al.*, 2005), *Oryza sativa* (Cao *et al.,* 2010). Onde há a ausência da UreE, sua função parece ter sido incorporada a UreG (Real-Guerra, 2011)

2.1.3 Proteína Acessória URE F (*URE 6)*

O papel da **UreF** parece ser como uma proteína ativadora da atividade de GTPase (GAP) da UreG no modelo *K. aerogenes* (Ligabue-Braun, *et. al.,* 2012). Mutações em resíduos altamente conservados em UreF comprometeram a plena ativação da urease, evidenciando que os resíduos formam uma superfície de ligação com UreG (Boer & Hausinger, 2012). Aparentemente, a ligação da UreF a UreD se dá por interação direta (Zambelli *et al.*, 2011).

2.1.4 Proteína Acessória URE G (*URE 7*)

Acredita-se que a **UreG** é responsável pela hidrólise de GTP associada a transferência de CO_2 para a lisina do sítio ativo. A proteína é intrinsecamente desordenada, ligando-se especificamente a Zn^{2+}, e apresenta baixa atividade de hidrólise de GTP *in vitro*, necessitando de interações com outras proteínas para alcançar a estrutura funcional final (Lam *et al.*, 2010).

A figura 2 mostra, esquematicamente, o processo de interação com proteínas acessórias para a formação da urease ativa no modelo *K. aerogenes*.

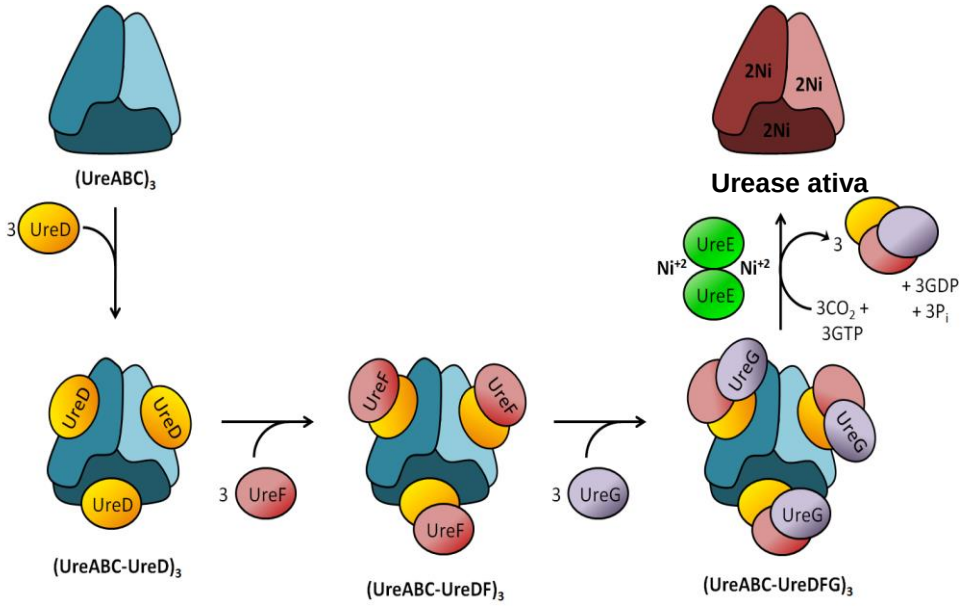

Figura 2: Representação esquemática da relação entre proteínas acessórias ureDEFG. Rota de ativação da urease com ligação de proteínas acessórias na apoenzima de *K. areogenes*. Modelo proposto por Quiroz-Valenzuela *et al.*, 2008. Apoproteína (Ure ABC) é formada por UreA, UreB e UreC. A representação trimérica considera UreABC como uma unidade funcional. A proteína acessória UreD, UreF, UreG sequencialmente liga-se para formar os complexos de ativação (UreABC–UreD)$_3$, (UreABC–UreDF)$_3$, and (UreABC–UreDFG)$_3$. A metalochaperona dimérica UreE carrega Ni^{2+} ao (UreABC–UreDFG)$_3$ (requerendo hidrólise de GTP). UreE e (UreDFG)$_3$ são então liberadas da enzima ativada. Extraído de Ligabue-Braun, *et. al.*, 2012).

OBJETIVO

Investigar o papel da urease como um fator de virulência de *C. gattii* e estabelecer se propriedades não enzimáticas da proteína podem contribuir para a criptococose

Objetivos Específicos

- Construção de mutantes de *C. gattii* R265 para os genes *URE 1* e uma ou mais proteínas acessórias *URE 4*, *URE 6* ou *URE 7*.
- Caracterização fenotípica dos mutantes *URE* de *C. gattii* e interferências destes genes na atividade ureolítica da levedura.
- Validar o potencial de virulência de genes *URE* na levedura patogênica *C. gattii* em ensaios de infecção experimental em camundongos.

JUSTIFICATIVA

Propriedades biológicas independentes da atividade enzimática são conhecidas ureases vegetais e bacterianos, no entanto, nenhum estudo foi realizado com ureases fúngicas. No modelo *Cryptococcus* sp. a importância da urease como fator de virulência para invasão e estabelecimento da criptococose no SNC já foi reconhecido, no entanto, ainda se faz necessário entender como a urease participa neste complexo sistema patogênico. Reconhecendo a relevância dessa enzima na criptococose, Casadevall, 2010, levantou a hipótese que, em sendo o efeito da urease extracelular, poderia ser possível proteger o parênquima cerebral contra a infecção criptococócica com anticorpos neutralizantes de urease.

Para tanto, é preciso aprofundar a compreensão do mecanismo de ação da urease e explorar todas as possibilidades de atuação desta enzima no estabelecimento da doença, a fim de compreender seu papel na patogenia da criptococose.

MANUSCRITO PARA SUBMISSÃO:

C*ryptococcus gattii* urease as virulence factor and influence of enzymatic activity on pathogenesis of cryptococosis.

Cryptococcus gattii urease as virulence factor and influence of enzymatic activity on pathogenesis of cryptococosis.

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ABSTRACT

Ureases (EC 3.5.1.5) are Ni^{2+} dependent metalloenzymes that hydrolyze urea to produce ammonia and CO_2. Ureases are produced plants, fungi and bacteria. The high sequence similarity suggests that all ureases posses similar tertiary structure and catalytic mechanisms. Our group has demonstrated that plant and bacterial ureases possess biological properties independent of their ureolytic activity that may potentially contribute to the pathogenicity of urease-producing microorganisms. The presence of urease in pathogenic bacteria (e.g. *Helicobacter pylori*, *Proteus mirabilis*) strongly correlates with pathogenesis in some human diseases. Some medically important fungi also produce urease, among which are *Cryptococcus neoformans*, *Cryptococcus gattii, Coccidioides immitis*, *Histoplasma capsulatum*, *Sporothrix schenckii*. *C. gattii* is one of the etiologic agents of cryptococcosis in humans and animals and most often affects immunocompetent individuals. Most isolates produce large amounts of urease, and several authors suggest that cryptococcal urease play an important role in pathogenesis of the disease related to transmigration of yeast cells. It has been proposed that ammonia produced by the urease action may cause damage to the endothelium of the host favoring permeability, which provides the transmigration of yeasts towards the central nervous system (CNS). To analyze the potential virulence of *C. gattii* ureases we constructed knockout mutants for the structural *URE* gene and for the genes encoding the accessory proteins (*URE*D, *URE*F- *ure4* and *ure6* respectively). All knockout mutants showed reduced multiplication within macrophages. In the mice intranasally infected model mutants *ure1*Δ (lacking urease protein) and *ure6*Δ (enzymatically inactive apourease) produced increased blood burden and reduced mortality. Analysis of *C. gattii* knockout mutants showed that *C. gattii* urease plays an important role in virulence independent of enzymatic activity.

Keywords: *Cryptococcus gattii* R265, urease, accessory proteins, mutants, virulence factor.

INTRODUCTION

Ureases (EC 3.5.1.5, urea amidohydrolase) are nickel-dependent metalloenzymes that catalyze the hydrolysis of urea to form ammonia and carbon dioxide [1]. Ureases are produced plants, fungi and bacteria. The high sequence similarity suggests that all ureases posses similar tertiary structure and catalytic mechanisms [2]. Accessory genes are required for activation of urease apoprotein, and roles for these accessory proteins in metallocenter assembly have been proposed [3]. Many fungi pathogenic to humans produce urease, among which are *Cryptococcus neoformans*, *Coccidioides immitis*, *Histoplasma capsulatum*, *Sporothrix schenckii*, [4] and species of *Trichosporon* and *Aspergillus*. The coccidioidal urease gene has been shown to be expressed in vivo, and plays a role in both sporulation and pathogenesis [5].

In microorganisms, urease enables the utilization of urea as a nitrogen source and, in some cases, contributes as a virulence factor to several human and animals diseases, such as gastroduodenal infection by *Helicobacter pylori* and urinary stone formation by *Proteus mirabilis* [6]. However, this protein may also play other important roles unrelated to its enzyme activity [7], including insecticidal [8,9], fungicidal [10] and exocytosis-inducing activities [11]. As a consequence of urease-induced secretion of ADP from dense granules, platelets from different species including humans undergo aggregation when exposed to nanomolar concentrations of ureases from plants (jack bean and soybean) and bacterial ureases (*Bacillus pasteurii, Helicobacter pylori*) [12]. The platelet-activating property of these ureases was shown to be independent of urea hydrolysis [12–14].

C. neoformans causes the most common fungal infection of the central nervous system (CNS) in HIV-1/AIDS populations with high mortality and morbidity. Cryptococcosis became one of the three most common opportunistic diseases associated with high doses steroids therapies, such as in transplanted patients, or immune disorders [15,16]. The tropical climate and the onslaught of the AIDS pandemic since the early 1990s has led to a sharp increase in the number of reported cases of cryptococcosis in the past decade [17]. The disease symptoms include severe pneumonia with development of lung cryptococcal cysts and tropism to central nervous system (CNS) [18,19].

Several virulence factors of *C. neoformans* are known, including a polysaccharide capsule, melanin production, and the ability to grow at 37°C. The majority of clinical isolates produce large amounts of urease. There are many discussions about the primary role of urease in *C. neoformans* infections The C. *neoformans* urease gene (*URE*1) was first cloned and used to disrupt the native *URE1* in the serotype A strain H99 in 2000. Interestingly urease-negative mutants inoculated in mice produced lower mortality as compared to urease positive H99 strain-infected mice. Comparison of ure1 mutant with H99 after murine intravenous and inhalational infection models revealed significant differences in survival. Mice infected with ure1 strain lived longer than mice infected with H99 in both models [20]. Further studies with direct inoculation of H99, ure1, and ure1+URE1-1 into the brain demonstrated that urease was not required to grow in the brain. However, the dissemination patterns in the brain, spleen, and other organs after intravenous inoculation indicated that cryptococcal urease contributes to the central nervous system invasion by enhancing yeast sequestration within microcapillary beds (such as within the brain) during hematogenous spread, thereby facilitating blood-to-brain invasion by *C. neoformans* [20]. More recently analysis by intravital microscopy reveals that brain invasion by *C. neoformans* follows capillary microembolic event. A major finding of this study is that fungal cells stop suddenly in capillary sites without rolling or tethering, in a manner similar to polystyrene microspheres. The similarity of the arresting process to that exhibited by inert microspheres suggests that the initial brain localization mechanism is mechanical and related to an inability of yeast cells to traverse narrow capillaries. If this is the case, the initial brain infection results from a microembolic event. It was observed that after stopping, *C. neoformans* cross the capillary wall in a process that requires viability but not replication, is associated with deformation of cell morphology, and is urease dependent. Finally, the investigators showed that inhibiting urease reduces brain fungal burden, suggesting that this might provide an entirely new approach toward protecting the brain in cryptococcal meningitis [21]. Based on these observations, pharmacological inhibition or urease-targeted antibody therapies have been proposed as potentially useful strategies to protect the brain against yeast invasion [20–22].

In the present work we aimed to contribute to investigations on the role of *C. gattii* urease as a virulence factor in cryptococcosis. For that purpose, mutant yeasts were constructed that lack urease protein or produced an enzymatically inactive apoprotein. Deletion mutants were phenotypically and molecularly characterized and tested for their virulence in mice.

MATERIAL AND METHODS

Ethics Statement

All animal studies were reviewed and approved by the Ethics Committee for Use of Animals of the Federal University of Rio Grande do Sul concerning housing and care of laboratory animals (CEUA – protocol number 19801). Mice were housed in groups of eight and kept in filtered top ventilated cages, with constant temperature and humidity, with food and water *ad libitum*, following the guidelines of the Brazilian National Council for Animal Experimentation (CONCEA) and Brazilian College of Animal Experimentation (COBEA). Experiments were carried out in a level 2 biosafety laboratory according to rules of the National Technical Committee on Biosafety (CTNBio). The safety procedures applied to experimental procedures as recommended by this committee are available at < http://www.ufrgs.br/cbiot/CS/CS_CBiot01.htm>.

Fungal Strains, plasmids and Media

Cryptococcus gattii hypervirulent strain R265, serotype B, mating type α, molecular type VGII, from the cryptococosis outbreak in Vancouver Island was kindly provided by Dr. Wieland Meyer (Sydney University - Australia). Cells were grown under continuous shaking (200 rpm) at 30°C in YPD medium (1% w/v yeast extract, 2% w/v peptone, 2% w/v dextrose), or at 30°C on agar plates (YPD medium with 1.5% w/v agar). YPD plates with either hygromicin (200 µg/mL) or nourseothricin (100 µg/mL) were use to select deletion and complementation transformants, respectively. Plasmid pJAF15 [23] was the source of a hygromycin resistance cassette and pAI4 [24] was the source of a nourseothricin resistance cassette. Plasmids were maintained in *Escherichia*

coli grown at 37^{o}C in LB broth or agar supplemented with 50 µg/ml of kanamycin.

Identification of target genes and sequences for *in silico* analysis

The putative *C. gattii URE1* (CNBG_4331.2) was identified by analysis of the Broad Institute *C. gattii* R265 genome database available at <http://www.broadinstitute.org>. Alignment of sequences to find protein accessory *URE4* and *URE6* genes was done using ClustalX2 [25]. Phylogenetic trees were developed with MEGA5 [26].

Disruption and complementation of the *URE* and Accessory *URE* genes

Structural urease gene (*URE1*) and accessory ure genes (*URE4* and *URE6*) were deleted in *C. gattii* strain R265, individually. Disruption was based on Delsgate methodology [27]. A Gateway cloning system donor vector (Invitrogen, Carlsbad, CA) containing the hygromycin selectable marker for *C. gattii* transformation was constructed cloning 2.2 kb PCR product spanning the hygromycin marker cassette fragment cloned into the EcoRV site of pDONR201 (Invitrogen, Carlsbad, CA) [28]. The 5′ and 3′ target genes flanks were PCR amplified and purified from agarose gels (illustra GFX PCR DNA and Gel Band Purification Kit, GE Healthcare, Bucking-Hampshire, UK). The cloning system was done according to manufacturer's instructions from BP clonase reaction (Invitrogen, Carlsbad, USA) and previously grounded by the group [28]. The product of this reaction was transformed into *E. coli* OmniMAX 2-T1. After confirmation of the correct deletion construct, the plasmid was linearized with I-SceI prior to *C. gattii* biolistic transformation [29]. The *C. gattii* mutants were submitted to a previous screening test adapted in solid YPD agar supplemented with urea (300 mM), $NiSO_4$ (1µm) and a phenol red as pH indicator [30]. The urease activity is expected to convert urea into ammonia, resulting in an increase in the pH of the medium. This feature is reflected by a colour change from yellow to bright pink. Negative urease producing colonies (no color change) were further screened by colony PCR, and the deletion was confirmed by Southern blot and semi quantitative RT-PCR analyses.

For complementation of *C. gattii* R265 *ure1*Δ, *ure4*Δ, and *ure6*Δ mutant, a ~1 kb genomic PCR fragment containing the WT respective genes was cloned into the SmaI site of pAI4 containing nourseothricin selection marker and submitted to in *vivo* recombination as previously described [31,32] in chemo-competent OmniMAX 2-T1™ prepared as established [33]. The resulting plasmid was used for transformation of the mutant strains. The *C. gattii* complemented mutants were submitted to a screening urease test as described above to confirm restored ureolytic activity. Genomic insertion of the complemented gene was confirmed by Southern blot and semi-quantitative RT-PCR analyses. The primers used in these plasmid constructions are listed in Table S1. The strategy used for generation of *C. gattii* mutant strains is summarized in Figure 1.

Southern blotting and quantitative real time RT-PCR analysis

Correct integration of the inactivation cassette into the WT, *URE1* or *URE4* and *URE6* locus was evaluated by Southern blotting and RT-PCR analysis. For Southern blotting, genomic DNA (10 µg) from strains was digested with the specific restriction enzyme for each mutant, as shown in Figure 1. The 3′ or 5´ flanking region was used as the Southern hybridization probe.

For RNA extraction, mutants and WT strains cultures were grown in YPD media (18-20 h/30°C) with shaking. RNA extraction and cDNA preparation were performed as previously described [34]. The Applied Biosystems (New York, USA) 7500 real-time PCR System was used for the real time PCR analysis and the PCR cycling conditions as well as the melting curves and relative expression determinations, were performed as described [34]. The experiments were performed with two biological samples and each cDNA sample was analyzed in triplicate with each primer pair. Actin cDNA levels were used to normalize each set of PCR experiments. The sequences of each primer used are listed in Table S1.

Phenotypic characterization assays

-Urease Activity

The rapid urea broth (RUH broth) developed by Roberts [35] and adapted by Kwon-chung [36] was used to detect ureolytic activity of wild type and mutants yeasts. *C. gattii* strains grown in solid YPD for 24h at 30°C were suspended in 2 ml of sterile phosphate buffer (PBS) pH 7.2. The optical density at 600 nm (OD_{600}) of the cell suspensions was measured and adjusted to 0.7. The cell suspensions were vortexed, mixed to an equal volume of 2x RUH broth mix, and kept at 37°C under shaking for 10 h. After 6, 8 and 10 h, the culture was centrifuged and the supernatant OD_{560} was determined. A sterile PBS blank was used as a control. The assay was performed in triplicates for each measured time.

-Urease inhibition in WT strain

C. gattii WT strain were grown for 20h in YPD, washed with phosphate-buffer-saline (PBS) and diluted for optical density of 1.0 in 1mL cell suspension at 600 nm. The suspension were incubated with different concentrations (0.5 to 20 mM) of the urease inhibitor acetohydroxamic acid (AHA) [84] and incubated for 18 h, under shaking at 7°C; a parallel control suspension of *C. gattii* with buffer and without AHA was used. After incubation cells were washed three times with PBS and macrophage interaction experiments were performed as described previously. Aliquots of all AHA inhibition experiments were plated on YPD agar for cell viability analysis. AHA-treated cells were monitored by Roberts test for 18 h in parallel to the macrophage interaction assay to certify the absence of urease enzymatic activity during the whole assay.

-Melanin production

Melanin production was assayed by yeast growth on Niger seed agar at 30°C and 37°C. Alternatively, *C. gattii* strains (wild type R265 and mutants)

were grown overnight at 30°C in YPD broth and the cells were pelleted by centrifugation, washed twice with sterile PBS and suspended in PBS at different OD_{600} dilutions (1, 0.1 and 0.01). Aliquots (5 μl) were spotted onto 1 mM L-DOPA agar [37], the plates were incubated at 30° C and 37°C and melanization was observed up to 7 days of incubation.

