The Ess1 prolyl isomerase is dispensable for growth but required for virulence in *Cryptococcus neoformans*

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Cryptococcus neoformans is an important human fungal pathogen that also serves as a model for studies of fungal pathogenesis. *C. neoformans* contains several genes encoding peptidyl-prolyl *cis*/*trans* isomerases (PPIases), enzymes that catalyse changes in the folding and conformation of target proteins. Three distinct classes of PPIases have been identified: cyclophilins, FK506-binding proteins (FKBPs) and parvulins. This paper reports the cloning and characterization of *ESS1*, which is believed to be the first (and probably only) parvulin-class PPIase in *C. neoformans*. It is shown that *ESS1* from *C. neoformans* is structurally and functionally homologous to *ESS1* from *Saccharomyces cerevisiae*, which encodes an essential PPIase that interacts with RNA polymerase II and plays a role in transcription. In *C. neoformans*, *ESS1* was found to be dispensable for growth, haploid fruiting and capsule formation. However, *ESS1* was required for virulence in a murine model of cryptococcosis. Loss of virulence might have been due to the defects in melanin and urease production observed in *ess1* mutants, or to defects in transcription of as-yet-unidentified virulence genes. The fact that Ess1 is not essential in *C. neoformans* suggests that, in this organism, some of its functions might be subsumed by other prolyl isomerases, in particular, cyclophilins Cpa1 or Cpa2. This is supported by the finding that *ess1* mutants were hypersensitive to cyclosporin A. *C. neoformans* might therefore be a useful organism in which to investigate crosstalk among different families of prolyl isomerases.

INTRODUCTION

Cryptococcus neoformans is an opportunistic fungal pathogen that occurs worldwide and causes fatal nervous system infections, predominantly in immunocompromised individuals (Mitchell & Perfect, 1995). It is a basidiomycete; sexual reproduction involves the formation of a specialized structure (basidium) in which nuclear fusion, meiosis and spore formation occur to generate infectious basidiospores. The diploid phase is transient and the cells grow vegetatively as haploids. *C. neoformans* also differentiates to produce several enzymes and structures that are crucial for virulence in animal models of cryptococcal meningitis. For example, production of the pigment melanin is important for virulence and is thought to protect the organism from toxic oxygen radicals produced by host phagocytes (Kozel, 1995), while production of the metalloenzyme urease, which catalyses the hydrolysis of urea to ammonia and carbamate, may function by altering host immune function (Cox *et al.*, 2000). A prominent structure associated with virulence is the polysaccharide capsule, which inhibits phagocytosis by host monocytes or macrophages (Fromtling *et al.*, 1982; Kozel & Cazin, 1971; Chang & Kwon-Chung, 1994).

Peptidyl-prolyl *cis*/*trans* isomerases (PPIases) catalyse the interconversion between *cis* and *trans* forms of the peptide bond preceding proline residues in proteins (Fischer, 1994; Fischer *et al.*, 1998; Schiene & Fischer, 2000). Conformational isomerization by PPIases is thought to control the activity of target proteins and their ability to interact with other proteins to form complexes (Schmid *et al.*, 1993; Hunter, 1998). Three families of PPIases that differ in structure and substrate specificity are known: the cyclophilins, the FK506-binding proteins (FKBPs) and the parvulins (Dolinski & Heitman, 1997; Arévalo-Rodríguez *et al.*, 2004). The PPIase activity of cyclophilin A is potently inhibited by the immunosuppressive drug cyclosporin A (CsA)

Abbreviations: CsA, cyclosporin A; FKBP, FK506-binding protein; 5-FOA, 5-fluoroorotic acid; PPIase, peptidyl-prolyl *cis*/*trans* isomerase.

The GenBank/EMBL/DDBJ accession number for the sequence reported in this paper is AF533511.

(Handschumacher *et al.*, 1984; Takahashi *et al.*, 1989). In *C. neoformans*, there are two cyclophilin A homologues, encoded by linked genes, *CPA1* and *CPA2* (Wang *et al.*, 2001). Mutations in *CPA1* and *CPA2* confer a spectrum of cell growth, mating and virulence phenotypes that indicate the homologues have distinct but overlapping roles in *C. neoformans* (Wang *et al.*, 2001). The second class of PPIases, the FKBPs, are inhibited by the immunosuppressants FK506 and rapamycin, drugs which also show antifungal activity. In *C. neoformans*, disruption of the *FKBP12* gene confers rapamycin and FK506 resistance but has no effect on growth, differentiation or virulence of *C. neoformans* (Cruz *et al.*, 1999, 2001). Prior to this report, no parvulin-class PPIases had to our knowledge been identified in *C. neoformans*.