-Laccase Activity

Assays for laccase activity followed [38] with slight modifications. Cells were grown overnight in YPD, washed twice, and transferred to asparagine media with 0.1% dextrose or galactose (7.6 mM L-asparagine, 22.1 mM KH_2PO4, 1 mM $MgSO_4 \cdot 7H_2O$, 3.0 mM thiamine-HCl, 4.1 mM biotin, pH 5.6). After 1 day, a total of 10^7 cells were inoculated into 50 mM sodium phosphate buffer containing 10 mM L-DOPA. After incubation at 30°C for 90 min, cells were pelleted and the OD of the supernatant was recorded at 475 nm (0.001 corresponds to 1 U laccase). Assays were run in triplicate.

-Capsule induction and Measurement

Capsule thickness and cell diameter ratio of mutants and wild type *C. gattii* strains were measured under microscopic examination of India ink preparations of yeast cells grown overnight in DMEM (GIBCO) with 10% fetal bovine serum at 30°C with 5% CO_2 which stimulate capsule production. Microscope Software AxioVision LE (Carl Zeiss ®) was used to determine the capsule measurements of at least 100 cells of each strain.

-Sensitivity to temperature (37°C) and oxidative conditions

To determine sensitivity of mutant strains to growth temperature 5 μL of diluted cultures (OD_{600} 1.0, 0.1 and 0.01) of each yeast strain were spotted onto solid YPD medium and incubated at 30°C or 37°C. Yeast growth was estimated visually after 24 and 48 hours growth.

To determine sensitivity of mutant strains to oxidative stress conditions, *C. gattii* strains were exposed to H_2O_2. Yeasts were grown in 25 mL YPD

overnight, pelleted, washed three times with sterile PBS and ressupended in PBS. Five microliters of diluted cultures (OD_{600} 1.0, 0.1 and 0.01) of each yeast strain were spotted onto solid YPD medium containing 2.0, 5.0 and 10 mM H_2O_2. Yeast growth was estimated visually after 24 and 48 hours growth at 30°C or 37°C.

Macrophage infection assay

A phagocytosis assay was performed to evaluate if *C. gattii* wild type and mutant *strains* were internalized by macrophages and if internalized yeast cells could multiply within macrophages.

Macrophage-like RAW264.7 cells (1×10^5 cells/100 µl of DMEM supplemented with 10% FBS) were placed into 96-well culture plates. After 24 h at 37°C and 5% CO_2, the medium was replaced with fresh medium containing 1×10^6 *C. gattii* (mutants or wild type strain) previously incubated 24-h in YPD and extensively washed in PBS. Macrophages were allowed phagocytosis of *C. gattii* cells during 1h (37°C, 5% CO_2), then the medium was removed and replaced with DMEM/FBS. After a second incubation of 18 h (37°C, 5% CO_2), yeast cells not associated with the macrophages were removed by PBS washes. To assert fungal survival after being phagocytosed, macrophages were lysed with sterile ice-cold Milli-Q water and the lysates were subsequently plated on YPD plates for CFU determination, after incubation for 48 h at 30°C. This assay was performed in triplicates for each strain.

Mice Survival assay and Organ fungal burden

Cryptococcus strains were grown for 24 h in YPD, washed three times with phosphate-buffered saline (PBS), counted in a Neubauer Chamber, and resuspended in PBS at a concentration of 2.0×10^5 yeast cells/ml. Confirmation of cell concentration was carried out by CFU determination in YPD agar. The mouse intranasal inoculation method of *Cryptococcus* sp has been described elsewhere [20,28]. Briefly, Balb-C female mice (5 weeks old, ~20g) were anesthetized with 100 mg/kg Ketamine and 16 mg/kg Xylazine given by intramuscular injection. Anesthetized mice then were suspended by their

incisors on a thread to fully extend their necks, and 50 µl of the yeast suspension (5×10^4 cells) was slowly pipetted into the nostrils of each mouse. The mice were kept suspended for an additional 10 min and then placed on ventilated cage to recover. Survival rate was accompanied in groups of 8 animals per *C. gattii* mutant or wild-type strain. For the organ fungal burden assay, mice were euthanized by CO_2 inhalation on the 7^{th} and 18^{th} day after inoculation, and their lungs, brain, spleen, kidney and blood were dissected under aseptic conditions. The organs were weight, macerated with 1 mL sterile PBS, and then appropriate dilutions of the homogenates were plated on YPD plates for CFU determination.

Statistical Analyses and Softwares

Kaplan–Meier analysis of survival was performed using GraphPad Software 5.0. One-way ANOVA followed by Bonferroni's test was used to evaluate statistical parameters of capsule size, organ burden and macrophage interaction.

RESULTS AND DISCUSSION

Identification of *URE* and Accessory proteins genes from *C. gattii*

A database search in the *C. gattii* R265 genome Fungal Genome Initiative database at the Broad Institute of MIT and Harvard, available at <http://www.broadinstitute.org/annotation/genome/cryptococcus_neoformans_b/MultiHome.html> (August 5, 2009) allowed the identification of the *C. gattii URE1* gene (CNBG_4331.2), as well the *URE4* gene (CNBG_0735.2) and *URE6* gene (CNBG_5207.2).

Phylogenetic analysis of *C. gattii* accessory proteins and significant Pfam matches [39] confirmed their similarity to orthologue proteins in plants and fungi (Supplementary Figures 1 and 2). *C. gattii* urease appears as a typical fungal urease with a single subunit of 833 amino acids, a molecular mass of 90kDa, and a theoretical pI of 5.5. While plant and fungal ureases are oligomers of identical subunits, typically of ca. 90 kDa, bacterial ureases are

complex multimers with two or three subunits [6,39,40]. Remarkably, though composed of different types of subunits, ureases from different sources extending from bacteria to plants and fungi exhibit high homology of amino acid sequences. When aligned with jack bean urease *C. gattii* Ure displays 58% identity. This suggests that all ureases are evolutionary variants of one ancestral enzyme [40]. Ure accessory proteins are not as conserved as Ure proteins in different species, with exception of Ure7, which presents regions of conserved sequence of different size. Multiple sequence alignment of *C. gattii* Ure accessory proteins with those of different organisms identified their characteristic domains, as detected by Pfam (Supplementary Figures 1-3).

Regarding phylogenetic inferences, urease accessory proteins Ure4, Ure6, and Ure7 in *C. gattii* seem to be ancestral to the orthologous proteins in fungi from Classes Agaricomycetes and Basidiomycetes. These proteins also show clear divergence from those from Ascomycota representatives. The Ure6 and Ure7 orthologues from plants seem to be the most distant from the clade containing the proteins from *C. gattii*, while the orthologs from *Wallemia sebi* (Class Wallemiomycetes) are the closest to the referred clade. This topology is somewhat different for the Ure4 tree, with proteins from photosynthesizing organisms grouping with the Basidiomycota clade. It is interesting to note that UreD is one of the less-conserved accessory proteins and the one for which very little information is available [41].

C. gattii transformants

The construction of vectors for transformations of *URE* and accessory proteins *URE*4, *URE*6, *URE*7 are summarized in Figure 1. Flanks from 5´and 3´ corresponding genes were fused to hygromycin cassette in pDONR-HYG [34,42] vector by Delsgate methodology [27]. The constructed and linearized target vectors were used to transform *C. gattii* R265 cells by biolistic [29]. The resulting Hygromicin resistant yeast colonies were selected to screening and correct insertion confirmation.

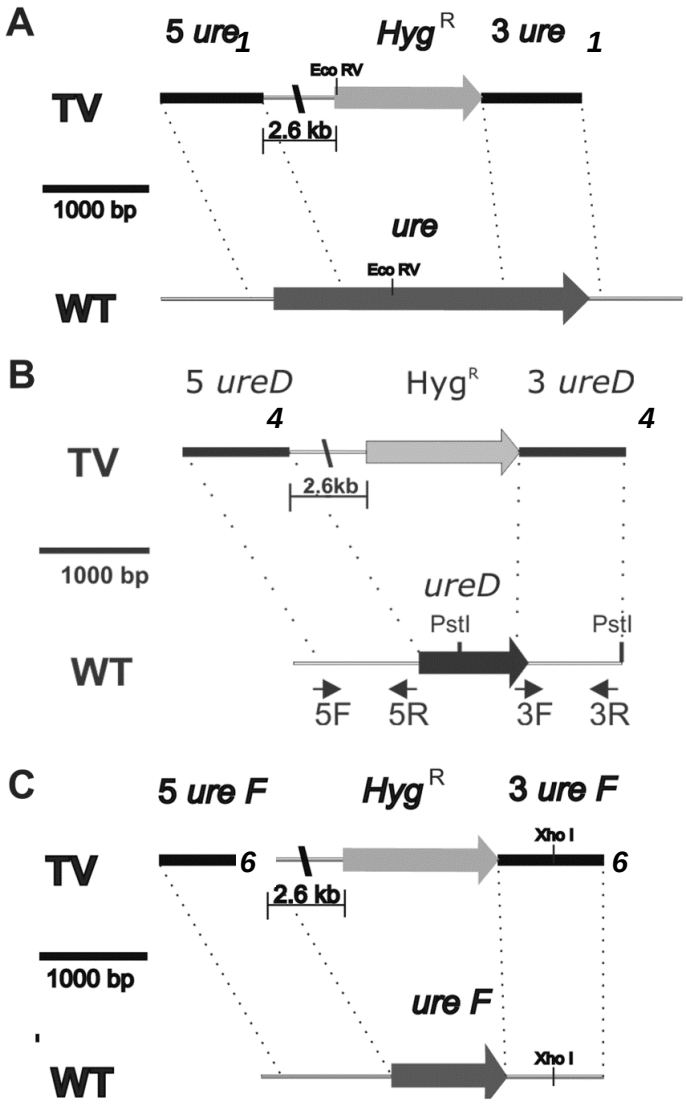

Figure 1: Ilustration of mutants constructions: target genes were replaced with hygromicin resistant mark cassette in light grey box (Hyg®). Target genes 5´and 3´flanks were fused with hygromicin cassette by Delsgate methodology [27]. The wild type locus of each gene and primers positions for dene disruption are indicated. The constructed target vector (TV) was used to *C. gattii* transformations. Restriction enzymes are also indicated for each TV.

The transformants were submitted to screening tests. Hygromicin resistant yeast colonies was spotted on YPD agar plates supplemented with urea, Ni^{2+} and phenol red pH indicator [30]. Wild type *C. gattii* strain was used as positive control. After 24 or 48h incubation at 30°C, colonies were selected based on the absence of a reddish color which indicated deletion mutants lacking ureolytic activity while colonies positive for urease production showed a pink magenta color (Figure 2).

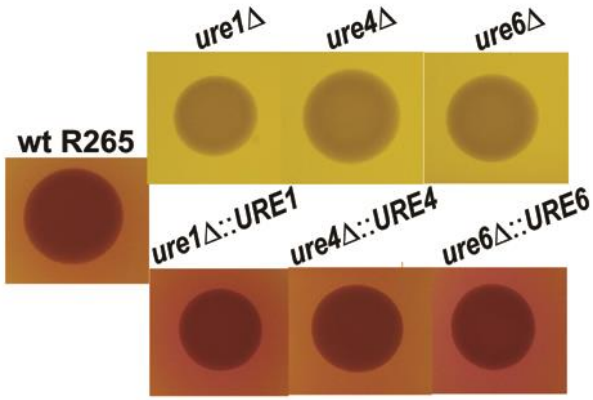

Figure 2: Urease activity screening test. Urease activity-screening test on YPD agar. To verify whether the transformants had deleted enzymatic activity or recovered activity (in the reconstituted mutants), spotted colonies on YPD agar, supplemented with urea and Ni^{2+}, denoted ammonia production by a pH increase indicated by a color change to pink/magenta. The tested strains were: WT R265, wild type *C. gattii* R265; Δ*ure1, ure* deleted *C. gattii* R265, Δ*ure*D, *ure*4 deleted *C. gattii* R265, Δ*ure*6, *ure*6 deleted *C. gattii* R265; Δ*ure1*::*URE1, ure1* reconstituted in Δ*ure1* deleted *C. gattii* R265; Δ*ure*4::*URE4, ure*4 reconstituted in Δ*ure4* deleted *C. gattii* R265; Δ*ure6*::*URE6, ure*6 reconstituted in Δ*ure*6 deleted *C. gattii* R265.

Selected yeast colonies of transformed *C. gattii* were submitted to colony PCR to confirm deletion of the target gene using primers corresponding to internal portions of the gene. The absence of amplification as compared to that obtained using genomic DNA from *C. gattii* R265 as template certifies total gene deletion from the transformants. Actin primers were used as control for negative PCR amplification samples (Figure 3).

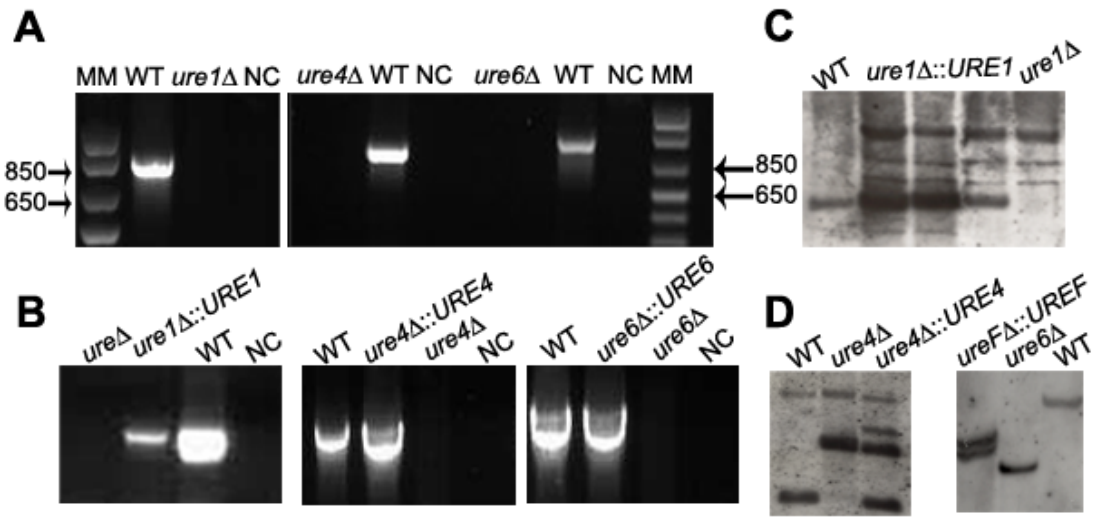

Figure 3: PCR confirmation of transformants. **A and B.** PCR of genomic DNA was used to confirm disruption and gene reconstitution with specifically designed primers. PCR test for target gene disruption and genes disruption and reconstitution. NC, negative control; MM, molecular marker. Arrows indicate the molecular marker sizes (base pairs). **C and D**. Southern blots of *URE1* (three distinct reconstituted clones), *URE4* and *URE6* mutants, confirming deletions and reconstituted genes.

Complementation of deletion mutants resulted in more than one clone. Ureolytic activity of the complemented clones was determined in yeast cell extracts using the rapid urea test [36]. The assay was developed to determine enzyme kinetics up to 10 hours (Figure 4).

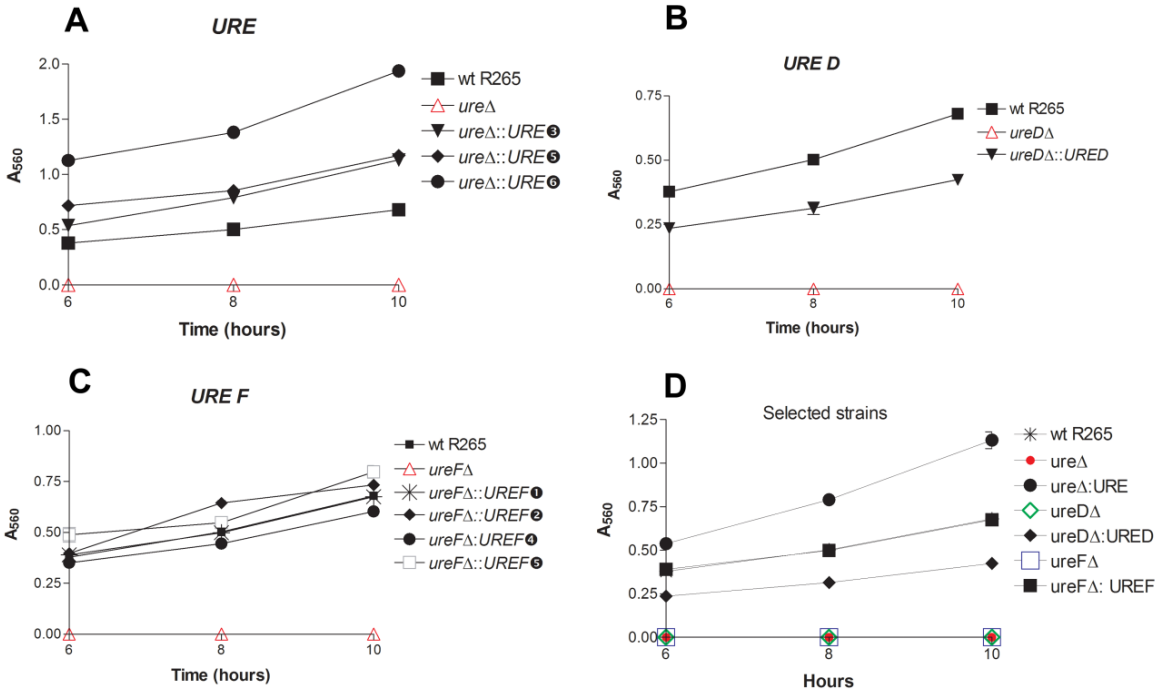

Figure 4: Ureolytic activity from transformants cells. *C. gattii*. Urease activity was measured in cell extracts incubated for 10 hours in urea buffer (see material and methods) in order to choose reconstituted transformants with ureolytic activity similar to wild type (WT) yeasts. Assay made in triplicates with end point readings at 6, 8 and 10 hours. **A- C** *URE1, URE4, URE6* reconstituted transformants **D**- Selected strains compared to WT and *ure1*Δ. Data shown are means ± s.d. of triplicates with end point readings at 6, 8 and 10 hours.

From the results shown in Figure 4, all reconstituted mutants showed close to or even higher levels of ureolytic activity as compared to wild type *C. gattii*. Based on these results *URE1* reconstituted clone 2 and *URE6* clone 1 were selected for the next experiments.

Semi quantitative PCR confirmed transformation of selected mutants strains (Figure 5) and successful reconstitution of the deleted genes. Amplification of actin was used as control of the PCR reaction.

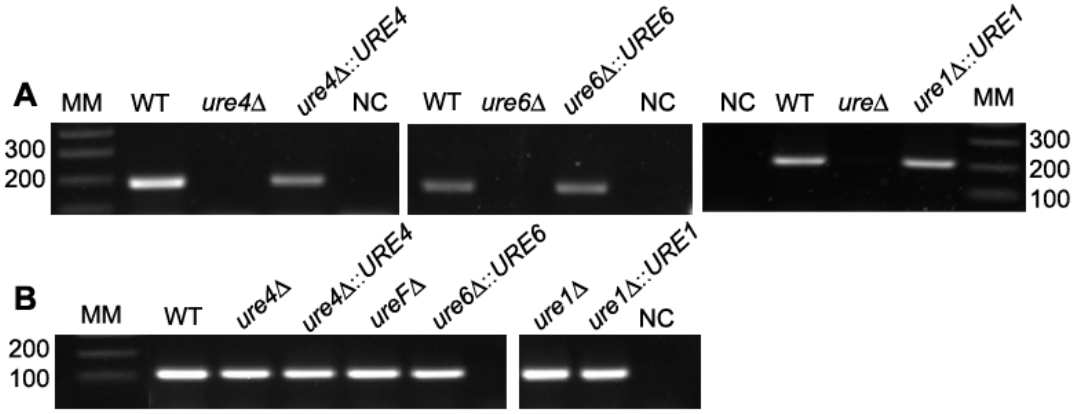

Figure 5: Semi quantitative RT-PCR from selected transformants. A. RT-PCR for the target genes **B**. Actin control, lane 1; WT, lane 2; *ure1*Δ, lane 3; *ure1*Δ::*URE1*, lane 4; *ure4*Δ, lane 5; *ure4*Δ::*URE4*, lane 6; *ure6*Δ, lane 7; *ure6*Δ::*URE6*, Negative Control (NC).

In order to determine which strain to use for *in vivo* experiments, the transformants were tested for survival under oxidative conditions. For that, yeasts were grown at 30°C or 37°C in the presence of H_2O_2. The results showed that URE4 mutants were unable to grow in the presence of 5mM H_2O_2 either at 30°C or 37°C (Figure 6).

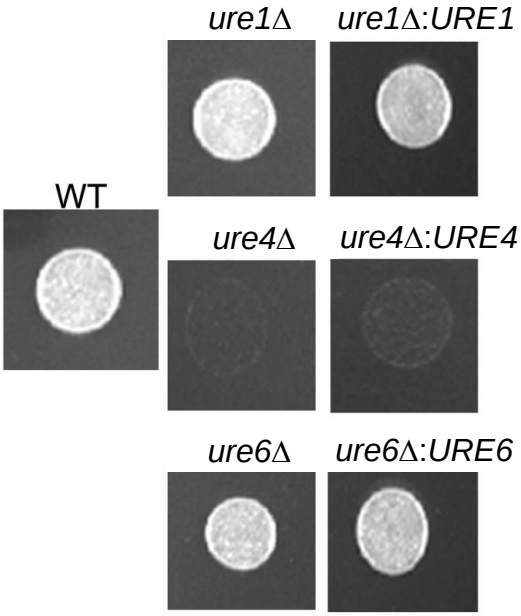

Figure 6: Decreased growth of URE D mutant in presence of 5 mM H_2O_2. Aliquots of 5 µL of the yeasts suspensions (OD_{600} 0.1) were incubated at 37°C for 24h on YPD agar plates with 5mM H_2O_2. The result was the same after 48h of incubation either with 5 and 10 mM H_2O_2 concentration.

Virulence Factors in *C. gattii URE* transformants.

All mutant strains were evaluated regarding the most studied virulence factors, namely the synthesis of capsular polysaccharide(s), yeast melanization, and ability to grow at 37°C [43]. Figure 7 illustrates that all mutants (*URE1*, and accessory proteins *URE4,* and *URE6*) showed normal growth at 37°C and homogeneous mean values of capsule size (Figure 8). Interestingly, *URE1* mutants showed increased melanization ability (Figure 9), indicating that the activity of laccase is somehow affected in *URE1* mutants (Figure 10).

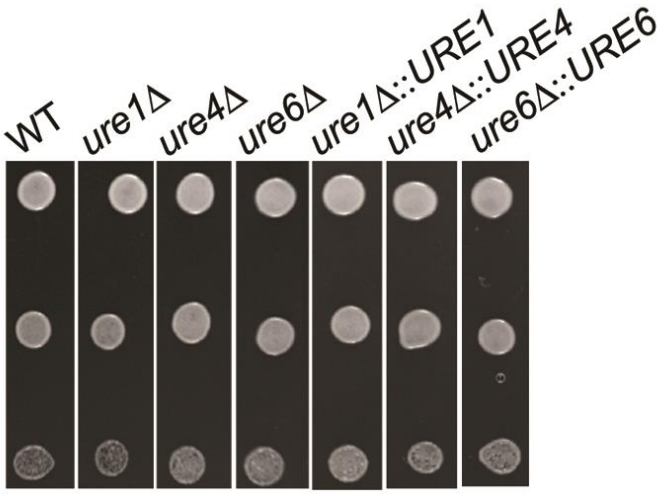

Figure 7: Ability of mutant strains to grow at 37°C. Wild-type (WT) and mutant strains (initial OD_{600} 0.1) were incubated at 30°C and 37°C in YPD medium. The pictures shows the cultures photographed after 24 h of incubation at 37°C.

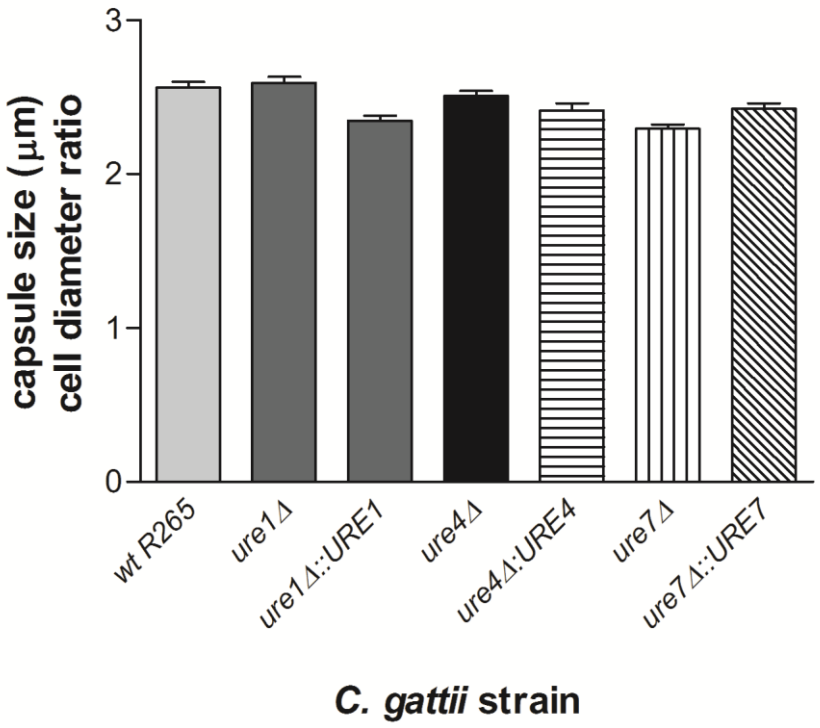

Figure 8: Evaluation of capsule ratio and in mutant strains: Capsule ratio (capsule tickness – cell diameter ratio) was measured for *C. gattii* wild-type and mutants after overnight growth at 30^0C, 5% CO_2.

Studies have shown that various cryptococcal virulence factors contribute to extrapulmonary dissemination, including the mating type, phenotypic switching, and production of capsular polysaccharide, mannitol, melanin, phospholipase, prostaglandins and urease [44]. Is well known that *Cryptococcus* yeasts have a thick cell wall with the deposition of the phenolic melanin, which has been proposed to protect cells from oxidation. Laccase, a key enzyme required for melanin biosynthesis, is also important for fungal virulence. Mutant strains lacking laccase production have attenuated virulence due to inability to escape from the lungs, without affecting their growth either in the blood or the brain, indicating that laccase plays a role in the dissemination of *Cryptococcus* into the bloodstream [45].

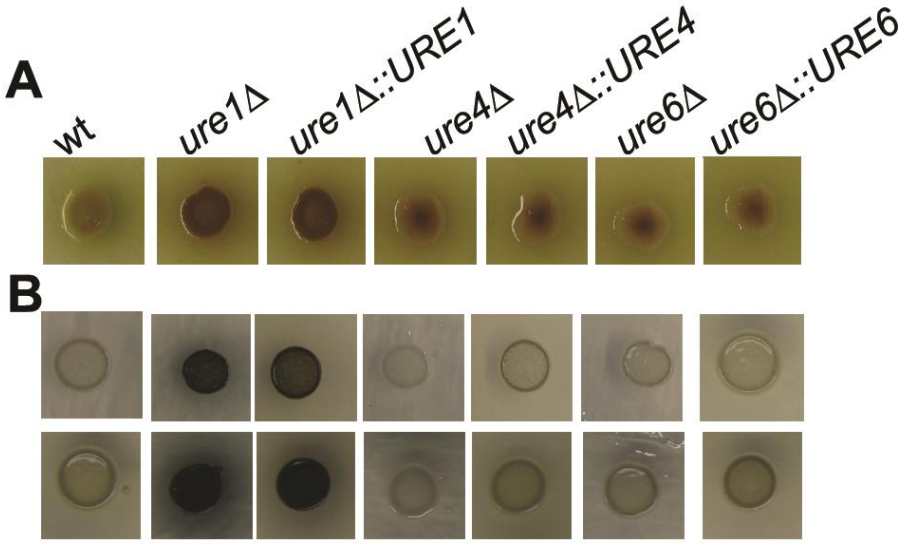

Figure 9: Increased melanization of *URE* mutants. **A**. Yeast strains (3 µL of a cell suspension with OD_{600} 1.0) were grown on Niger seed agar for 48 h. Melanization of both *URE* mutant strains, *ure1*Δ and *ure1*Δ::*URE*1, started earlier than other strains and could be visualized after 24 h. **B**. Melanization of yeast strains (5 µL of cell suspensions with OD_{600} 1, 0.1 and 0.01) was accompanied during 7 days in solid minimal medium supplemented with 1 mM L-DOPA. Mutants *ure*Δ and *ure1*Δ::*URE1* showed enhanced melanization either at 30° C and 37°C.

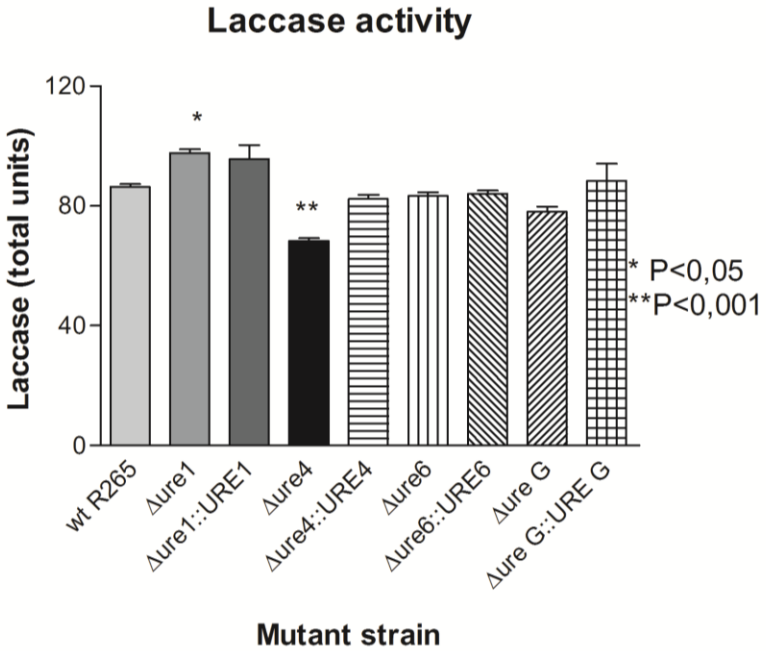

Figure 10: Evaluation of laccase activity in mutant strains: Laccase activity measured in cell suspensios of mutant yeasts. Results are means ± sd of triplicates.

Next we evaluated the phagocytic activity of macrophages towards *C. gattii* mutant strains and the ability of the yeast cell to survive inside macrophages. Figure 11 shows that all yeast strains were equally phagocytosed by macrophages after 1 hour of exposition and survived within the phagocytes for at least 18 h. However, *URE1* deletion mutants did not multiply within macrophages, despite their increased ability to melanin (figures 9 and 10), contrasting to wild-type *C. gattii*, which almost quadruplicated the number of cells after 18 h inside the phagocytes. The ability to survive phagocytosis and multiply within the macrophages was partially restored in the reconstituted mutants (Figure 11B).

Our data thus show that mutants that lack the urease protein (*ure1*Δ) or express enzymatically inactive apoureases (*ure4*Δ and *ure6*Δ) did not multiply within macrophages. Similarly, treatment of WT with acetohydroxamic acid, a Ni^{2+} chelant inhibitor of urease activity [38], did not affect phagocytosis by macrophages but blocked the ability of the yeast to multiply within the cell in a dose-dependent fashion (Figure 11C). Taken together, these data indicate a relevant role of the ureolytic activity in survival and/or multiplication of yeasts within the macrophages. Previously, it was proposed that urease-mediated alkalinization and phagosomal cathepsins inhibition, which are promoted by urease-producing microorganisms, are important factors allowing the survival and multiplication of such microorganisms within phagosomes [46].

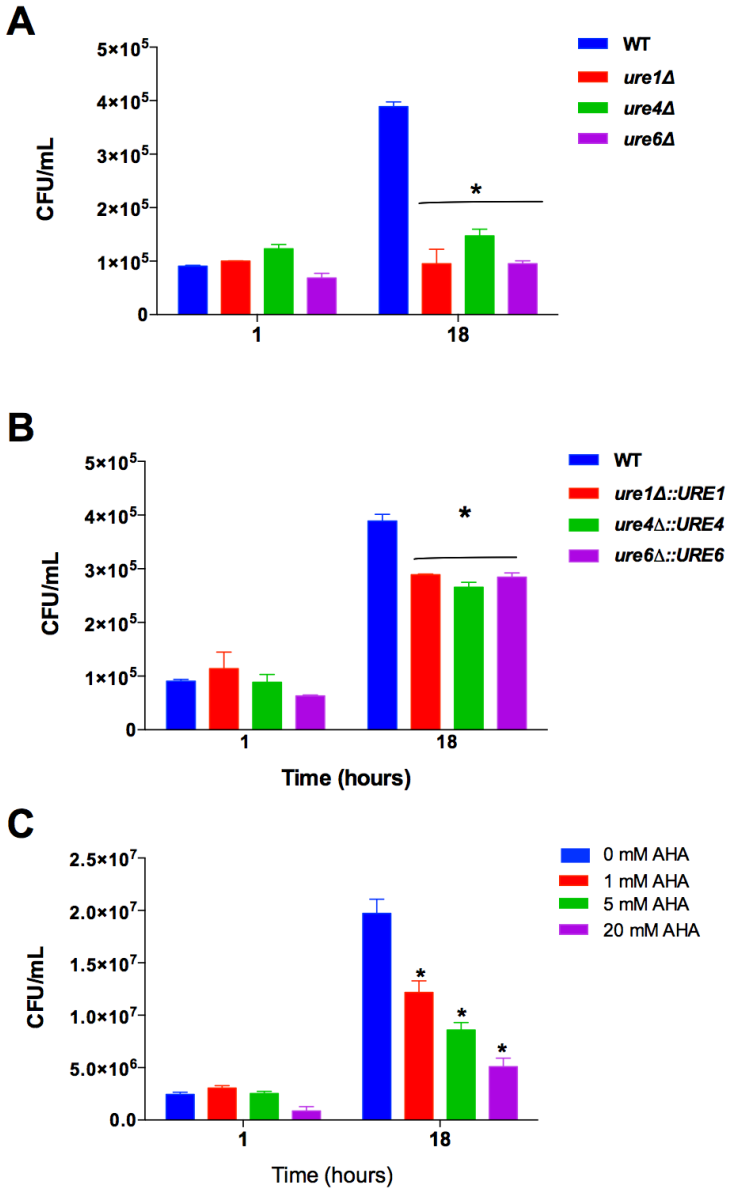

Figure 11: Role of urease on cryptococcal survival and multiplication in macrophages. Macrophage-like RAW264.7 cells were incubated with *C. gattii* cells for 1h (37°C, 5% CO_2) to allow phagocytosis. The medium was then removed and replaced with DMEM/FBS. After a second incubation for 18 h (37°C, 5% CO_2), yeast cells not associated with macrophages were removed with PBS washes. Fungal survival was evaluated after macrophage lysis with sterile ice-cold Milli-Q water and subsequent plating of the lysates on YPD plates for colony formation unit (CFU) determination. **A**. Results obtained with deleted mutants, compared to WT strain. **B**. Results obtained with reconstituted strains, compared to WT strain. **C**. Results obtained with the WT strain pretreated with different concentrations of acetohydroxamic acid (AHA), an urease inhibitor. The error bars represent standard deviation from technical triplicates of two independent biological replicates. The statistical analyses were conducted with the T-test. *, $P<0.05$ in relation to WT or control.

Experimental infection in mice by intranasal inoculation was carried out with wild-type *C. gattii*, the deletion mutants *URE1* (lack of protein) and *URE*6 (inactive apourease) and correspondent reconstituted strains. *C. gattii* cells were reported to produce smaller capsules in general and are more efficient in crossing the blood-brain barrier and causing CNS infection. Preliminar experiments using a inoculum of 10^7 cells showed no significant difference in mortality when comparing all mutant strains to wild-type yeast, with mean value of 13 days to death (data not shown). This high inoculum infection, previously used in studies with other strains and virulence factors in *C. neoformans* [28,49] proved inconclusive to discriminate differences among the *C. gattii* strains for brain invasion observation.

Considering that brain invasion, peculiarly to *C. gattii* R265 – induced cryptococosis, is subsequent to acute and severe lung infection, this results agrees with a previous comparison between mice infected via the pulmonary route with *C. neoformans* strain H99 or *C. gattii* R265, in which mice infected with H99 appeared to have been killed by brain infection, while those infected with R265 were killed by lung infection [47]. Thus, another experimental infection was carried out using a lower inoculum (2 x 10^5 yeast cells). Animals euthanized at an early infection stage (7^{th} day) showed low burden in lungs for all strains, even for wild-type yeast. However at the 18^{th} day post-inoculation, significant differences could be seen when comparing *URE* mutants (lacking urease protein) to wild-type strain, with a much lower lung or almost null blood yeast burden (Figure 12 C and D). On the other hand, mutants producing inactive apourease behaved similarly to the wild-type strains, pointing to an ureolysis-independent contribution of *C. gattii* urease to cryptococosis. The lower spread of *URE* mutants from lung (Figure 12C) to blood at this stage of infection and their impaired ability to survive and multiple within phagocytes *in vitro* suggest an involvement of urease as a virulence factor starting right after lungs infection.