Parvulin-class PPIases are named after an *Escherichia coli* protein called parvulin (Rahfeld *et al.*, 1994). The first eukaryotic parvulin to be discovered was the Ess1 protein from *Saccharomyces cerevisiae* (Hanes *et al.*, 1989; Hani *et al.*, 1995). Ess1 is the only PPIase that is essential for growth in *S. cerevisiae* (Hanes *et al.*, 1989; Dolinski *et al.*, 1997). Cells depleted of Ess1 arrest in mitosis and undergo nuclear fragmentation (Lu *et al.*, 1996; Wu *et al.*, 2000). Some evidence suggests that Ess1 and its homologue in humans, Pin1, interact with cell cycle proteins to control mitotic progression (Crenshaw *et al.*, 1998; Shen *et al.*, 1998; Stukenberg & Kirschner, 2001), while other evidence shows that Ess1 binds to the carboxy-terminal domain (CTD) of the large subunit of RNA polymerase II and is important for mRNA transcription (Morris *et al.*, 1999; Wu *et al.*, 2000, 2001). It is thought that Ess1-mediated isomerization of the CTD controls multiple steps in transcription, including initiation and elongation, and pre-mRNA processing (Shaw, 2002; Wu *et al.*, 2003; Xu *et al.*, 2003; Wilcox *et al.*, 2004).

Ess1 homologues have been studied in metazoans such as *Drosophila melanogaster* (Maleszka *et al.*, 1996; Hsu *et al.*, 2001), Xenopus (Winkler *et al.*, 2000), mice and humans (Fujimori *et al.*, 1999; Lu *et al.*, 1996). In most of these organisms, Ess1/Pin1 is not essential for growth. However, in two other fungi, *Candida albicans* and *Aspergillus nidulans*, Ess1 was shown to be essential (Devasahayam *et al.*, 2002; Joseph *et al.*, 2004). In *Ca. albicans*, mutants with a reduced gene dosage showed defects in filamentation (Devasahayam *et al.*, 2002) and reduced virulence in a mouse model of candidiasis (Li *et al.*, 2005). Here, we sought to identify an Ess1/Pin1 homologue in *C. neoformans*, a distantly related human fungal pathogen, and to characterize its requirement for growth, differentiation and virulence. Unlike the case for *S. cerevisiae* and *Ca. albicans*, disruption of *ESS1* in *C. neoformans* was not lethal. No defects were observed in growth rate, capsule formation, response to mating pheromones, or haploid fruiting. However, *ess1*Δ mutants were avirulent when tested in a murine model of cryptococcosis, and showed reduced levels of melanin production and urease activity, factors known to be important for virulence (Salas *et al.*, 1996; Cox *et al.*, 2000). These results, together with studies in *Ca. albicans*, suggest that the Ess1 PPIase might be a useful target for the development of broad-spectrum antifungal drugs.

METHODS

Strains, media and transformations. Strains used in this paper are listed in Table 1. Standard yeast media, i.e. YPD (yeast extract peptone glucose [dextrose]), and CSM (complete synthetic medium) with relevant amino-acid dropouts were made as described by Guthrie & Fink (1991). Niger seed agar (Alspaugh *et al.*, 1997; Salkin, 1979), urea agar (McGinnis, 1980), and limited-iron medium with EDTA and bathophenanthroline disulphonic acid (Pierini & Doering, 2001) were used for detecting melanin production, urease activity, and capsule formation, respectively. *S. cerevisiae* transformations were done using the standard lithium acetate procedure (Ito *et al.*, 1983) and *C. neoformans* transformations were done using biolistic DNA delivery as described by Toffaletti *et al.* (1993) and Davidson *et al.* (2000). For biolistic transformation, approximately 5 μg linear DNA was used and transformants were selected on CSM lacking uracil. Cyclosporin A solution (Novartis) was a gift of Dr David Porter (University of Pennsylvania Medical Center, Philadelphia, PA, USA). FK520 was purchased from Calbiochem and dissolved in DMSO.