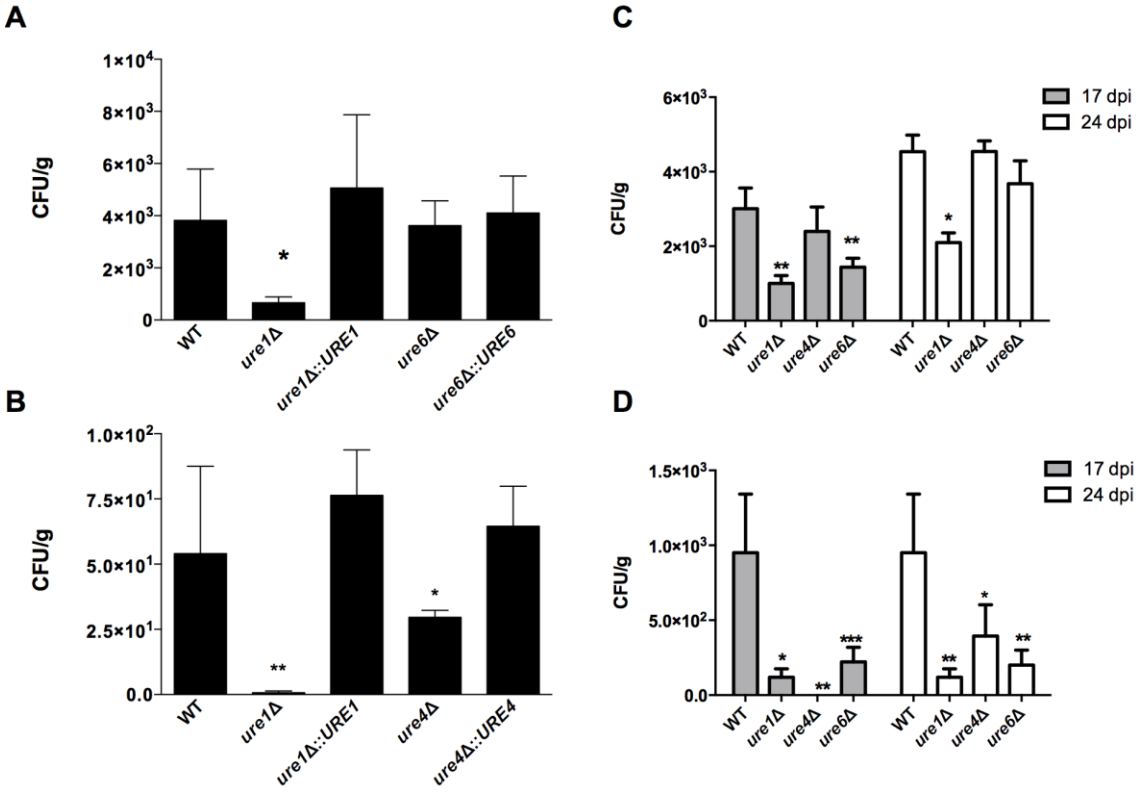

Figure 12: Urease activity alters organ fungal burden in murine models of cryptococcosis. The fungal burden was assessed in lungs (A, C) and blood (B, D) of mice infected with WT, mutant and complemented *C. gattii* strains. BALB/c mice were inoculated via intranasal instillation of 1 x 10^7 (A, B) or 5 x 10^4 (C, D) of *C. gattii* strains, and the CFU determined from organs collected from infected mice at 18 days post-infection (dpi) (A, B) or 17 and 24 dpi (C, D). The statistical analyses were conducted with the T-test. Samples that showed difference to WT or control were assigned with * (P<0.05) or ** (P<0.01).

Using an intranasal model of murine infection, we found that mice infected with the *URE1* and *URE6* null mutant survived longer (median survival 34 days and 31 days respectively) than those infected with the WT (24 days) and complemented strains median survival 25 and 26 days, respectively (Figure 13). Besides, mutants infected mice presented clearly attenuated symptoms showing locomotion activity, photophobia and food consumption comparable to control non-infected mice.

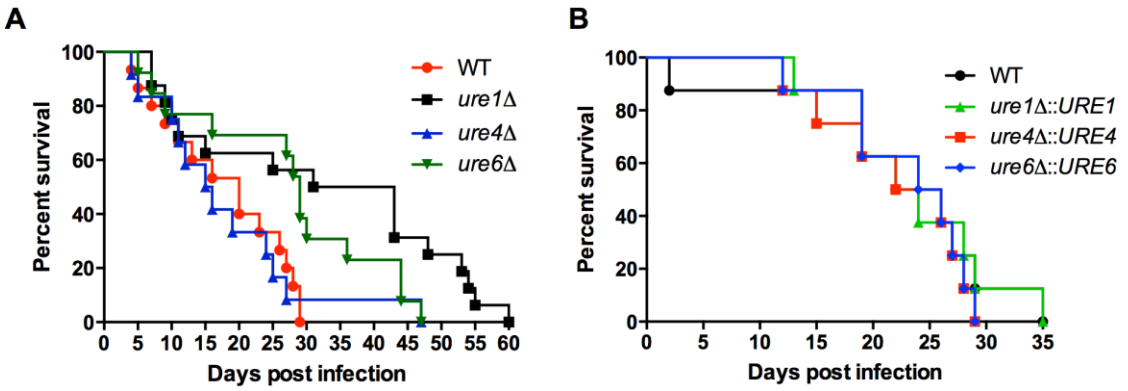

Figure 13: Some *URE* genes are necessary for proper cryptococcal virulence. Survival analysis of the WT, null mutants or complemented mutants in a murine model. **A**. Virulence assays of the WT, *ure1*Δ, *ure4*Δ and *ure6*Δ mutant strains in an intranasal inhalation infection BALB/c mouse model. **B**. Virulence assay performed with complemented strains.

Results from survival assay clearly point to a role of urease as virulence factor in *C. gattii* cryptococosis, as previously suggested for *C. neoformans* infections. Moreover, our data indicate that ureolysis by cryptococal ureases is minor contribution to its role as a virulence factor, since mutants producing an enzymatically inactive apourease showed no significant differences in blood or lung yeast burden, or even mortality, as compared to wild-type *C. gattii*.

CONCLUDING REMARKS

The present work corroborated previous studies pointing to a role of *Cryptococcus* urease as relevant virulence factor in cryptococosis. Furthermore, this is the first report to indicate that fungal ureases also display enzyme independent biological activities. In agreement with these findings, previous studies of our group showed that the bacterial urease of *Helicobacter pylori* displays enzyme-independent properties, such as platelet-activating and pro-inflammatory activities, which contribute to its role as an essential virulence factor of this gastric pathogen [8,12,13]. Knockout mutants of the structural gene or of accessory proteins were essential to discriminate between enzyme-dependent and independent effects of *C. gattii* urease. Additional studies are still necessary to understand the mechanism by which urease induces

transmigration of yeast cells. Further characterization of urease mutants yeast should explore their ability to colonize the CNS upon alternative inoculation routes (i.v., intracerebral). Even confirming recent study that improve lungs as first target of *C. gattii* R265 [47], this work was able to begin new and necessary discussions about the role of Cryptococcus urease beyond its ureolytic activity. Finally, our data support the proposition that targeting Cryptococcus urease might provide an entirely new therapeutical approach to cryptococosis and cryptococcal meningitis [20–22].

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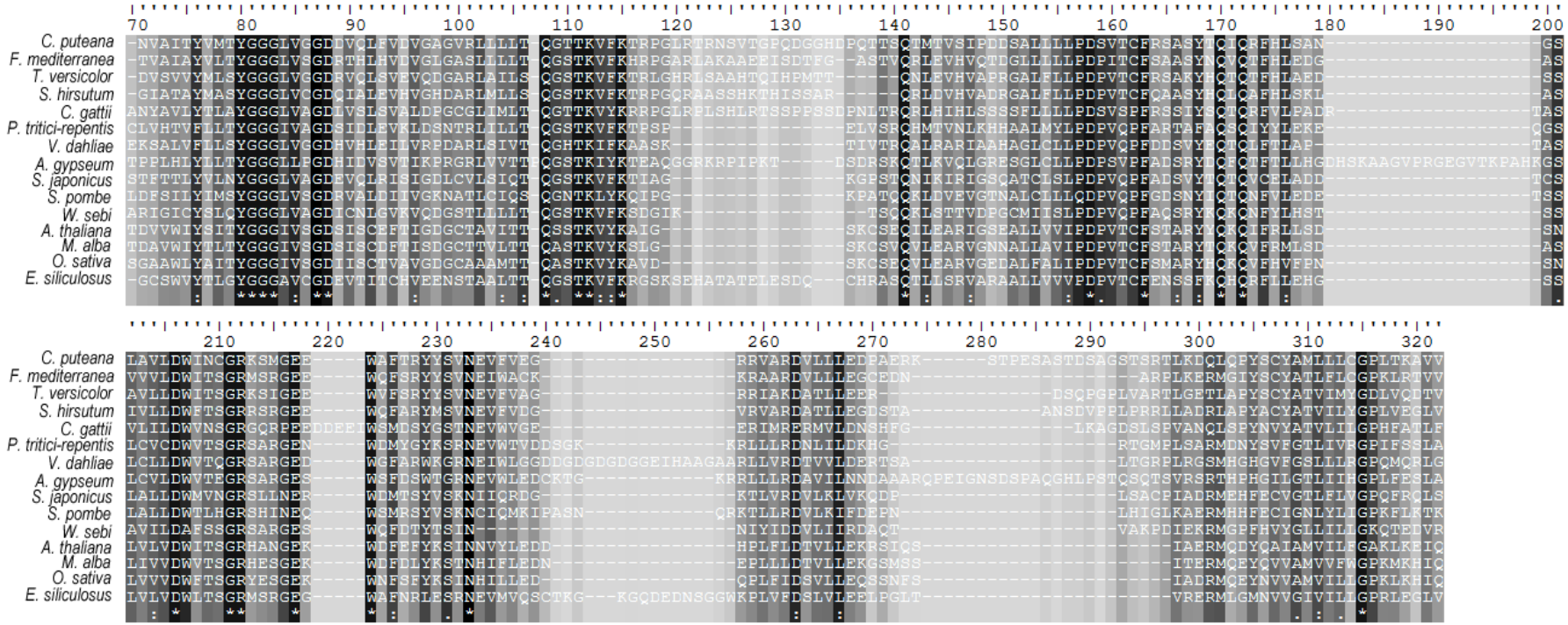
SUPPLEMENTARY MATERIAL

A


C. puteana
F. mediterranea
T. versicolor
S. hirsutum
C. gattii
P. tritici-repentis
V. dahliae
A. gypseum
S. japonicus
S. pombe
W. sebi
A. thaliana
M. alba
O. sativa
E. siliculosus

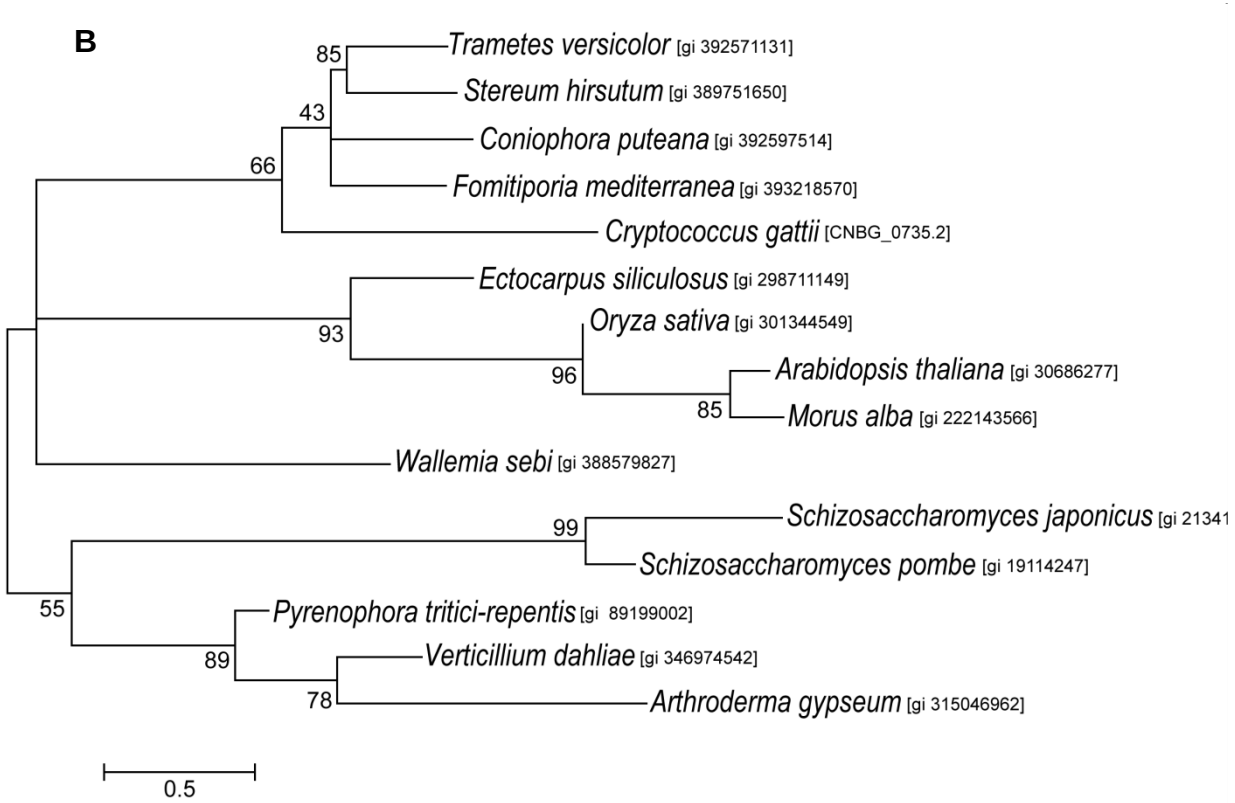


Figure 1. (A) Multiple sequence alignment of the Ure4 characteristic domain in different proteins, as detected by Pfam. **(B)** Molecular Phylogenetic anaylsis of Ure4 orthologs by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the Whelan and Goldman model with discrete Gamma distribution and some evolutionarily invariable sites (WAG+G+I). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

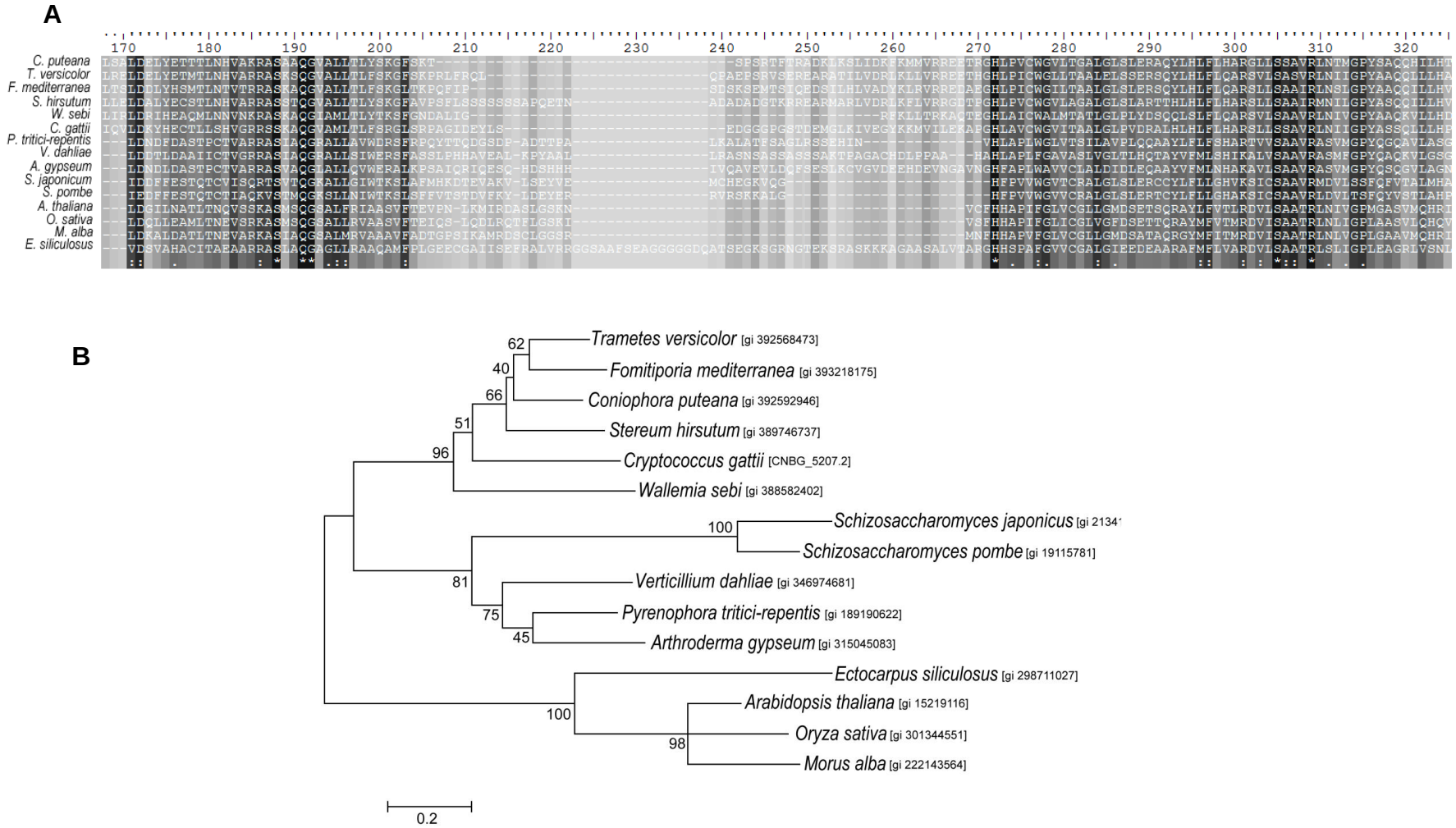


Figure2. (A) Multiple sequence alignment of the Ure6 characteristic domain in different proteins, as detected by Pfam. **(B)** Molecular Phylogenetic anaylsis of Ure6 orthologs by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the Whelan and Goldman model with discrete Gamma distribution (WAG+G). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

Suplementary Table 1: List of primers used during this work.

Primer Name	Sequence (5´- 3´)	Size (bp)	Function
Ure Del 5´F	AAAATAGGGATAACAGGGTAATGCATCTCCTCCCAAGAGAAACG	798	Disruption construct for URE 5´flank
Ure Del 5´R	GGGGACAAGTTTGTACAAAAAAGCAGGCTATTTGTCACAGTTTTCTTCTCCC		
Ure Del 3´F	GGGGACCACTTTGTACAAGAAAGCTGGGTACTCTCGTATCCGCTCTGAAACC	791	Disruption construct for URE 3´flank
Ure Del 3´R	AAAAATTACCCTGTTATCCCTAGCGCGCACATCGTAGGTCTCG		
Ure D Del 5´F	AAAATAGGGATAACAGGGTAATTTGCTTTACCCTTTCCTCCTAC	688	Disruption construct for URE 4 5´flank
Ure D Del 5´R	GGGGACAAGTTTGTACAAAAAAGCAGGCTATCATCGGCGGGGTAATATAGATA		
Ure D Del 3´F	GGGGACCACTTTGTACAAGAAAGCTGGGTATTTGACATGGAGTTTTAGTGA	679	Disruption construct for URE 4 3´flank
Ure D Del 3´R	AAAAATTACCCTGTTATCCCTAACGAAATCGATGCGAACTTGG		
Ure F Del 5´F	AAAATAGGGATAACAGGGTAATCAGAGTGATGGATAAGCTCAC	676	Disruption construct for URE 6 5´flank
Ure F Del 5´R	GGGGACAAGTTTGTACAAAAAAGCAGGCTAAGACGGAGGGAGTTTGTAATAA		
Ure F Del 3´F	GGGGACCACTTTGTACAAGAAAGCTGGGTAAAAAATGGGAAGGAAGAAGAT	690	Disruption construct for URE 6 3´flank
Ure F Del 3´R	AAAAATTACCCTGTTATCCCTACGATGGCCAGTTCATCTGATG		
confDel Ure	TCCGCTTCCCAGACCACTTC	803	Amplification of URE1 internal fragment to confirm transformants
	Combined with Ure Del 5´F		
UreD confF	GCTCGAGAGCTGAAGGACATC	683	Amplification of URE 4 internal fragment to confirm transformants
UreD confR	CGAGGAAAGATGTATGTGCAG		
UreF confF	GTCAAATGACGATATTGTGGG	794	Amplification of URE 6 internal fragment to confirm transformants
UreF confR	TTTTGATGATCGTCGACCAAC		
ureCompF	TTTCTCCTTTTTGTCGACGAGG	5034	Amplification of URE 1 for reconstitution
ureCompR	GGTTGCAAGATTTGTGAGGCT		
ureDCompF	CAAGGCCGTTGACGACCTCAT	2819	Amplification of URE 4 for

ureDCompR	TCCATTGGGGAAAGGAGAGTT		reconstitution
ureFCompF	GGGACCGAAGTATCGATGATCGATGGAG	2950	Amplification of URE 6 for reconstitution
ureFCompR	AATACTCAAGCTATGCATCAACTTACACCGGCTGCGGAGGT		
RTUreF	GGTCCTGCAGATGGCTCCAA	210	Amplification of URE1 for RT-PCR
RTUreR	AATAACTTCGGGCGTGGCGC		
RTUreDF	CCCTCTCAAACTTCTCTCTCCA	190	Amplification of URE 4 for RT-PCR
RTUreDR	GCCTGGACGACGCTTGTATAC		
RTUreFF	GGTTCCTTTCTTCACAATACTC	192	Amplification of URE 6 for RT-PCR
RTUreFR	TCCCAGCTTTGAAAGCTTTAC		
RTActF	CGGTATCGTCACAAACTGG	92	Amplification of ACT for RT-PCR
RTActR	GGAGCCTCGGTAAGAAGAAC		

DISCUSSÃO

A importância da atividade ureásica na patogenicidade de fungos é tema de debates. Até o momento, dos gêneros de fungos patogênicos produtores de urease já estudados, atribuiu-se um papel na patogenicidade apenas para as ureases de *Coccidioides* e *Cryptococcus* (Mirbod et al., 2002).