Identification, cloning and sequence analysis of the *C. neoformans ESS1* gene. The *C. neoformans ESS1* gene was identified using BLAST on the genome sequence at the Stanford Genome Technology Center Genome Project (http://www-sequence.stanford.edu/group/C.neoformans). Oligonucleotides with the sequences 5′-GGAATTCCATATGTCATCAACCGGTTGGGAGATTC-3′ and 5′-TCATTACTCGAGCCAACTTTGATTTCCATAGCCGTTC-3′ were synthesized and used to amplify the *ESS1* gene by PCR from *C. neoformans* B-3501 genomic DNA. Standard reaction conditions were used (94 °C 1 min, 59 °C 45 s, 72 °C 1 min) and a fragment of approximately 0·7 kb was amplified, cloned and sequenced. Two introns in the *ESS1* gene were identified by alignment of the predicted Ess1 protein with other members of Ess1 family of parvulin-class prolyl-isomerases: *S. cerevisiae* Ess1, *Ca. albicans* Ess1, *A. nidulans* PinA, *Drosophila melanogaster* Dodo and *Homo sapiens* Pin1. The introns were deleted using the 'gene splicing by overlap extension' method (Horton *et al.*, 1990) and the uninterrupted ORF cloned into the yeast expression vector pJG4-1ΔE (2μ, *TRP1*; Gyuris *et al.*, 1993), which has an ADH promoter and terminator. The resulting intron-less clone is pCnESS1-ΔI. The GenBank accession number for *C. neoformans ESS1* is AF533511. Phylogenetic analysis of Ess1/Pin1 amino acid sequences was done with the PAUP v4.0b4a program (Swofford, 2000) using a bootstrap method with a neighbour-joining search (options: number of bootstrap replicates = 1000; ties in trees resolved randomly).

***S. cerevisiae* strain construction.** To construct haploid strain YPR-57 (*ess1*Δ : : *HIS3* pGD-CaESS1), a high-copy plasmid carrying *Ca. albicans ESS1*, pGD-CaESS1 (*URA3*) (Devasahayam *et al.*, 2002), was transformed into the heterozygous *ESS1*/*ess1*Δ : : *HIS3* (YSH-55) strain. Cells were induced to sporulate, tetrads dissected, and His$^+$ Ura$^+$ segregants were selected. To generate YPR-34 (*ess1*Δ : : *URA3*/*ESS1*), a PCR product of the *URA3* gene flanked by 46 nucleotides of homology to 5′ and 3′ *ESS1* flanking sequences was transformed into diploid *S. cerevisiae* strain W303-1A × B and uracil prototrophs were selected. The *ess1* : : *URA3* disruption was confirmed by PCR.

Complementation experiments. The temperature-sensitive *S. cerevisiae* strain *ess1*$^{\mathrm{H164R}}$ (YGD-ts22W; Wu *et al.*, 2000) was transformed with the plasmid pCnESS1-ΔI, a positive-control vector (pRS424-ESS1, which contains a *Bam*HI–*Xho*I fragment of *S. cerevisiae ESS1* from plasmid pRS413-ESS1), or vector alone (pJG4-1ΔE),

Table 1. Strains used in this study

Strain	Parent	Genotype	Source
S. cerevisiae			
W303-1A × B		*MAT***a***/MATα ura3-1/ura3-1 leu2-3,112/leu2-3,112 trp1-1/trp1-1 can1-100/can1-100 ade2-1/ade2-1 his3-11/his3-11* [*phi*$^{+}$]	R. Rothstein*
YSH-55	W303-1AxB	*MAT***a***/MATα ade2-1/ade2-1 can1-100/can1-100 his3-11,15/his3-11,15 leu2-3,112/LEU2 ura3-1/ura3-1 ESS1/ess1Δ : : HIS3*	Wu *et al.* (2000)
YGD-ts22W	W303-1A	*MAT***a** *ura3-1 leu2-3,112 trp1-1 can1-100 ade2-1 his3-11,15* [*phi*$^{+}$] *ess1-H164R*	Wu *et al.* (2000)
YPR-57	YSH-55	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112/LEU2 ura3-1 ess1Δ : : HIS3* + pCaESS1	This study
YPR-34	W303-1A × B	*MAT***a***/MATα ura3-1/ura3-1 leu2-3,112/leu2-3,112 trp1-1/1-1 can1-100/can1-100 ade2-1/ade2-1 his3-11/his3-11 ESS1/ess1Δ : : URA3*	This study
***C. neoformans* var. *neoformans* (serotype D)**			
B-3501		Wild-type *MATα*	Kwon-Chung (1978)
JEC21		Wild-type *MATα*	Kwon-Chung *et al.* (1992a)
JEC20		Wild-type *MAT***a**	Kwon-Chung *et al.* (1992a)
JEC43	JEC21	*MATα ura5*	Moore & Edman (1993)
CnPR68	JEC43	*MATα ura5 ess1 : : URA5*	This study
CnPR37	CnPR68	*MATα ura5 ess1 : : ura5*	This study
CnPR170	CnPR37	*MATα ura5 ess1 : : ura5 ESS1 : : URA5*	This study

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and the ability of cells to grow at the restrictive temperature (37 °C) was tested by streaking on appropriate solid media. For plasmid-curing experiments, a haploid *S. cerevisiae ess1* deletion strain, YPR-57, carrying a plasmid-borne copy of *ESS1* was transformed with pCnESS1-ΔI. Transformants were serially passaged in liquid CSM lacking tryptophan for 3 days, i.e. selecting for the pCnESS1-ΔI (*TRP1*) but not for the pGD-CaESS1 (*URA3*) plasmid. Cells were plated and the phenotypes of individual colonies were scored by replica-plating to appropriate selective media. For segregation analysis, the heterozygous disruption strain YPR-34 (*ess1Δ : : URA3/ESS1*) was transformed with pCnESS1-ΔI. Cells were induced to undergo sporulation on 1 % potassium acetate plates, tetrads were dissected and haploid segregants were grown on rich medium. Growth was scored after 3 days, and segregation of the Ura^{+} and Trp^{+} phenotypes monitored by replica-plating to detect presence of the *ess1 : : URA3* disruption and the pCnESS1-ΔI plasmid (*TRP1*) respectively.

Disruption of *ESS1* in *C. neoformans* and reconstitution of mutant strains. The *ESS1* gene was disrupted by homologous recombination using an *ess1 : : URA5* disruption allele. To generate the disruption allele the following strategy was used. A 2·0 kb fragment containing the *C. neoformans URA5* gene was PCR amplified from *C. neoformans* B-3501 genomic DNA. The primers used incorporated *Spe*I and *Mfe*I sites at the 5′ and 3′ ends of *URA5*, respectively. The fragment was digested with these enzymes and cloned into the same sites of plasmid pUC-CnESS1-SpeI. pUC-CnESS1-SpeI contains a modified version of *ESS1* in which the initiator codon was destroyed and replaced with stop codons in all three frames and included a *Spe*I cloning site (P. Ren & S. D. Hanes, unpublished). The final disruption construct, pUC-Cness1-URA5, replaces the first 124 nucleotides of the *ESS1* ORF with the *URA5* gene. To use this disruption construct, a 4·1 kb fragment was released by *Eco*RI and *Xba*I digestion and used for biolistic transformation of JEC43. Uracil-prototrophic transformants were selected and colony purified. Approximately 60 transformants per μg DNA were obtained. As determined by PCR and Southern analysis, about 1 % of the transformants carried homologous gene replacements at the *ESS1* locus, resulting in strain CnPR68.

A CnPR68 *ura5* prototrophic revertant, CnPR37, was obtained by counterselection using 5-fluoroorotic acid (5-FOA) medium (Kwon-Chung *et al.*, 1992b). Strain CnPR37 was used to generate a reconstituted strain by biolistic transformation with plasmid pCn-tel-CnESS1 that was linearized with *Not*I. pCn-tel-CnESS1 was constructed by insertion of a 2·1 kb *Eco*RI–*Bam*HI fragment (consisting of 1·4 kb of upstream untranslated region plus 0·7 kb of the *C. neoformans ESS1* gene) into plasmid pCn-tel1. pCn-tel contains a *URA5* selection marker and was kindly supplied by Ping Wang and Joseph Heitman (Duke University, Durham, NC, USA; Davidson *et al.*, 2000; Edman & Kwon-Chung, 1990). The 2·1 kb fragment had been obtained by PCR from *C. neoformans* B-3501 genomic DNA. The DNA sequence of the *C. neoformans ESS1* gene in this construct was confirmed by sequence analysis. In the reconstituted strain, CnPR170, the *Not*I-linearized pCn-tel-CnESS1 was integrated at random into the genome (i.e. not at the *ess1 : : ura5* locus). The integrated copy of *ESS1* contained its own promoter region so that it could be expressed normally. The presence of intact *ESS1* in the reconstituted strain was confirmed using four PCR reactions with different sets of primers.

Cell morphology, capsule formation, melanin production and urease biosynthesis assays. To observe the cell morphology, cells were incubated in YPD broth at 25 °C to mid-exponential phase and photographed at × 400 magnification using a compound microscope equipped with a digital camera. For capsule formation, the wild-type, *ess1* mutant and *ess1* + *ESS1* reconstituted strains were inoculated into 10 ml limited-iron medium with 100 μM EDTA and 100 μM bathophenanthroline disulphonic acid, and incubated at 30 °C for 5 to 7 days with agitation. Cells from these cultures were mixed with a standard India ink preparation and photographed (×400 magnification). To detect melanin or urease production, wild-type, *ess1* mutant and *ess1*+*ESS1* reconstituted strains were freshly cultured to mid-exponential phase in YPD broth, and 5 μl of each culture was spotted onto Niger seed agar medium and incubated for 4 days at 30 °C, or onto urea agar medium for 2 days at 30 °C, respectively.