Cox et al., (2000) demonstraram que camundongos infectados com mutantes urease-negativos de *C. neoformans* apresentaram uma maior porcentagem de sobrevivência em relação aos infectados com cepas urease positiva. Olszewski et al. (2004) realizaram estudos com linhagens de *C. neoformans* positivas e negativas para urease e diferentes vias de inoculação por vias distintas em camundongos e constataram o papel da urease como facilitadora de infestação no sistema nervoso central, promovendo sequestro das leveduras nos microcapilares cerebrais. Em outro estudo, a identificação de um gene URE 2, que possivelmente codifica uma proteína acessória envolvida na biossíntese no metalocentro da enzima, isolado de uma cepa urease positiva de um paciente com meningite criptocócica, levantou mais perguntas em relação à urease como fator de virulência (Varma et al., 2006).

Ainda não se conhece o mecanismo pelo qual a urease facilitaria o sequestro do *C. neoformans* em microcapilares. Postula-se que a hidrólise da uréia próxima ao endotélio poderia promover adesão do fungo ao endotélio, ou que a liberação de amônia resultaria em toxicidade celular, afetando astrócitos e fragilizando as junções celulares da barreira hemato-encefálica. Uma função da urease como fator de virulência através de um mecanismo não dependente da atividade ureolítica também foi sugerido por (Olszewski et al., 2004 e Varma et al., 2006).

Mais recentemente, estudos com microscopia intravital que a invasão de *C. neoformans* no SNC se dá pelo evento de micromebolia. Após chegar aos capilares cerebrais, a levedura atravessa a barreira hemato-encefálica penetrando no SNC, num processo urease-dependente e que leva a uma deformidade celular da levedura. O sequestro da levedura no córtex dos capilares pode produzir uma ruptura mecânica e liberação de enzimas com a urease para participarem deste evento (Charlier *et al.* 2009; Shi *et al.* 2010). Casadevall (2010) reforça a hipótese de que um excepcional neurotropismo de *C. neoformans* parece abranger mais de um mecanismo envolvendo tanto uma invasão no SNC diretamente pelo *C. neoformans*, bem como uma invasão associada à fagocitose, em um evento tipo “Cavalo de Tróia” .

A compreensão do mecanismo de ação da urease de *C. gattii*, assim como a distinção entre fatores dependentes ou não da atividade enzimática aumenta a

abrangência e grau de envolvimento desta proteína no complexo sistema de funcionamento da criptococose no hospedeiro.

Recentemente foi realizado um estudo comparativo entre as linhagens R265 de *C. gattii* e H99 de *C. neoformans* demonstrando que os pulmões são o alvo principal para a linhagem R265 enquanto o cérebro é o principal alvo na linhagem H99. Enquanto C. neoformans H99 cresce mais rápido no cérebro causando morte por meninogoencefalite, em C. gattii R265 crescem mais rapidamente nos pulmões causando morte sem meningoencefalite fulminante. A inoculação de 10^4 células de *C. gattii* por via intravenosa produziu severa meningoencefalite, demonstrando que a levedura *C. gattii* pode eficientemente atravessar a barreira hemato encefálica (Ngamskulrungroj *et al.*, 2012b). Este trabalho sugere que mesmo a urease sendo citada até o momento como fator de virulência para invasão no sistema nervoso central, ainda há contribuição da proteína na fase intermediária da doença na fase de pós-infecção pulmonar. Os mutantes de urease (Δure) apresentaram aumento da atividade de lacase, relacionado a hipermelanização da levedura que é conhecida como importante fator de virulência para disseminação da criptococose pós infecção pulmonar (Liu, et al., 2012; Frases *et al.*, 2006). Estes dados reforçam a importância da urease como fator de virulência, pois estes mutantes apresentaram aumento da sobrevida após infecção experimental, mesmo estando com aumento de atividade de um importante fator de virulência que poderia compensar o efeito da deleção do gene URE gerando resultados não significativos. No entanto, estes efeitos só foram observados em infecção de $2x10^5$ células por via intranasal, o mesmo experimento realizado com alta carga de infecção (10^7 células por via intranasal) não revelou alteração na virulência dos mutantes comparada ao tipo selvagem. Estes dados sugerem a confirmação dos dados de Ngamskulrungroj e colaboradores (2012) que *C. gattii* R265 tem como alvo principal os pulmões além de que a urease começa a desempenhar sua função como fator de virulência após a infecção pulmonar, não sendo essencial em quadros pulmonares graves com infecções maciças.

Outro forte indicativo de que a urease apresenta ações distintas não correlacionadas a atividade enzimática, diz respeito a interação da levedura com macrófagos. O trabalho demonstrou que todos os mutantes tem a habilidade de serem internalizados pelo macrófago em comparação com o tipo selvagem. No entanto, os mutantes da urease (ure1Δ) e das proteínas acessórias (*ure4*Δ, *ure6*Δ) apresentaram significativa redução de se multiplicarem dentro do macrófago. Estes dados sugerem a presença de mais de um mecanismo de ação: um relacionado à atividade enzimática,

outro relacionado à proteína estrutural e outro ainda possivelmente desencadeado pela estrutura molecular da proteína na célula. O tratamento das células do tipo selvagem pelo inibidor de atividade enzimática ácido aceto hidroxâmico, atuando como quelante de Ni^{2+}, não afetou a fagocitose dos macrófagos, mas interferiu na multiplicação da levedura no macrófago num perfil dose-dependente. Esses dados reforçam os achados com os mutantes.

Este estudo abre novas vertentes de exploração a respeito de mecanismo independente da atividade ureolítica, como já proposto pelo nosso grupo para a urease de *H. pylori* (Wassermann, et. al, 2010), urease de *C. ensiformis* (Follmer, *et. al.*, 2001) receberam tratamento com inibidor irreversível *p*-hydroximercuriobenzoato que anula a atividade ureolítica não afeta a capacidade de induzir agregação plaquetária, assim como ocorreu com a urease de *Bacillus pausterii* (Follmer *et al.*, 2004).

Muitos estudos têm focado na compreensão a respeito do mecanismo de invasão cerebral pela levedura em questão. Uma complexa organização com múltiplos fatores parece estar envolvida, uma vez que *Cryptococcus* é maior do que bactérias e vírus comumente conhecidos como agentes etiológicos de encefalites e meningoencefalites. Para que isto ocorra, vários fatores de virulência foram identificados por contribuírem para a disseminação extrapulmonar, incluindo a cápsula de polissacarídeos, manitol, transportadores de inositol, *mating type*, melanina, fosfolipase e urease (Liu, *et al*., 2012).

O uso de ferramentas de biologia molecular na construção de mutantes tem sido amplamente empregado para compreensão de mecanismos de ação de fatores de virulência, bem como a compreensão do metabolismo celular de *Cryptococcus.* Este recurso levou a compreensão mais profunda do funcionamento do patógeno e sua interação com o hospedeiro e no ambiente (Kmetzsch *et al.*, 2011a;Kmetzsch *et al.*, 2011b;Schneider *et al.*, 2012;Olszewski *et al.*, 2004). Estes estudos moleculares têm apresentado respostas chaves sobre o funcionamento do sistema patógeno-hospedeiro durante a infecção, devendo ser considerado prioridade para compreensão de mecanismos que podem resolver estratégias de controle da criptococose.

CONCLUSÃO GERAL E PERSPECTIVAS

Este trabalho demonstrou a importância da urease como fator de virulência na levedura *Cryptococcus gattii*, linhagem hipervirulenta R265. Estes dados reforçam que mesmo a linhagem R265 ter um curso de patogenicidade preferencial no quadro respiratório pulmonar, há uma participação efetiva da urease e proteínas acessórias na manifestação da criptococose, sendo que tanto linhagens mutantes da proteina estrutural (*URE1*) e da proteína acessória *URE6* escolhida para ensaios de sobrevivência em camundongos infectados demonstrou ter atenuação na virulência destas linhagens.

Este foi o primeiro estudo a respeito de proteínas acessórias de urease em *Cryptococcus gattii* e seus efeitos na criptococose, abrindo uma série de questões a serem expandidas para maiores esclarecimentos sobre o mecanismo de ação destas proteínas na virulência.

A possibilidade de expressão da urease recombinante de Cryptococcus e da expressão da urease com suas proteínas acessórias recombinantes pode esclarecer várias possíveis atuações da urease em células do hospedeiro em análises *in vitro* (macrófagos, neutrófilos, plaquetas, p.e)

A extensão dos ensaios de infecção e análise de virulência com os mutantes contruídos de *C. gattii* neste estudo podem complementar questões ainda desconhecidas sobre o comportamento destas linhagens no SNC. Isto pode ser analisado através de outras vias de infecção com diferentes concentrações de inoculo, além de análise de carga de infecção por órgãos em diferentes níveis de infecção experimental.

Com isso pretende-se iniciar um novo capítulo sobre o mecanismo de ação da urease classificando propriedades farmacológicas dependentes e independentes da atividade enzimática.

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Cryptococcus gattii urease as a virulence factor and the relevance of enzymatic activity in cryptococcosis pathogenesis

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Ureases (EC 3.5.1.5) are Ni^{2+}-dependent metalloenzymes produced by plants, fungi and bacteria that hydrolyze urea to produce ammonia and CO_2. The insertion of nickel atoms into the apo-urease is better characterized in bacteria, and requires at least three accessory proteins: UreD, UreF, and UreG. Our group has demonstrated that ureases possess ureolytic activity-independent biological properties that could contribute to the pathogenicity of urease-producing microorganisms. The presence of urease in pathogenic bacteria strongly correlates with pathogenesis in some human diseases. Some medically important fungi also produce urease, including *Cryptococcus neoformans* and *Cryptococcus gattii*. *C. gattii* is an etiological agent of cryptococcosis, most often affecting immunocompetent individuals. The cryptococcal urease might play an important role in pathogenesis. It has been proposed that ammonia produced via urease action might damage the host endothelium, which would enable yeast transmigration towards the central nervous system. To analyze the role of urease as a virulence factor in *C. gattii*, we constructed knockout mutants for the structural urease-coding gene *URE1* and for genes that code the accessory proteins Ure4 and Ure6. All knockout mutants showed reduced multiplication within macrophages. In intranasally infected mice, the *ure1*Δ (lacking urease protein) and *ure4*Δ (enzymatically inactive apo-urease) mutants caused reduced blood burdens and a delayed time of death, whereas the *ure6*Δ (enzymatically inactive apo-urease) mutant showed time and dose dependency with regard to fungal burden. Our results suggest that *C. gattii* urease plays an important role in virulence, in part possibly through enzyme activity-independent mechanism(s).

Introduction

Ureases (EC 3.5.1.5, urea amidohydrolase) are Ni^{2+}-dependent metalloenzymes that catalyze the hydrolysis of urea to form ammonia and carbon dioxide [1]. Ureases are found in plants, fungi, and bacteria. Their high sequence similarities suggest that all ureases possess similar tertiary structures and catalytic

Abbreviations
AHA, acetohydroxamic acid; CFU, colony-forming unit; CNS, central nervous system; DMEM, Dulbecco's modified Eagle's medium; WT, wild-type.

mechanisms [2]. Accessory proteins are required to activate the urease apoprotein and to assemble its metallocenter [3]. Many fungi that are pathogenic to humans produce urease, including *Cryptococcus neoformans*, *Coccidioides immitis*, *Histoplasma capsulatum*, *Sporothrix schenckii* [4], and species of *Trichosporon* and *Aspergillus*. The coccidioidal urease gene has been shown to be expressed *in vivo* and to play a role in both sporulation and pathogenesis [5].

In microorganisms, urease enables the utilization of urea as a nitrogen source and, in some cases, contributes as a virulence factor to several human and animal diseases, such as gastroduodenal infections by *Helicobacter pylori*, and *Proteus mirabilis*-induced urinary stone formation [6]. However, urease might also play other important roles that are unrelated to its enzymatic activity [7], such as insecticidal [8,9], fungicidal [10] and exocytosis-inducing activities [11]. As a consequence of urease-induced ADP secretion from dense granules, platelets from different species, including humans, undergo aggregation upon exposure to nanomolar concentrations of plant (jack bean and soybean) or bacterial (*Bacillus pasteurii* and *H. pylori*) [12] ureases. The platelet-activating properties of these ureases were shown to be independent of urea hydrolysis [12–14]. Ureases also show potent proinflammatory activity. For instance, canatoxin, an isoform of *Canavalia ensiformis* urease [15], and *H. pylori* urease were shown to induce inflammation *in vivo* (mouse paw edema). Additionally, *H. pylori* urease at nanomolar concentrations activates human neutrophils to produce reactive oxygen species and inhibits their apoptosis, thus potentially contributing to the gastritis pathogenesis caused by this bacterium [16].

C. neoformans causes the most common central nervous system (CNS) fungal infection in HIV-1/AIDS populations, with high mortality and morbidity rates. Cryptococcosis has become one of the three most common opportunistic diseases associated with high-dose steroid therapies such as those administered to patients with transplants or immune disorders [17,18]. The risk of cryptococcosis has assumed global proportions over the years. An increasing number of AIDS cases since the early 1990s has led to a sharp increase in the number of reported cryptococcosis cases in the past decade [18]. The disease symptoms include severe pneumonia with the development of lung cryptococcal cysts, and tropism to the CNS, producing neurological symptoms of meningoencephalitis [19,20].

Several *C. neoformans* virulence factors are known, including a polysaccharide capsule, melanin production, enzymes (urease and phospholipase) [5,21], and the ability to grow at 37 °C. The majority of clinical isolates produce large amounts of urease. The primary role of urease in *C. neoformans* infections has been a matter of ongoing debate. *URE1*, which encodes *C. neoformans* urease, was first cloned in 2000, and used to disrupt native *URE1* in the serotype A strain H99. A comparison of the survival rates observed for the null mutant and the H99 urease-positive strains in murine models of intravenous and inhalational infections revealed significant differences. Mice infected with the *ure1*Δ mutant lived longer than those infected with H99 in both models [5]. Additional studies on the inoculation of H99, *ure1*Δ and reconstituted *ure1*Δ::*URE1* strains directly into the brain demonstrated that urease was not required for yeast growth in the brain. However, the dissemination patterns in the brain, spleen and other organs after intravenous inoculation suggested a contribution of cryptococcal urease to the process of CNS invasion by enhancing yeast sequestration within microcapillary beds (such as those within the brain) during hematogenous spread, thereby facilitating *C. neoformans* blood-to-brain invasion [20]. More recently, intravital microscopy analysis revealed that brain invasion by *C. neoformans* follows a capillary microembolic event. A major finding of this study was that fungal cells arrest suddenly at capillary sites without rolling or tethering, in a manner similar to that of polystyrene microspheres. The similarity of the arresting process to that shown by inert microspheres suggested that the initial mechanism leading to brain localization is mechanical and related to an inability of yeast cells to traverse narrow capillaries. In this scenario, the initial brain infection would result from a microembolic event. It was observed that, after arresting at capillary sites, *C. neoformans* cells cross the capillary wall in a process that requires viability and cell morphology alteration, but not replication. Moreover, this process was shown to be urease-dependent, because the deletion of *URE1* reduced the brain fungal burden in infected mice [22]. On the basis of these observations, pharmacological inhibition or urease-targeted antibody therapies have been proposed as potentially useful strategies to protect the brain against yeast invasion in cryptococcal meningitis [5,20–23]. Recently, Singh *et al.* [24] used *C. neoformans* mutants with *URE1*, *URE4*, *URE6* and *URE7* deletions to infect mice via intravenous injection. Yeast colony-forming units (CFUs) were determined in the brain tissues at 3 and 24 h postinfection, and the results demonstrated that the enzymatic activity, rather than the Ure1 protein, was crucial for brain invasion by *C. neoformans* [24].

It is well established that *C. neoformans* infects mainly immunosuppressed patients, although the majority of reports of *Cryptococcus gattii* infection are in non-HIV patients. Whereas cryptococcosis in patients infected with *C. gattii* is mainly characterized by pulmonary disease, meningoencephalitis is the main complication resulting from *C. neoformans* infections. In line with the epidemiological data, assays employing animal models of cryptococcosis revealed that, despite the fact that *C. gattii* has the molecular mechanisms needed to cross the blood–brain barrier; fatal lung infection is the main cause of death in mice infected with *C. gattii* [25].

In the present study, we aimed to contribute to the elucidation of the role(s) of *C. gattii* urease as a virulence factor in cryptococcosis. To that end, mutant yeast strains were constructed that lacked the urease protein or produced an enzymatically inactive apoprotein. Deletion mutants were phenotypically and molecularly characterized, and their virulence was tested in mice.

Results and Discussion

Identification of *URE* and accessory protein genes from *C. gattii*

A search of the *C. gattii* R265 genome Fungal Genome Initiative database at the Broad Institute of MIT and Harvard, available at http://www.broadinstitute.org/annotation/genome/cryptococcus_neoformans_b/MultiHome.html (5 August 2009), allowed the identification of *C. gattii URE1* (CNBG_4331.2), as well *URE7* (CNBG_1252.2 – ortholog of bacterial *ureG*), *URE4* (CNBG_0735.2 – ortholog of bacterial *ureD*), and *URE6* (CNBG_5207.2 – ortholog of bacterial *ureF*). Analysis of the predicted protein sequences in the Pfam database [26] confirmed their similarities to orthologous proteins in plants and fungi.

C. gattii urease appears to be a typical fungal urease, with a single subunit of 833 amino acids, a molecular mass of 90 kDa, and a theoretical pI of 5.5. Whereas plant and fungal ureases are oligomers of identical and typically ~ 90-kDa subunits, bacterial ureases are complex multimers with two or three subunits [6,26–28]. Remarkably, despite comprising different types of subunits, ureases from different sources, ranging from bacteria to plants and fungi, show high amino acid sequence similarity. For instance, *C. gattii* Ure1 shows 58% identity with jack bean urease. This suggests that all ureases are evolutionary variants of a single ancestral enzyme [27].