Virulence in a mouse model of cryptococcal meningitis. Cells of wild-type strain (JEC21), the *ess1* mutant strain (CnPR68) and the reconstituted strain (CnPR170) were grown to mid-exponential phase in liquid YPD medium and washed and resuspended in 15 mM PBS. BALB/c male mice (Jackson Laboratory, Bar Harbour, ME; USA, six in a group for each strain) weighing 20–25 g (about 6–8 weeks old) obtained from the Griffin Laboratory (Wadsworth Center) were infected by lateral tail vein injection using 100 μl *C. neoformans* at 10^7 cells ml^{-1}. The cell concentrations were determined using a haemocytometer before injection and confirmed by

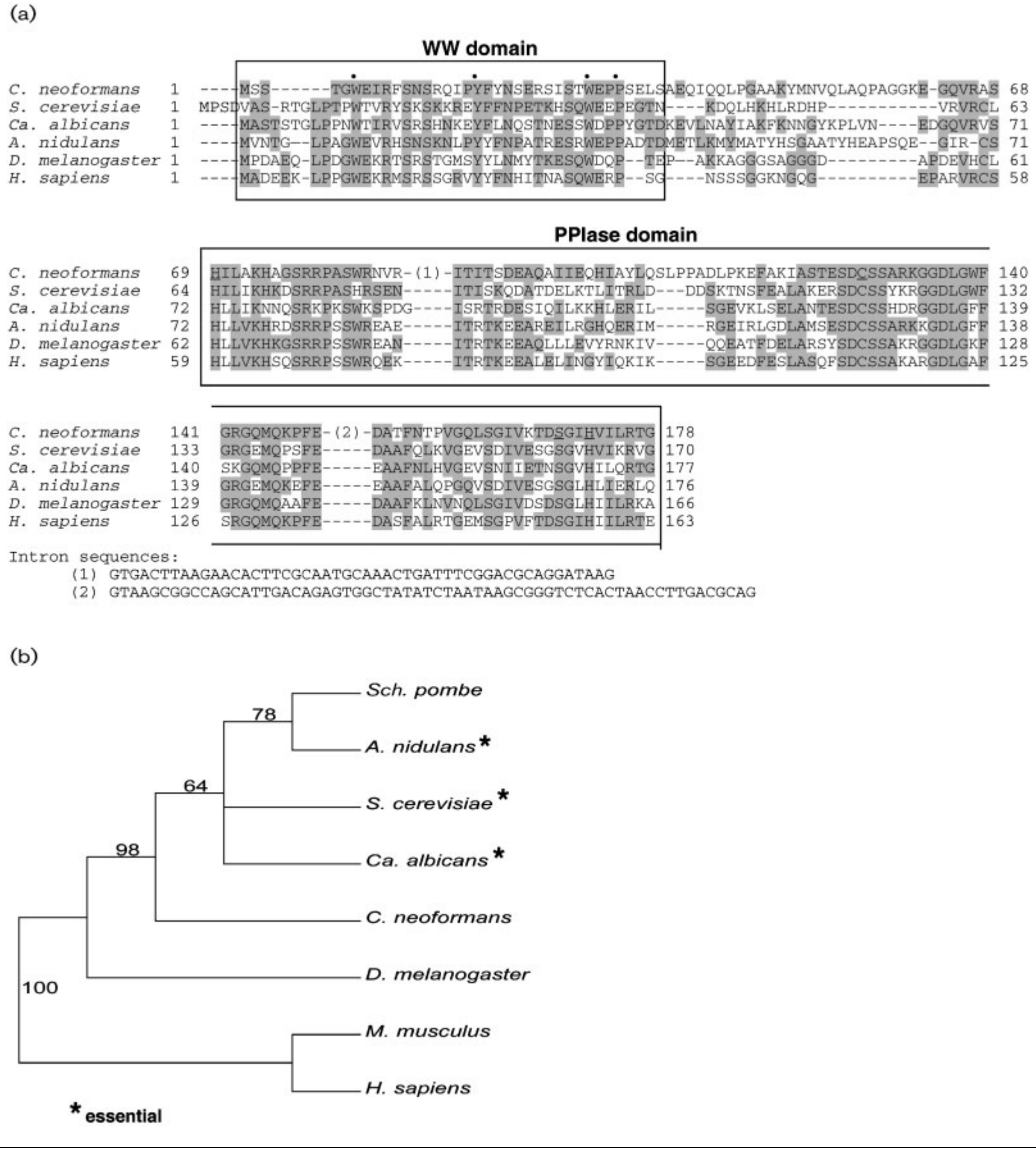

Fig. 1. (a) Alignment of *C. neoformans* Ess1 (AF533511) with selected members of the Ess1 family of parvulin-class prolyl isomerases. Dashes indicate gaps. Shaded areas indicate regions of identity with the *C. neoformans* protein or identity among others. The signature tryptophans of the WW domain (Trp6 and Trp29), and conserved residues Tyr18 and Pro32, are indicated by dots. (b) Phylogenetic relationship among Ess1/Pin1 sequences. The phylogenetic tree is derived using a bootstrap method with neighbour-joining. The numbers are the bootstrap values given as a percentage of 1000 replications (1000 trials). Organism sources and NCBI database accession numbers for the sequences are: *C. neoformans* Ess1 (AF533511), *S. cerevisiae* Ess1 (S52764), *Ca. albicans* Ess1 (AAK00626), *A. nidulans* PINA (AAC49984), *Sch. pombe* Pin1 (NP_587913), *D. melanogaster* Dodo (P54353), *M. musculus* Pin1 (BAB22743) and *H. sapiens* Pin1 (NP_006212). The asterisk (*) indicates organisms in which Ess1 has been shown to be essential for cell growth and viability.

plating 100 µl of 10^{-4} dilutions of cell suspensions onto YPD plates. Mice were monitored once daily and those that were moribund or in apparent discomfort were sacrificed by CO_2 inhalation. Survival was analysed using the Kaplan–Meier method with SAS software, version 6.12 (SAS Institute, Cary, NC, USA). Brain tissue pieces dissected from the dead mice were spread on Niger seed agar medium and incubated at 30 °C for 5 to 7 days to detect the presence of *C. neoformans* by microscopic examination after staining with India ink. To confirm the presence of the *ess1*::*URA5* disruption allele in strains from infected brains, colony isolates were subjected to diagnostic PCR.

RESULTS

Isolation of the *C. neoformans* homologue of *ESS1*

C. neoformans genomic DNA sequences were searched using BLAST for ORFs with similarity to *S. cerevisiae* Ess1 protein. A unique ORF was identified, and PCR primers were designed and used to amplify the corresponding DNA sequences (see Methods). No other *ESS1*-related sequences were identified. After conceptual removal of two small introns, of 50 bp and 64 bp, this region encoded a predicted protein of 178 aa with 39 % identity to *S. cerevisiae* Ess1, 38 % identity to *Ca. albicans* Ess1, 39 % to *A. nidulans* Pin1, 40 % to *Drosophila* Dodo and 42 % to human Pin1 (Fig. 1a). The overall structure of the encoded protein is conserved with that of all known Ess1/Pin1 homologues; it has an N-terminal WW domain, a short linker region (19 amino acids) and a C-terminal PPIase domain.