Ure accessory proteins are not as well conserved as Ure1 proteins across different species. A multiple sequence alignment of *C. gattii* Ure4 (Fig. 1) and Ure6 (Fig. 2) accessory proteins with those from different organisms allowed the identification of their characteristic domains, as detected by Pfam. As discussed below, Ure7 was not subjected to phylogenetic analysis, owing to its unique features. Regarding the phylogenetic inference of urease accessory proteins in *C. gattii*, Ure4 and Ure6 from this species did not cluster with the orthologous proteins in fungi from the Agaricomycetes (i.e. *Fomitiporia mediterranea* and *Trametes versicolor*) and Basidiomycetes (i.e. *Coniophora puteana* and *Stereum hirsutum*). These proteins also show clear divergence from those from members of the Ascomycota (i.e. *Arthroderma gypseum*, *Pyrenophora tritici-repentis*, *Schizosaccharomyces japonicus*, *Schizosaccharomyces pombe*, and *Verticillium dahliae*). This suggests that ancestors of cryptococcal Ure4 and Ure6 diverged from the ancestor of the corresponding proteins in the other fungal species analyzed. The Ure6 orthologs from plants seem to be the most distant from the clade that contains *C. gattii* proteins, whereas the orthologs from *Wallemia sebi* (Wallemiomycetes) are the closest to the referred clade. The topology is somewhat different in the Ure4 tree, as proteins from photosynthesizing organisms group with the Basidiomycota clade. It is noteworthy that Ure4 is one of the less-conserved accessory proteins, and less information is available for it [29].

In eukaryotes, the Ure7 accessory proteins also bind metals, possessing a dual activity that combines properties of the metallochaperone UreE in bacteria [30–32]. Ure7 was shown to be an intrinsically disordered protein [33], with structural fluctuations that respond to the cellular environment [29–35]. These facts led us to exclude Ure7 from our mutant analysis.

Construction of *C. gattii* mutant strains

We deleted the *URE* genes by employing inactivation cassettes harboring a hygromycin resistance construct. To evaluate the urease proficiency of the hygromycin-resistant yeast colonies that arose from transformation with *URE* inactivation cassettes, tests in urea media (YPD agar plates supplemented with urea, Ni^{2+}, and phenol red pH indicator) were conducted [36]. The wild-type (WT) *C. gattii* strain was used as a urease-positive control. After 24 or 48 h of incubation at 30 °C, colonies were selected on the basis of the absence of a reddish color, as this indicated deletion mutants lacking ureolytic activity. Urease-positive colonies were visible as a pink/magenta color, observed in WT and in com-

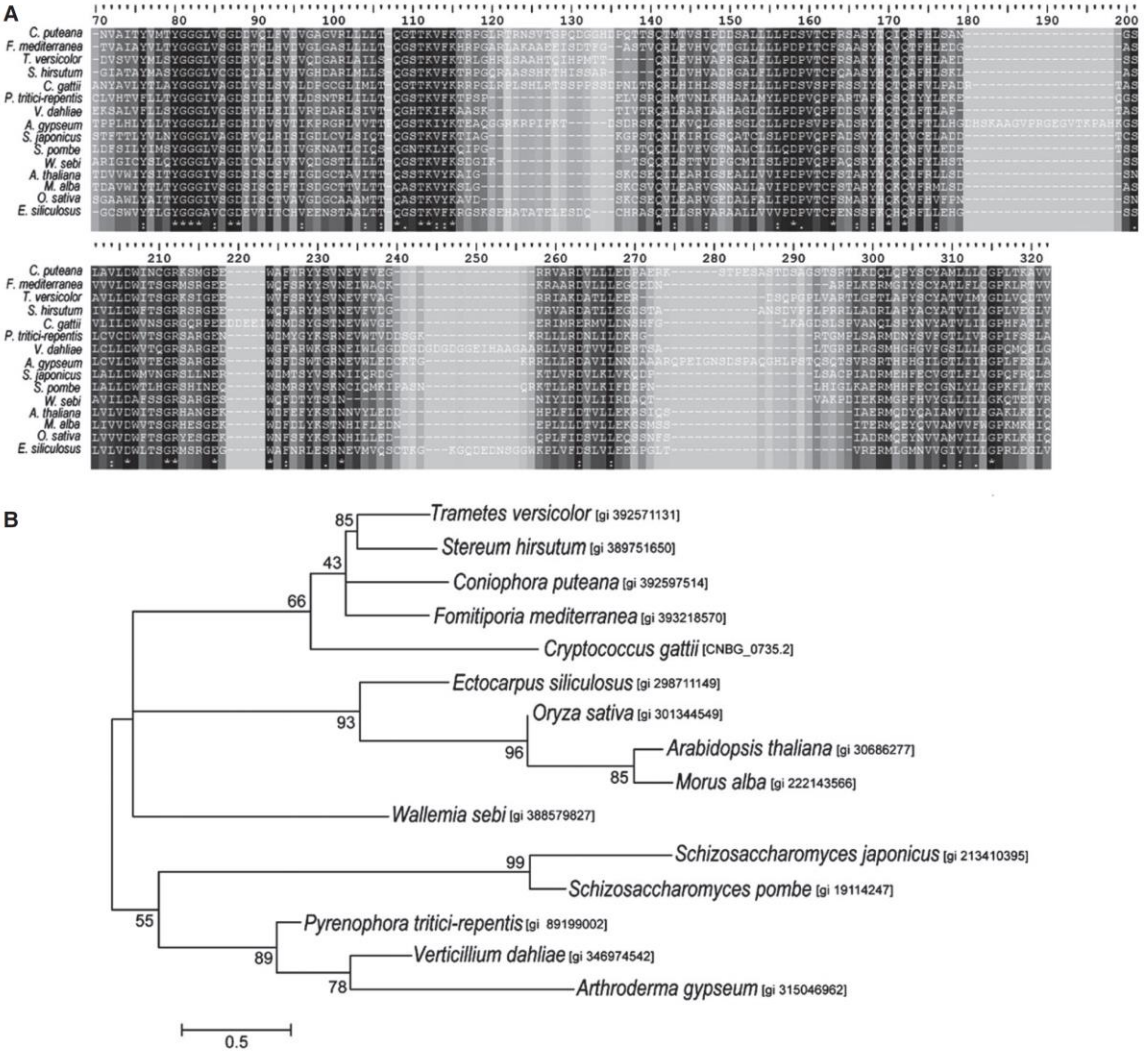

Fig. 1. Phylogeny of Ure4. (A) Multiple sequence alignment of the Ure4 characteristic domain in different proteins, as detected by Pfam. (B) Molecular phylogenetic analysis of Ure4 orthologs with the maximum likelihood method. The evolutionary history was inferred by use of the maximum likelihood method based on the Whelan and Goldman model with discrete gamma distribution and some evolutionarily invariable sites (WAG + G + I). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

plemented mutant *URE* strains (Fig. 3A). The ureolytic activities of the mutants and complemented clones were also determined in yeast cell extracts with the rapid urea test [37]. According to the results shown in Fig. 3B, all reconstituted mutants showed levels of ureolytic activity that were nearly equivalent to or even higher than those observed in WT *C. gattii*, whereas this activity could not be detected in mutant strains. The integration of the inactivation and complementation cassettes was confirmed (Fig. 4).

Virulence factors in *C. gattii URE* mutant strains

We evaluated the phagocytic activity of macrophages towards the *C. gattii* mutant strains, and the ability of the WT, mutant and complemented strain yeast cells to survive inside macrophages. Figure 2A shows that all yeast strains were equally phagocytosed by macrophages after 1 h of exposure, and survived within the phagocytes for at least 18 h. However, the *ure1*Δ mutant did not multiply within macro-

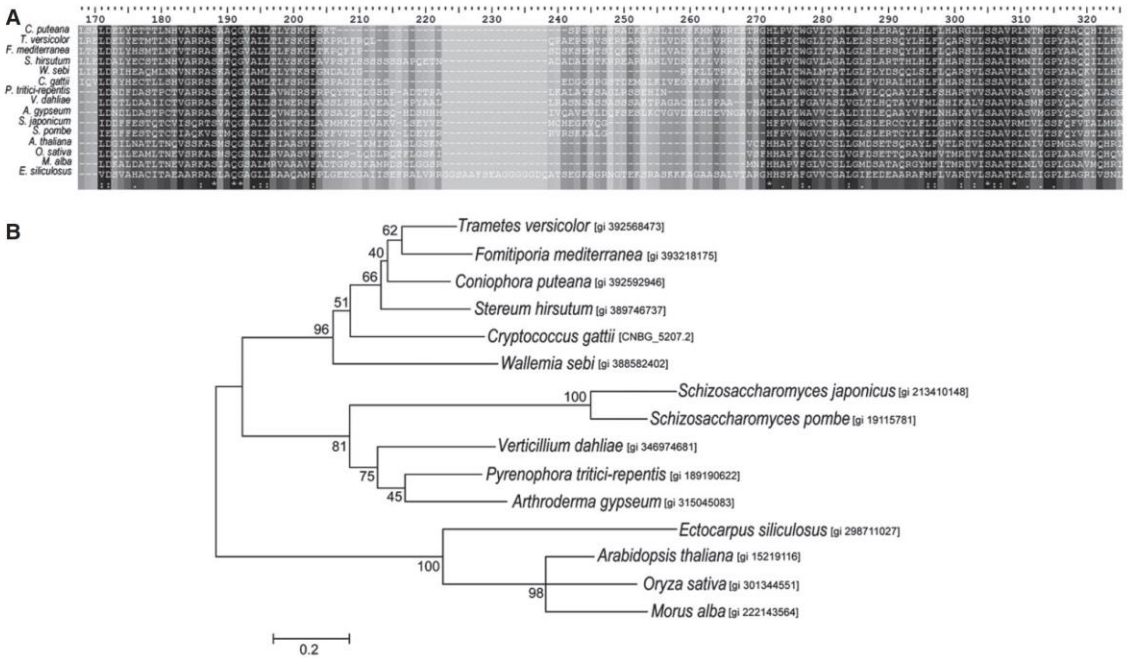

Fig. 2. Phylogeny of Ure6. (A) Multiple sequence alignment of the Ure6 characteristic domain in different proteins, as detected by Pfam. (B) Molecular phylogenetic analysis of Ure6 orthologs with the maximum likelihood method. The evolutionary history was inferred by use of the maximum likelihood method based on the Whelan and Goldman model with discrete gamma distribution (WAG + G). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

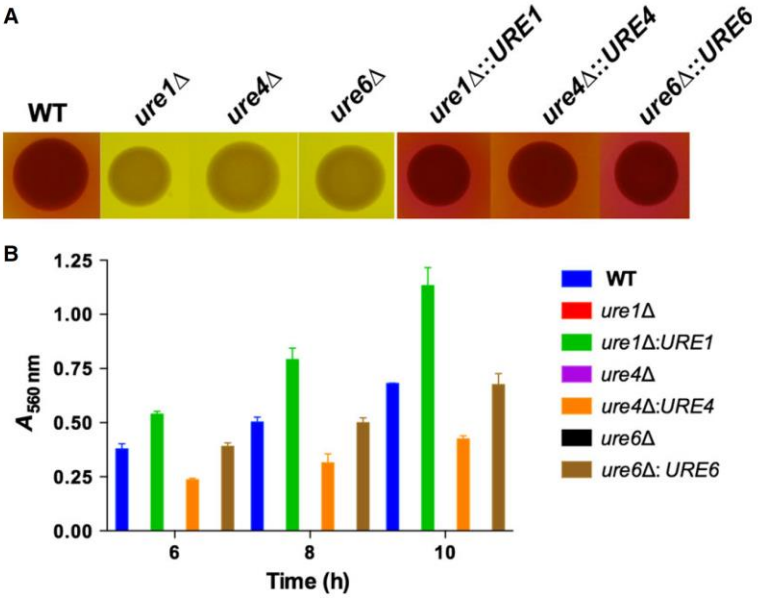

Fig. 3. Inactivation of *URE* genes abolishes *C. gattii* urease activity. (A) Urease activity-screening test on YPD agar with the WT, mutant and complemented strains. (B) Ureolytic activity in transformed *C. gattii* strains. Urease activity was measured in the extracts of cells that were incubated for up to 10 h in urea buffer. Data shown are means ± standard deviations of triplicates with endpoint readings at 6, 8, and 10 h.

phages (Fig. 5A). This contrasts with the WT *C. gattii* cells, which nearly quadrupled in number after 18 h inside the phagocytes. The ability to survive phagocytosis and multiply within macrophages was partially restored in the reconstituted mutants (Fig. 5B).

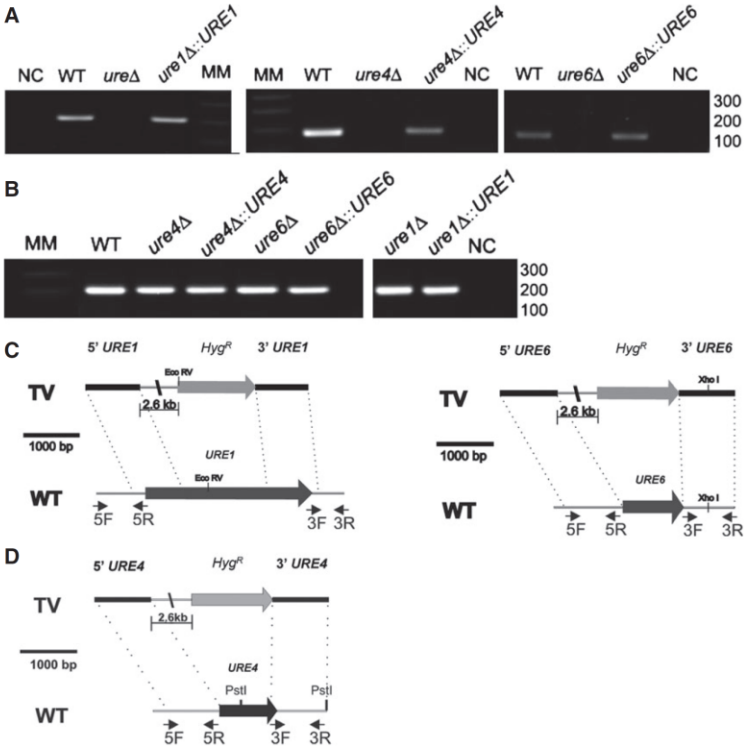

Fig. 4. Inactivation and complementation of *C. gattii URE* genes. (A) RT-PCR was performed to evaluate the expression of *URE1*, *URE4* and *URE6* in the respective mutants and complemented strains with *URE*-specific primers. (B) As a loading control, the expression of *ACT1* was also evaluated. NC, negative control; MM, molecular marker. (C–E) The cassettes for the generation of the null mutants for *URE1* (C), *URE4* (D) and *URE6* (E) are also illustrated. Target genes were replaced with hygromycin-resistant marker cassettes, as indicated by the light gray box (Hyg^R). The target gene 5′ and 3′ flanks were fused with the hygromycin cassette according to the Delsgate methodology. The WT locus of each gene and the primer positions for gene disruption are indicated. The constructed target vector (TV) was used for the *C. gattii* transformations. The restriction enzymes are also indicated for each TV.

Our data thus show that mutants lacking the urease protein (*ure1*Δ) or expressing enzymatically inactive apoureases (*ure4*Δ and *ure6*Δ) did not multiply within macrophages. Similarly, treatment of WT cells with acetohydroxamic acid, an Ni^{2+} chelant inhibitor of urease activity [38], did not affect phagocytosis by macrophages, but blocked the ability of the yeast to multiply within the cell in a dose-dependent fashion (Fig. 5C). Taken together, these data indicate a relevant role of the ureolytic activity in survival and/or multiplication of yeasts within the macrophages. Previously, it was proposed that urease-mediated alkalinization and phagosomal cathepsin inhibition, which are promoted by urease-producing microorganisms, are important factors allowing the survival and multiplication of such microorganisms within phagosomes [39].

Experimental infections in mice via intranasal inoculation were performed to follow the virulence of the WT, mutant and complemented strains. Fungal burdens were analyzed in lungs and blood with intranasal inoculation of 1×10^7 yeast cells of the WT, *ure1*Δ and *ure6*Δ strains, or the corresponding reconstituted strains, to simulate an acute infection. Mice were killed at an early stage of infection (day 7), as well as at a late stage (day 18). The mice had low yeast burdens in lungs after 1 week, regardless of the yeast strain (data not shown), but significant differences in lungs (Fig. 6A) and blood (Fig. 6B) burdens appeared at day 18 postinfection in these conditions. Another experimental infection was then performed to analyze the fungal burdens with a lower inoculum (5×10^4), and the fungal burden was analyzed at different stages of the infection process (days 17 and 24). This experiment confirmed that significant differences could be observed when *ure1*Δ mutant-infected mice were compared with the WT strain; specifically, lower lung or nearly absent blood yeast burdens were observed in the *ure1*Δ mutant-infected mice (Fig. 6C,D). This difference persisted until day 24 postinfection. On the other hand, the inactive apo-urease-producing mutants behaved similarly to the WT strain, at least in lungs and in the late stages of infection (Fig. 6C), suggesting that a ureolysis-independent contribution of *C. gattii* urease to pulmonary cryptococcosis could occur in specific stages of the infection. The same scenario could not be observed for fungal burdens in blood, where *URE4* and *URE6* are important for cryptococcal survival (Fig. 6D). The lower extent of spread of *ure1*Δ mutants from the lung (Fig. 6C) to the blood at

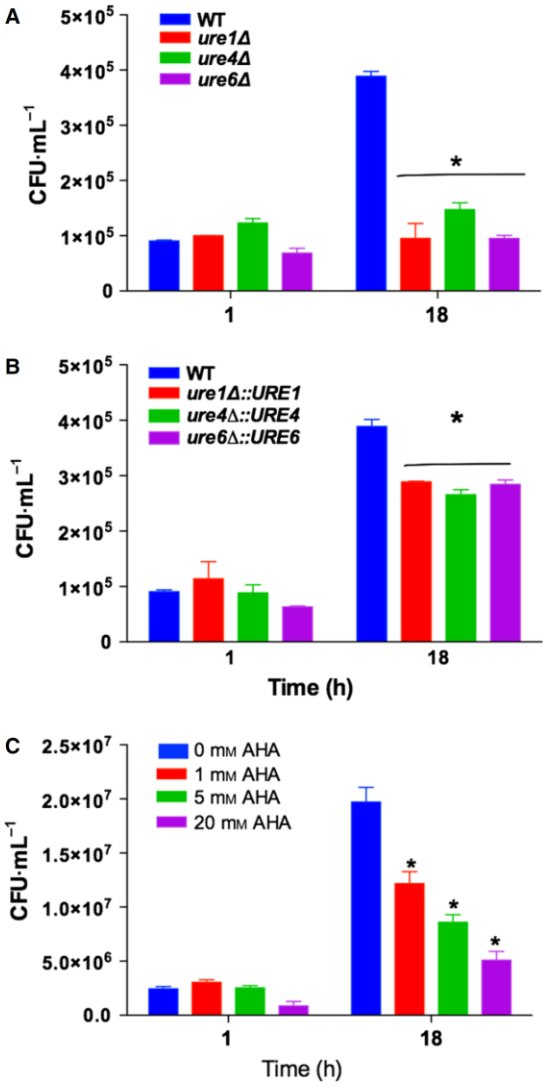

Fig. 5. Role of urease in cryptococcal survival and multiplication in macrophages. Macrophage-like RAW264.7 cells were incubated with *C. gattii* cells for 1 h (37 °C, 5% CO_2) to allow phagocytosis. The medium was then removed and replaced with DMEM/FBS. After a second incubation for 18 h (37 °C, 5% CO_2), yeast cells not associated with macrophages were removed with PBS washes. Fungal survival was evaluated after macrophage lysis with sterile ice-cold Milli-Q water and subsequent plating of the lysates on YPD agar plates for CFU determination. (A) Results obtained with deleted mutants, as compared with the WT strain. (B) Results obtained with reconstituted strains, as compared with the WT strain. (C) Results obtained with the WT strain pretreated with different concentrations of AHA, a urease inhibitor. The error bars represent standard deviation from technical triplicates of two independent biological replicates. The statistical analyses were conducted with the *t*-test. *$P < 0.05$ in relation to the WT or control.

this stage of infection and the impaired ability of these mutants to survive and multiply within phagocytes suggest that urease acts as a virulence factor immediately after lung infection. The *ure4*Δ and *ure6*Δ mutants appear to be similar to the WT strain in the lung at late stages of the infection process (24 days), indicating that, at this stage, ureolytic-independent activity of urease is important for virulence. On the other hand, such mutants showed CFUs in the blood that were very similar to those of the *ure1*Δ mutant, indicating that, in this case, urease activity is important. Mice from all strains showed low or no burdens in the kidneys, spleen and brain at all dilutions and and in all experiments (data not shown). A previous study using a lower inoculum by the intravenous route showed a low burden of *C. gattii* R265 in the blood, but detectable burdens in other organs (spleen, brain, and kidneys) [25]. Comparing our study with the results of Ngamskulrungroj *et al.* [25], we suggest that different mechanisms of infection or progression of the infection could be related to distinct inoculation routes of the pathogen. This corroborates with the statement by the same group implying that the paucity of meningoencephalitis upon inhalation of *C. gattii* may therefore be partly attributable to an unknown factor(s) in the host's blood coupled with immune protection that reduces dissemination to the brain and fosters lung infection.