WW domains are proline-binding modules found in a variety of signalling proteins and are characterized by two precisely spaced tryptophan residues (Sudol, 1996). The predicted *C. neoformans* Ess1 protein contains these signature tryptophan residues, properly spaced (by 23 residues), along with other highly conserved residues (e.g. Tyr18 and Pro32 in *C. neoformans* Ess1). In the PPIase domain, the four putative active-site residues (His157, Cys113, His59 and Ser154 in Pin1) that are predicted on the basis of the crystal structure of human Pin1 (Ranganathan *et al.*, 1997) are conserved (Fig. 1a; underlined residues). These features indicate that the *C. neoformans ESS1* encodes a structural homologue of Ess1/Pin1.

To examine the evolutionary relatedness of the predicted *C. neoformans* Ess1 protein to Ess1/Pin1 homologues in other organisms, the protein sequences were analysed using the neighbour-joining method. We chose seven other fungal and metazoan species for which at least some functional data on Ess1/Pin1 exist. In the resulting phylogenetic tree (Fig. 1b), the Ess1/Pin1 sequences clearly show the fungal clades to be distinct from those of the fly and mammals. Within the four ascomycetes species, *S. cerevisiae* and *Ca. albicans* were grouped in one clade and *Schizosaccharomyces pombe* and *A. nidulans* were grouped in another clade. That *C. neoformans* (a basidiomycete) appeared as a monophyletic group, a sister to the other four fungal species, was as expected. These results support the idea that *C. neoformans* Ess1 is homologous to Ess1/Pin1 proteins from other organisms.

C. neoformans ESS1 is the functional homologue of *S. cerevisiae ESS1*

Although the primary sequence features suggest that *C. neoformans ESS1* encodes a homologue of Ess1/Pin1, it was necessary to demonstrate a conserved function. To do this, we tested whether *C. neoformans ESS1* would functionally complement *S. cerevisiae ess1* mutants. This was done using several different methods. After the two introns were deleted from the original isolate of the *C. neoformans ESS1* gene (see Methods), the intron-less version, carried on an episomal plasmid (pCnESS1-ΔI), was introduced into a conditional-lethal strain of *S. cerevisiae* ($ess1^{H164R}$; Wu *et al.*, 2000). This strain cannot grow at the restrictive temperature of 37 °C due to a mutation in *ESS1* ($ess1^{H164R}$) that renders it temperature-sensitive. The no-growth phenotype of the *S. cerevisiae* temperature-sensitive strain was fully complemented at the restrictive temperature (37 °C) by the *C. neoformans ESS1* gene (data not shown).