Comparisons of *C. neoformans* (H99) and *C. gattii* (R265) infection patterns in mice revealed distinct primary organ targets [25]. Mice infected with H99 via the pulmonary route were killed by brain infection, whereas those infected with R265 were killed by lung infection. *C. gattii* cells have been reported to produce smaller capsules and to cross the blood–brain barrier more efficiently than *C. neoformans* [40] cells. Although *C. gattii* can cross the blood–brain barrier, the host immune system interferes more competently in infections caused by this yeast, and death is therefore frequently subsequent to severe lung infections before fatal meningoencephalitis can occur. A preliminary experiment with a high inoculum (10^7 cells) showed no significant differences in mortality among all mutant strains and WT yeast, with a mean time to death of 13 days (data not shown). In the present study, this infection model, which has been used in other studies with *C. neoformans* virulence factors [5,41,42], proved inconclusive in discriminating differences among the *C. gattii* strains with regard to the survival time after infection. We believe that this high inoculum favored an acute and severe lung infection that killed the animals before any yeast spread to other organs.

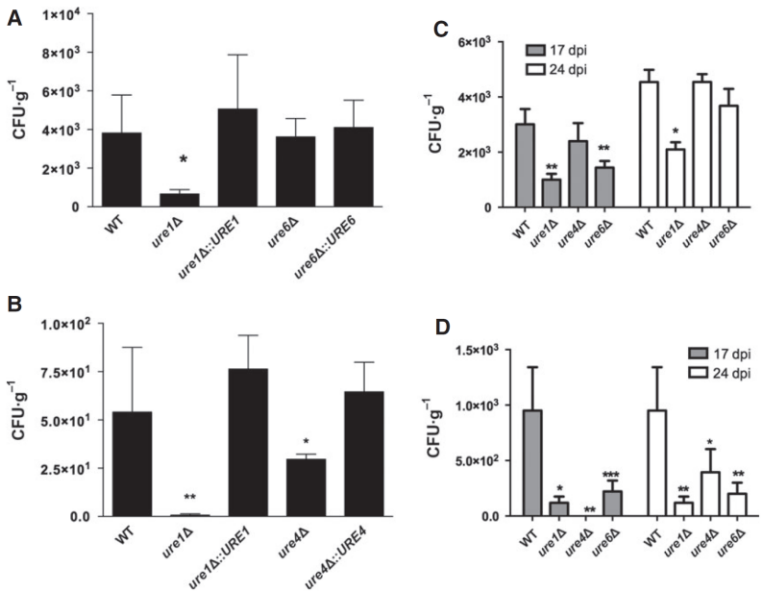

Fig. 6. Urease activity alters organ fungal burden in murine models of cryptococcosis. The fungal burden was assessed in lungs (A, C) and blood (B, D) of mice infected with WT, mutant and complemented *C. gattii* strains. BALB/c mice were inoculated via intranasal instillation of 1×10^7 (A, B) or 5×10^4 (C, D) *C. gattii* cells, and the CFUs were determined from organs collected from infected mice at 18 days postinfection (dpi) (A, B) or 17 and 24 dpi (C, D). The statistical analyses were conducted with the *t*-test. For samples that showed differences from the WT or control: $^*P < 0.05$ or $^{**}P < 0.01$.

Figure 7A shows that mice infected with the *ure1*Δ and *ure6*Δ mutants survived for longer periods (median survival times of 34 days and 29.5 days, respectively) than those infected with the WT strain (median survival of 20 days). The median survival times of mice infected with the complemented strains were not significantly different from that of mice infected with the WT strain (Fig. 7B). The mean survival time of mice infected with the *ure*4Δ mutant was not significantly different from that of mice infected with WT *C. gattii*. We also evaluated the pivotal cryptococcal virulence factors in the WT and mutant strains. No differences in the capsule size or growth at 37 °C could be found between the mutant strains and the WT strain (data not shown).

The results from the survival assay clearly indicated a role for urease as a virulence factor in *C. gattii*-induced cryptococcosis, in agreement with previous suggestions regarding *C. neoformans* infections [19,24]. The results showed that the *ure1*Δ and *ure6*Δ mutants present similar survival rates, higher than in the case of WT strains. However, our data suggested that cryptococcal urease-mediated ureolysis is not the only contributor to its role as a virulence factor. Although this activity is apparently necessary for initial lung colonization, the urease-mediated ureolysis is not fully necessary in the late stages of infection, as the enzymatically inactive apo-ureases present in the *ure4*Δ and *ure6*Δ mutants led to cryptococcal virulence comparable to that of the WT strain. However, this could not be observed for blood fungal burden. This suggests that Ure1 may be developing some urease-independent functions at specific stages of infection.

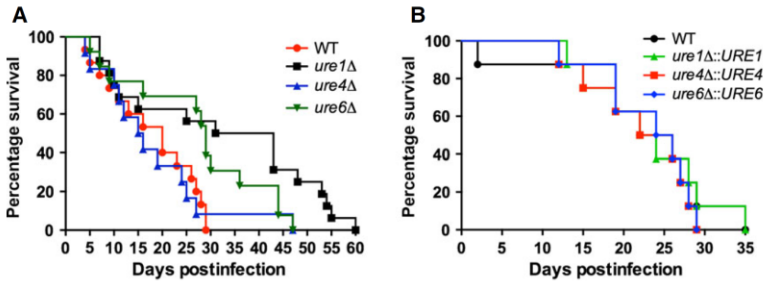

Fig. 7. Some *URE* genes are necessary for normal cryptococcal virulence. Survival analysis of the WT, null mutants or complemented mutants in a murine model. (A) Virulence assays of the WT, *ure1*Δ, *ure4*Δ and *ure6*Δ strains in an intranasal inhalation infection BALB/c mouse model. (B) Virulence assay performed with complemented strains.

Previous studies have demonstrated the importance of *C. neoformans* urease in CNS pathogenesis [20]. The use of artificial routes (injection versus inhalation) to deliver the yeast strains probably overrides most of the steps of the infection process that we have shown herein to involve ureolysis-independent cryptococcal urease activities. Unfortunately, several studies [20,22,24] did not show the survival rates of injected mice or assays to determine the interactions between mutated yeast strains and macrophages, which would have allowed a better comparison with our results.

Conclusions

Here, by using urease-deleted and urease-inactive yeast mutants and a simulated natural infection (inhalation) process in immunocompetent animals, we were able to distinguish the ureolysis-independent and ureolysis-dependent mechanisms by which urease might contribute to cryptococcosis. This study has clarified some matters regarding the contribution of urease to yeast invasion of the lung. Our data also highlight the importance of overall cryptococcal urease inhibition, rather than of its enzymatic activity alone, for the future development of therapeutic tools targeting cryptococcosis, either from *C. neoformans* or from *C. gattii* infections.

Experimental procedures

Ethics statement

All animal studies were reviewed and approved by the Ethics Committee for the Use of Animals of the Federal University of Rio Grande do Sul, concerning the housing and care of laboratory animals (CEUA – protocol number 17535). Mice were housed in groups of eight, and kept in filtered top-ventilated cages with constant temperature and humidity, and food and water *ad libitum*, according to the guidelines of the Brazilian National Council for Animal Experimentation (CONCEA) and the Brazilian College of Animal Experimentation (COBEA). Experiments were carried out in a Level 2 biosafety laboratory according to the rules of the National Technical Committee on Biosafety (CTNBio). The safety procedures that were applied to the experimental procedures as recommended by this committee are available at http://www.ufrgs.br/cbiot/CS/CS_CBiot01.htm.

Fungal strains, plasmids, and media

The *C. gattii* hypervirulent strain R265, serotype B, mating type α, molecular type VGII, from the cryptococcosis outbreak in Vancouver Island [43], was kindly provided by W. Meyer (Sydney University, Australia). Cells were grown at 30 °C with continuous shaking (200 r.p.m.) in YPD medium (1% w/v yeast extract, 2% w/v peptone, and 2% w/v dextrose), or at 30 °C on YPD agar (YPD medium with 1.5% w/v agar). YPD agar plates with either hygromycin (200 $\mu g \cdot mL^{-1}$) or nourseothricin (100 $\mu g \cdot mL^{-1}$) were used to select the deletion and complementation transformants, respectively. The plasmid pJAF15 [43] was the source of the hygromycin resistance cassette, and pAI4 [44] was the source of the nourseothricin resistance cassette. The plasmids were maintained in *Escherichia coli* cells that were grown at 37 °C in Luria–Bertani broth or agar supplemented with 50 $\mu g \cdot mL^{-1}$ kanamycin.

Identification of target genes and sequences for *in silico* analysis

The putative *C. gattii URE1* (CNBG_4331.2) was identified from the Broad Institute *C. gattii* R265 genome database, available at http://www.broadinstitute.org. A BLAST search sequence alignment was performed to find the accessory protein-coding genes *URE4*, *URE6*, and *URE7*, with the sequences from the respective *C. neoformans* orthologs as queries, and then compared by the use of CLUSTALX2 [45]. Phylogenetic trees were developed with MEGA5 [46]. The evolutionary history was inferred by use of the maximum likelihood method based on the Whelan and Goldman model with discrete gamma distribution [47]. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed.

Disruption and complementation of the *URE* genes

The construction of vectors for transformations of *URE1* and the accessory protein-coding genes *URE4* and *URE6* are summarized in Fig. 4. These genes were individually inactivated in the *C. gattii* genome. Disruption was based on the DelsGate methodology [48]. The 5′ and 3′ target gene flanks were PCR-amplified (Table S1) and purified from agarose gels (Illustra GFX PCR DNA and Gel Band Purification Kit; GE Healthcare, Uppsala, Sweden). The cloning reaction was performed with the BP clonase (Life Technologies, Carlsbad, CA, USA) and the 5′ and 3′ target gene flanks, as well the pDONRHYG vector [42,49,50]. The product of this reaction was transformed into the *E. coli* OmniMAX 2-T1 strain. After confirmation of the correct deletion construct, the plasmid was linearized with I-*Sce*I prior to *C. gattii* biolistic transformation [51]. The *C. gattii* mutants were subjected to a previously adapted screening test in YPD agar plates supplemented with urea (300 mM), $NiSO_4$ (1 μM), and phenol red as a pH indicator [36]. The urease activity would be expected to convert urea into ammonia, resulting in an increase in the medium pH

and causing a color change from yellow to bright pink. Urease activity negative-colonies (no color change) were further screened by colony PCR, and deletions were confirmed by Southern blotting and semiquantitative RT-PCR analyses.

For complementation of the *ure1*Δ, *ure4*Δ and *ure6*Δ mutants, fragments of approximately 5, 2.8 and 2.9 kb containing the respective WT genes, respectively, were cloned into the *Sma*I site of pAI4, which has a nourseothricin selection marker, and subjected to *in vivo* recombination, as previously described [52,53], in chemocompetent Omni-MAX 2-T1 *E. coli*, prepared as previously established [54]. The resulting plasmid was used for transformation into the respective mutant strains. The complemented mutants were subjected to a urease-screening test, as described above, to confirm the restored ureolytic activity. The primers used in these plasmid constructions are listed in Table S1.

Southern blotting and RT-PCR analysis

Southern blotting and RT-PCR analysis were used to evaluate the integration of the inactivation cassettes into the WT *URE1* or *URE4* and *URE6* locus. For Southern blotting, genomic DNA (10 μg) from strains was digested with the specific restriction enzyme for each mutant. The 3′ or 5′ flanking region was used as the Southern hybridization probe.

For RNA extraction, mutant and WT strain cultures were grown in YPD medium (18–20 h/30 °C) with shaking. RNA extraction and cDNA preparation were performed as previously described [49]. The Applied Biosystems 7500 real-time PCR system was used for the real-time PCR analysis, and the PCR cycling conditions, melting curves and relative expression determinations were as described previously [49]. The experiments were performed with two biological samples, and each cDNA sample was analyzed in triplicate with each primer pair. Actin cDNA levels were used to normalize each set of PCR experiments. The sequences of the primers used are listed in Table S1.

Phenotypic characterization assays

Urease activity

The rapid urea broth (RUH broth) developed by Roberts [55] and adapted by Kwon-Chung [37] was used to detect ureolytic activity in the WT and mutant yeast strains. *C. gattii* strains grown in solid YPD agar for 24 h at 30 °C were suspended in 2 mL of sterile PBS at pH 7.2. The $D_{600\ nm}$ of the cell suspensions was adjusted to 0.7. The cell suspensions were vortexed, mixed with an equal volume of 2× RUH broth mix, and maintained at 37 °C with shaking for 10 h. After 6, 8, and 10 h, the cultures were centrifuged (5000 ***g*** - 5 min), and $A_{560\ nm}$ values of the supernatants were determined. A sterile PBS blank was used as a control. The assay was performed in triplicate for each time point.

Urease inhibition assays

C. gattii WT cells were grown for 20 h in YPD medium, washed with PBS, and diluted to give an $D_{600\ nm}$ of 1.0 in 1 mL of cell suspension. The suspension were incubated with different concentrations (0.5–20 mM) of the urease inhibitor acetohydroxamic acid (AHA) [56], and incubated for 18 h, with shaking at 7 °C; a parallel control suspension of *C. gattii* with buffer and without AHA was used. After incubation, cells were washed three times with PBS, and macrophage interaction experiments were performed as described previously. Aliquots of all AHA inhibition experiments were plated on YPD agar for cell viability analysis. AHA-treated cells were monitored with the Roberts test for 18 h in parallel with the macrophage interaction assay to certify the absence of urease enzymatic activity throughout the whole assay.

Capsule induction and measurement

The capsule thicknesses and cell diameter ratios of the mutant and WT *C. gattii* strains were measured during a microscopic examination of India ink preparations that were grown overnight in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Carlsbad, CA, USA) with 10% FBS (Life Technologies) at 30 °C in 5% CO_2 to stimulate capsule production. AXIOVISION LE microscope software (Carl Zeiss, Jena, Germany) was used to determine the capsule thicknesses of at least 100 cells per strain.

Sensitivity to temperature (37 °C)

To determine sensitivities of the mutant strains to the growth temperature, 5 μL of diluted cultures ($D_{600\ nm}$ values of 1.0, 0.1, and 0.01) from each yeast strain were spotted onto YPD agar plates and incubated at 30 or 37 °C. Yeast growth was estimated visually after 24 and 48 h of growth.

Macrophage infection assay

A phagocytosis assay was adapted from [57] and [49], with slight modifications, to evaluate whether the *C. gattii* WT and mutant strains were internalized by macrophages and whether the internalized yeast cells could multiply within macrophages. Macrophage-like RAW264.7 cells (1×10^5 cells/100 μL of DMEM supplemented with 10% FBS) were placed in 96-well culture plates. After 24 h of incubation at 37 °C in 5% CO_2, the medium was replaced with fresh medium containing 1×10^6 *C. gattii* cells (WT and mutant strains, as well the WT strain inhibited with

different concentrations of AHA) that had been previously incubated for 24 h in YPD and extensively washed in PBS. Macrophages were allowed to interact with *C. gattii* cells for 1 h (37 °C, 5% CO_2), after which the medium was removed and replaced with DMEM/FBS. After a second 18-h incubation (37 °C, 5% CO_2), yeast cells that were not associated with macrophages were removed by PBS washes. To evaluate fungal survival after phagocytosis, the macrophages were lysed with sterile ice-cold water, and the lysates were subsequently plated onto YPD plates for CFU determination, after 48 h of incubation at 30 °C. This assay was performed in triplicate for each strain.

Mouse survival assay and organ fungal burdens

Cryptococcal strains were grown for 24 h in YPD, washed three times with PBS, counted in a Neubauer chamber, and suspended in PBS. BALB/c female mice (5 weeks of age, ~20 g) were anesthetized with 100 $mg \cdot kg^{-1}$ ketamine and 16 $mg \cdot kg^{-1}$ xylazine administered by intraperitoneal injection. Anesthetized mice were suspended on a thread by their incisors, and 50 μL of the yeast suspension (5×10^4 or 2×10^6 cells) was slowly pipetted into the nostrils of each mouse. The mice were kept suspended for an additional 10 min, and then placed in a ventilated cage to recover. The survival rates were determined for groups of eight animals per strain. For the organ fungal burden assay, mice were killed by CO_2 inhalation on different days after inoculation, and their lungs, brains, spleens, kidneys and blood were obtained under aseptic conditions. The organs were weighed, and macerated in 1 mL of sterile PBS; appropriate dilutions of the homogenates were then plated on YPD agar plates for CFU determinations.

Statistical analyses and software

A Kaplan–Meier survival analysis was conducted with GRAPHPAD 5.0 (GraphPad Software, La Jolla, CA, USA). One-way ANOVA, followed by Bonferroni's test, was used to evaluate the statistical parameters of capsule size, fungal burden, and macrophage interaction.

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Author contributions

V. Feder, L. Kmetzsch, C. C. Staats, C. R. Carlini, and M. H. Vainstein: conceived and designed the experiments. V. Feder, L. Kmetzsch, C. C. Staats, and N. Vidal-Figueiredo: performed the experiments. V. Feder, L. Kmetzsch, C. C. Staats, R. Ligabue-Braun, C. R. Carlini, and M. H. Vainstein: analyzed the data:. V. Feder, L. Kmetzsch, C. C. Staats, R. Ligabue-Braun, C. R. Carlini, and M. H. Vainstein: contributed reagents/materials/analysis tools. V. Feder: wrote the paper. V. Feder, L. Kmetzsch, R. Ligabue-Braun, C. C. Staats, C. R. Carlini, and M. H. Vainstein: reviewed the paper.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web site:

Table S1. List of primers used during this work.

Suplementary Table 1: List of primers used during this work.