While these results are highly suggestive of functional homology, it could be argued that the *C. neoformans ESS1*-encoded protein (Ess1) was simply stabilizing or restoring the activity of the mutant $ess1^{H164R}$ protein. To rule out this possibility, we tested whether *C. neoformans ESS1* could complement *S. cerevisiae* strains bearing a complete deletion of the *ESS1* gene. Since *ESS1* is essential in *S. cerevisiae*, certain manipulations were necessary to create the appropriate genetic background. First, the pCnESS1-ΔI plasmid was introduced into a heterozygous *ess1* mutant strain of *S. cerevisiae* (*ess1*Δ::*URA3*/*ESS1*). The diploid cells were induced to undergo sporulation and the resulting tetrads were dissected. As expected, cells transformed with the vector alone showed a 2 : 2 segregation for viable : inviable spores (Table 2). In contrast, about half of the tetrads derived from diploids transformed with the pCnESS1-ΔI plasmid yielded a 4 : 0 segregation of viable : inviable spores,

Table 2. *C. neoformans ESS1* complements an *S. cerevisiae ess1*Δ mutation during spore germination and outgrowth

Tetrad analysis of a heterozygous *ESS1*/*ess1*Δ::*URA3 S. cerevisiae* strain transformed with two isolates of plasmid pCnESS1-ΔI (*TRP1*) and induced to undergo sporulation. All viable spores that were Ura^+ (i.e. *ess1*Δ) were also Trp^+, indicating that they contained the complementing plasmid (data not shown).

Plasmid	Tetrads dissected	Viable spores per tetrad				
		0	1	2	3	4
Vector	40	2	13	25	0	0
pCnESS1-ΔI-1	60	1	1	26	12	20
pCnESS1-ΔI-2	60	0	1	22	10	27

Table 3. *C. neoformans ESS1* complements an *S. cerevisiae ess1Δ* mutation in vegetatively growing cells

Percentage loss of the pCaESS1 plasmid (*URA3*) in a haploid disruption strain (*ess1Δ::HIS3*) harbouring the indicated plasmid constructs (*TRP1*). Cells were grown in the absence of selection for the pCaESS1 plasmid for 2 days at 30 °C and plated on medium containing 5-FOA.

Plasmid	His^+ Trp^+	Ura^+	$5\text{-}FOA^R$	Percentage loss of pCaESS1
Vector	200	200	0	0
pCnESS1-ΔI-1	200	83	117	58·5
pCnESS1-ΔI-2	200	79	121	60·5

indicating that the *C. neoformans ESS1* gene complements *ess1Δ* haploid cells to allow spore outgrowth and cell viability. Note that not all of these tetrads showed 4 : 0 segregation for viability. This was presumably due to failure of the plasmid to be uniformly maintained during sporulation, a commonly observed phenomenon. As expected, all of the cells that carried the *ess1::URA3* disruption (i.e. were Ura^+) also carried the pCnESS1-ΔI plasmid (i.e. were Trp^+; data not shown).

To test whether *C. neoformans ESS1* complemented an *S. cerevisiae* deletion mutation (*ess1Δ::HIS3*) in vegetatively growing cells, we used plasmid shuffling (Table 3). Here, the pCnESS1-ΔI plasmid was introduced into *ess1* deletion cells that carried a plasmid expressing *Ca. albicans ESS1*. Cells were then grown in the absence of selection for the *Ca. albicans ESS1* plasmid, which carried a *URA3* marker, and plated onto medium containing 5-FOA. If the *C. neoformans ESS1* complements the *ess1* deletion, then cells would lose the *Ca. albicans ESS1* plasmid (*URA3*) and grow on 5-FOA medium (which selects against Ura^+ cells). Indeed, this is what we observed. About 60 % of cells showed growth on 5-FOA medium, indicating complementation by *C. neoformans ESS1*. Thus, the results of all three complementation tests indicate that *C. neoformans ESS1* encodes a *bona fide* (functional) homologue of Ess1/Pin1.