Primer Name	Sequence (5´- 3´)	Size (bp)	Function
Ure1Del 5´F	AAAATAGGGATAACAGGGTAATGCATCTCCTCCCAAGAGAAACG	798	Disruption construct for *URE1* 5´flank
Ure1Del 5´R	GGGGACAAGTTTGTACAAAAAAGCAGGCTATTTGTCACAGTTTTCTTCTCCC		
Ure1Del 3´F	GGGGACCACTTTGTACAAGAAAGCTGGGTACTCTCGTATCCGCTCTGAAACC	791	Disruption construct for *URE1* 3´flank
Ure1Del 3´R	AAAAATTACCCTGTTATCCCTAGCGCGCACATCGTAGGTCTCG		
Ure4Del 5´F	AAAATAGGGATAACAGGGTAATTTGCTTTACCCTTTCCTCCTAC	688	Disruption construct for *URE4* 5´flank
Ure4Del 5´R	GGGGACAAGTTTGTACAAAAAAGCAGGCTATCATCGGCGGGGTAATATAGATA		
Ure4Del 3´F	GGGGACCACTTTGTACAAGAAAGCTGGGTATTTGACATGGAGTTTTAGTGA	679	Disruption construct for *URE4* 3´flank
Ure4Del 3´R	AAAAATTACCCTGTTATCCCTAACGAAATCGATGCGAACTTGG		
Ure6Del 5´F	AAAATAGGGATAACAGGGTAATCAGAGTGATGGATAAGCTCAC	676	Disruption construct for *URE6* 5´flank
Ure6Del 5´R	GGGGACAAGTTTGTACAAAAAAGCAGGCTAAGACGGAGGGAGTTTGTAATAA		
Ure6Del 3´F	GGGGACCACTTTGTACAAGAAAGCTGGGTAAAAAATGGGAAGGAAGAAGAT	690	Disruption construct for *URE6* 3´flank

Ure6Del 3´R	AAAAATTACCCTGTTATCCCTACGATGGCCAGTTCATCTGATG		
confDel Ure1	TCCGCTTCCCAGACCACTTC	803	Amplification of *URE1* internal fragment to confirm transformants
	Combined with Ure1Del 5´F		
Ure4 confF	GCTCGAGAGCTGAAGGACATC	683	Amplification of *URE4* internal fragment to confirm transformants
Ure4 confR	CGAGGAAAGATGTATGTGCAG		
Ure6 confF	GTCAAATGACGATATTGTGGG	794	Amplification of *URE6* internal fragment to confirm transformants
Ure6 confR	TTTTGATGATCGTCGACCAAC		
Ure1CompF	TTTCTCCTTTTTGTCGACGAGG	5034	Amplification of *URE1* for reconstitution
Ure1CompR	GGTTGCAAGATTTGTGAGGCT		
Ure4CompF	CAAGGCCGTTGACGACCTCAT	2819	Amplification of *URE4* for reconstitution
Ure4CompR	TCCATTGGGGAAAGGAGAGTT		
Ure6CompF	GGGACCGAAGTATCGATGATCGATGGAG	2950	Amplification of *URE6* for reconstitution
Ure6CompR	AATACTCAAGCTATGCATCAACTTACACCGGCTGCGGAGGT		
RTUre1F	GGTCCTGCAGATGGCTCCAA	210	Amplification of *URE1* for RT-PCR
RTUre1R	AATAACTTCGGGCGTGGCGC		

Curriculum Vitae: Vanessa Feder

Julho/2012

Vanessa Feder Soares
Curriculum Vitae

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CPF 742.214.340-15

Formação acadêmica/titulação

2008 Doutorado em Biologia Celular e Molecular.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil
Título: Propriedades biológicas da Urease de Cryptococcus gattii como fator de virulência
Orientador: Célia R. Carlini; Marilene H. Vainstein
Bolsista do(a): Conselho Nacional de Desenvolvimento Científico e Tecnológico

2007 - 2008 Mestrado em Programa de Pós-Graduação em Biologia Celular e Molecular - UFRGS.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil
Título: Isolamento e Caracterização de Urease de Cryptococcus gattii, Ano de obtenção: 2008
Orientador: Célia R. Carlini ; Marilene H. Vainstein
Bolsista do(a): Conselho Nacional de Desenvolvimento Científico e Tecnológico

2001 - 2002 Especialização em Clínica e Cirurgia de Cães e Gatos Lato Sensu.
Universidade Luterana do Brasil, ULBRA, Canoas, Brasil
Título: Leishmaniose Tegumentar e Visceral: uma contribuição à clínica veterinária e saúde pública no Brasil
Orientador: Dr Celso Bittencourt dos Anjos

1993 - 1998 Graduação em Medicina Veterinária.
Universidade Luterana do Brasil, ULBRA, Canoas, Brasil
Título: Relatório de Conclusão de Curso em Clínica e Cirurgia de Caninos, Felinos e Animais Silvestres
Orientador: Dra Carla Hennemann

Formação complementar

1997 - 1997 Extensão universitária em Doença de Chagas, Leishmaniose e Esquistossomose.
Fundação Oswaldo Cruz, FIOCRUZ, Rio De Janeiro, Brasil

1996 - 1996 Curso de curta duração em Medicina Veterinária Em Animais Silvestres.
Universidade Luterana do Brasil, ULBRA, Canoas, Brasil

1996 - 1996 Curso de curta duração em Atualização Em Clínica Cirúrgica e Atualização Em.
Universidade Federal de Santa Maria, UFSM, Santa Maria, Brasil

1996 - 1996 Curso de curta duração em Curso de Atualização Em Clínica Cirúrgica.
Universidade Federal de Santa Maria, UFSM, Santa Maria, Brasil

1996 - 1996 Extensão universitária em Atendimento Clínico Veterinário na XIX Expointer.
Universidade Luterana do Brasil, ULBRA, Canoas, Brasil

1995 - 1995 Extensão universitária em Serviço de Saúde de Defesa Sanitária e Zootecnia.
Universidade Luterana do Brasil, ULBRA, Canoas, Brasil

1995 - 1995 Curso de curta duração em Manutençao e Reprodução de Répteis Em Cativeiro.
Sociedade dos Zoológicos do Brasil, SZB, Brasil

1994 - 1994 Extensão universitária em Campanha de Vacinação Antirábica de Novo Hamburgo.
Universidade Luterana do Brasil, ULBRA, Canoas, Brasil

Atuação profissional

1. Universidade Federal do Rio Grande do Sul - UFRGS

Vínculo institucional

2008 - Atual Vínculo: Bolsista Doutorado CNPq , Enquadramento funcional: Doutorado acadêmico , Carga horária: 40, Regime: Dedicação exclusiva

2007 - 2008 Vínculo: bolsista CNPq , Enquadramento funcional: mestrado acadêmico , Carga horária: 40, Regime: Dedicação exclusiva

2005 - 2005 Vínculo: estágio , Enquadramento funcional: estagiário , Carga horária: 30, Regime: Dedicação exclusiva

2005 - 2008 Vínculo: bolsista , Enquadramento funcional: bolsa de apoio técnico nível 1 , Carga horária: 40, Regime: Dedicação exclusiva

Atividades

01/2006 - Atual Pesquisa e Desenvolvimento, Instituto de Biociências, Departamento de Biofísica
Linhas de pesquisa:
Ação fungicida de urease de soja em fungos fitopatogênicos , Purificação de Proteínas , Biologia Molecular , Mecanismo de ação de fungos de importância médica

05/2005 - Atual Serviço Técnico Especializado
Especificação:
serviço técnico especializado

02/2005 - 04/2005 Estágio, Centro de Biotecnologia
Estágio:
acompanhamento de atividades dos pesquisadores do laboratório de proteínas tóxicas

2. Dog Doc Veterinários Associados - DOG DOC

Vínculo institucional

1997 - 2001 Vínculo: Prestação de Serviço Autônomo , Enquadramento funcional: Clínica e Cirurgiã da equipe , Carga horária: 44, Regime: Integral

Atividades

07/2000 - 08/2000 Outra atividade técnico-científica, Centro de Informações Toxicológicas, Intoxicações

Especificação:
publicação em congresso

05/2000 - 05/2001 Direção e Administração
Cargos ocupados:
Coordenadora Clínica

05/2000 - 05/2001 Serviço Técnico Especializado
Especificação:
Clínica e Cirurgiã

05/2000 - 05/2001 Treinamento
Especificação:
Treinamento de Estagiários Plantonistas

07/1999 - 08/1999 Outra atividade técnico-científica, Centro de Informações Toxicológicas, Informações
Especificação:
Publicação em Congresso

01/1999 - 01/1999 Serviço Técnico Especializado
Especificação:
Clínica e Cirurgiã de caninos, felinos e silvestres

06/1998 - 10/1998 Estágio, Universidade Luterana do Brasil, Medicina Veterinária
Estágio:
Estágio Currículeular Obrigatório

06/1997 - 11/1997 Estágio
Estágio:
plantonista voluntária

05/1997 - 06/1997 Outra atividade técnico-científica, Centro de Informações Toxicológicas, Acidente Com Animais Peçonhentos
Especificação:
PUBLICAÇÃO EM CONGRESSO

3. Semefertil comércio de produtos veterinários - SEMEFERTIL

Vínculo institucional

2003 - 2003 Vínculo: profissional liberal , Enquadramento funcional: representante comercial , Carga horária: 40, Regime: Dedicação exclusiva

Atividades

07/2003 - 12/2003 Serviço Técnico Especializado
Especificação:
representação comercial , suporte técnico na área de nutriçao e farmacologia, palestras de orientação a profissionais e leigos.

07/2003 - 01/2004 Treinamento
Especificação:
Instrução sobre fármacos e princípios de nutrição em caninos.

4. Pet Store Serviços Veterinários Ltda - PET STORE

Vínculo institucional

1999 - 2000 Vínculo: Prestação de Serviço Autônomo , Enquadramento funcional:

Responsável Técnica, Clínica e Cirurgiã , Carga horária: 36, Regime: Parcial

Atividades

08/1999 - 04/2000 Serviço Técnico Especializado
Especificação:
Responsabilidade Técnica, Clínica e Cirurgiã

5. Miauau Comércio de Serviços e Produtos Veterinários Ltda - MIAUAU

Vínculo institucional

2001 - 2003 Vínculo: prestação de Serviço autônomo , Enquadramento funcional: Clínica e cirurgiã de caninos, felinos e anim , Carga horária: 25, Regime: Parcial

Atividades

12/2001 - 07/2003 Serviço Técnico Especializado
Especificação:
clínica e cirurgiã de caninos, felinos e animais silvestres

Linhas de pesquisa

1. Ação fungicida de urease de soja em fungos fitopatogênicos

2. Biologia Molecular

3. Mecanismo de ação de fungos de importância médica

4. Purificação de Proteínas

Idiomas

Inglês Compreende Bem , Fala Bem , Escreve Bem , Lê Bem

Espanhol Compreende Razoavelmente , Fala Razoavelmente , Escreve Pouco , Lê Bem

Português Compreende Bem , Fala Bem , Escreve Bem , Lê Bem

Producão

Produção bibliográfica
Artigos completos publicados em periódicos

1. Menegassi, Angela, Menegassi, Angela, WASSERMANN, German E., Olivera-Severo, Deiber, Becker-Ritt, Arlete B., Martinelli, Anne Helene S., Carlini, Celia R., Carlini, Celia R., FEDER, Vanessa, Olivera-Severo, Deiber, Martinelli, Anne Helene S., WASSERMANN, German E., Becker-Ritt, Arlete B.
Urease from Cotton (Gossypium hirsutum) Seeds: Isolation, Physicochemical Characterization, and

Antifungal Properties of the Protein. Journal of Agricultural and Food Chemistry. , v.56, p.4399 - 4405, 2008.

2. Becker-Ritt, A. B, MARTINELLI, A. H.S., MITIDIERI, S., FEDER, Vanessa, WASSERMANN, G. E., SANTI, L., VAINSTEIN, M. H., OLIVEIRA, J., FIUZA, L. M., PASQUALI, G., CARLINI, C. R.
Antifungal Activity of Plant and Bacterial Ureases. Toxicon. , v.008, p.1 - 14, 2007.

Trabalhos publicados em anais de eventos (resumo)

1. Feder, Vanessa, KMTEZSCH, L., Staats, C., DEMARTINI, D. R., CARLINI, C. R., VAINSTEIN, M. H.
Urease de Cryptococcus gattii: análises físicos químicas e construção de mutantes para deleção do gene estrutural de proteínas acessórias na linhagem R265 In: XIII Reunião Anual PPGBCM UFRGS, 2011, porto alegre.
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2. SILVEIRA, C. P., LANDELL, M., KMTEZSCH, L., Feder, Vanessa, KRUG, M., PIFFER, A., Staats, C., SCHRANK, A., VAINSTEIN, M. H.
Aplicação de Proteínas Imunogênicas Associadas a Flagelina no tratamento de criptococose In: XII Reunião Anual do Programa de Pós Graduação em Biologia Celular e Molecular do Centro de Biotecnologia da UFRGS, 2010, Porto Alegre.
Aplicação de proteínas imunogênicas associadas a flagelina no tratamento de criptococose. , 2010.

3. LIMA, T. M. F., FEDER, Vanessa, OLIVERA-SEVERO, D., Becker-Ritt, A. B, PINTO, P. M., VAINSTEIN, M. H., CARLINI, C. R.
Characterization and Structural Aspects from Purified Urease of Cryptococcus gattii – R265 In: XXXIX Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular - SBBq; 1st Latin American Symposium on the Molecular Mechanisms of Skeletal Mineralization, 2010, Foz do Iguaçu - PR.
Anais do Congresso. , 2010.

4. FEDER, Vanessa, ROSA e SILVA, L. K., Staats, C., LIMA, T. M. F., CARLINI, C. R., VAINSTEIN, M. H.
DelsGate method for inactivating genes encoding Urease and Acessory Proteins of Cryptococcus gattii. In: XXXIX Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular - SBBq;1st Latin American Symposium on the Molecular Mechanisms of Skeletal Mineralization, 2010, Foz do Iguaçu - PR.
Anais do Congresso. , 2010.

5. Feder, Vanessa, KMTEZSCH, L., Staats, C., LIMA, T. M. F., VAINSTEIN, M. H., CARLINI, C. R.
Influência de Proteínas Acessórias na Construção de Mutantes de Urease em Cryptococcus gattii linhagem R265 In: XII Reunião Anual do PPGBCM UFRGS, 2010, Porto Alegre.
Livro de Resumos. Porto Alegre: UFRGS, 2010. p.97 - 97

6. PELEGRINI, A. L., BARBIERI, N., FILLIPI CHIELA, E. C., FIUZA, L. M., MABONI, F., Pinhati, V. R., SACHETT, L. G., SALTON, G. D., VARGAS, J. E., LOPES, P. L. C., OLIVEIRA, J., VELHO, R. V., VILLDORE, E. S., WEBER, S. S., FEDER, Vanessa, MARGIS, R., CARLINI, C. R.
Curso de Férias: Uma alternativa didádica ou uma didática alternativa? In: XI Reunião Anual do Programa de Pós Graduação em Biologia Celulare Molecular - UFRGS, 2009, Porto Alegre - RS.
Livro de Resumos. , 2009.

7. FEDER, Vanessa, OLIVERA-SEVERO, D., Becker-Ritt, A. B, SCHROEDER, E., VAINSTEIN, M. H., CARLINI, C. R.
Purification and Characterization of a Urease from Pathogenic Yeast Cryptococcus gattii- R265 In: XVI World Congress of the International Society on Toxinology, X Congresso da Sociedade Brasileira de Toxinologia, 2009, Recife- PE.

Livro de Resumos. , 2009.

8. FEDER, Vanessa, ROSA e SILVA, L. K., LIMA, T. M. F., CARLINI, C. R., VAINSTEIN, M. H.
Uso da Técnica DelsGate para deleção do gene Urease e Proteínas Acessórias de Cryptococcus gattii - linhagem R265 In: XI Reunião Anual PPGBCM, Porto Alegre.
XI Reunião Anual PPGBCM-UFRGS. , 2009.

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X Reunião Anual PPGBCM - UFGRS. , 2008.

10. Becker-Ritt, A. B, MARTINELLI, A. H.S., MITIDIERI, S., FEDER, Vanessa, WASSERMANN, G. E., PASQUALI, G., CARLINI, C. R.
Antifungal activity of ureases from Glycine max and Canavalia ensiformis seeds and a recombinant Helicobacter pylori urease In: Plant Biology and Botany 2007 Joint Congress, 2007, Chicago.
Antifungal activity of ureases from Glycine max and Canavalia ensiformis seeds and a recombinant Helicobacter pylori urease. , 2007.

11. FEDER, Vanessa, VAINSTEIN, M. H., CARLINI, C. R.
Purificação e Caracterização da Urease de Cryptococcus neoformans In: IX Reunião Anual do Programa de Pós Graduação em Biologia Molecular e Celular- UFRGS, 2007, Porto Alegre - RS.
Resumos. , 2007.

12. FEDER, Vanessa, CHULA, Silvia, MARQUES, Ana Cândida, RAMOS, Carla, MARQUES, Maria da Graça
Mamona: Ralato de caso de intoxicação em cão Poodle In: XXI Congresso Brasileiro e Conferência Sul-americana de Medicina Veterinária, 2000, Rio de Janeiro.
Anais do XXI Congresso Brasileiro e Conferência Sul-americana de Medicina Veterinária. Rio de Janeiro: , 2000. p.54 - 54

13. FEDER, Vanessa, CHULA, Silvia, RAMOS, Carla, MARQUES, Maria da Graça, MARQUES, Ana Cândida
Metoclopramida: intoxicação em cão - relato de caso In: XXI Congresso Brasileiro e Conferência Sul-americana de Medicina Veterinária, 2000, Rio de Janeiro.
Anais do XXI Congresso Brasileiro e Conferência Sul-americana de Medicina Veterinária. Rio de Janeiro: , 2000. p.55 - 55

14. FEDER, Vanessa, CHULA, Silvia, RAMOS, Carla, MARQUES, Maria da Graça, MARQUES, Ana Cândida
A importância dos Centros de Informação Toxicológica na Clínica Veterinária - Relato de Casos. In: Brasiltox 99 - XI Congresso Brasileiro de Toxicologia Internacional. International Congress of Clinical Toxicology, 1999, Guarujá.
Brasiltox 99 - XI Congresso Anais - Brasileiro de Toxicologia Internacional. International Congress of Clinical Toxicology. , 1999. p.70 - 70

15. FEDER, Vanessa, CHULA, Silvia, RAMOS, Carla, MARQUES, Maria da Graça
Araneísmo: acidentes com animais do gênero Phoneutria spp. em pequenos animais - relato de caso In: II Congresso de Medicina Veterinária do Conesul. XIII Congresso Estadual de Medicina Veterinária. XXV Congresso Brasileiro de Medicina Veterinária, 1997, Gramado.
Anais doII Congresso de Medicina Veterinária do Conesul. XIII Congresso Estadual de Medicina Veterinária. XXV Congresso Brasileiro de Medicina Veterinária. , 1997. p.63 - 63

16. FEDER, Vanessa, CHULA, Silvia, RAMOS, Carla, MARQUES, Maria da Graça
Ofidismo: acidentes com animais do gênero Bothrops spp. em pequenos animais- relato de caso. In: II Congresso de Medicina Veterinária do Conesul. XIII Congresso Estadual de Medicina Veterinária. XXV Congresso Brasileiro de Medicina Veterinária, 1997, Gramado.
II Congresso de Medicina Veterinária do Conesul. XIII Congresso Estadual de Medicina

Veterinária. XXV Congresso Brasileiro de Medicina Veterinária. Gramado: , 1997.

Produção técnica
Trabalhos técnicos

1. FEDER, Vanessa
Trabalho de Conclusão de Curso: relatório de estágio em clínica e Cirurgia de cães, gatos e animais silvestres, 1998

Demais produções técnicas

1. PELEGRINI, A. L., BARBIERI, N., FILLIPI CHIELA, E. C., VILLDORE, E. S., LOPES, F. C., MABONI, F., SALTON, G. D., VARGAS, J. E., SACHETT, L. G., LOPES, P. L. C., VELHO, R. V., WEBER, S. S., FEDER, Vanessa, MARGIS, R., CARLINI, C. R.
Curso de Férias - Microorganismo: Mocinhos ou bandidos?, 2009. (Extensão, Curso de curta duração ministrado)

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Orientações e Supervisões

Orientações e supervisões

Orientações e supervisões concluídas

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