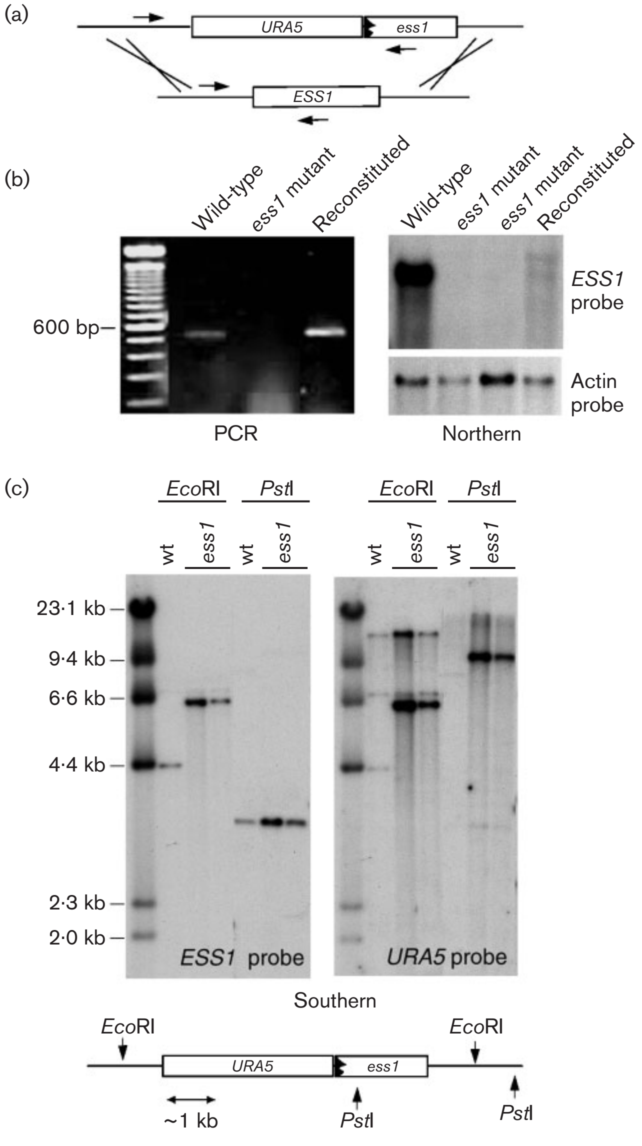

Fig. 2. Isolation of *ess1* mutant strains of *C. neoformans*. (a) Schematic illustration of the *ESS1* gene-specific disruption. The *ess1::URA5* allele fragment was transformed into the *ura5* strain JEC43. Also indicated are approximate positions of diagnostic PCR primers used in (b). (b, left panel) Verification of *ess1* gene disruption mutant and reconstituted strains by PCR with *ESS1* gene-specific primers. A band of 517 bp indicates an intact *ESS1* gene. The large PCR product (~2·5 kb) predicted for the disruption strain is not made under these conditions. (b, right panel) Northern analysis showing the absence of *ESS1* RNA in two *ess1* mutant strain isolates. Twenty-five micrograms of total RNA was loaded per lane, and the same filter was probed sequentially with the indicated ^{32}P-labelled probes. Film exposure was about five times longer and the specific activity was seven times higher for the *ESS1* probe (~600 bp) vs the actin probe (~1400 bp). (c) Southern blot analysis of genomic DNA prepared from isogenic *C. neoformans* strains containing wild-type or mutant allele of *ESS1*. Genomic DNA was cleaved with *Eco*RI or *Pst*I and electrophoresed in a 0·8 % agarose gel. The same blot was probed sequentially with *ESS1* and *URA5* probes. Lanes 1 to 3 contain 1–2 μg *Eco*RI-digested genomic DNA and lanes 4 to 6 contain 1–2 μg *Pst*I-digested genomic DNA from the following strains: (1, 4) wild-type *C. neoformans* JEC43; (2, 5 and 3, 6) two $Ura5^+$ isolates of JEC43 generated by biolistic transformation with *ess1::URA5* allele fragment. Using an *ESS1* probe (left panel), the wild-type *ESS1* gene yields a 4·4 kb *Eco*RI fragment and a 3·2 kb *Pst*I fragment, and the *ess1::URA5* disruption allele yields a 6·4 kb *Eco*RI fragment and 3·2 kb *Pst*I fragment. Using a *URA5* probe (right panel), the *ess1::URA5* disruption allele yields a 6·4 kb *Eco*RI fragment and a 9·4 kb *Pst*I fragment. The banding pattern using each of the two probes is as predicted by the restriction map based on genomic DNA sequence, thus confirming the expected genotypes.

The *ESS1* gene is not essential in *C. neoformans*

To determine whether *ESS1* is essential in *C. neoformans*, we disrupted it by homologous recombination (Fig. 2a). An *ess1*::*URA5* disruption allele was transformed into a *ura5* strain of *C. neoformans* (JEC43) by biolistic transformation. From a total of about 170 Ura$^+$ transformants, two isolates were identified that appeared to have undergone homologous recombination to generate *ess1* mutations. The structure of the *ESS1* genomic locus disrupted in these mutants was verified by PCR (Fig. 2b, left panel) and Southern analysis (Fig. 2c). In contrast to wild-type cells, neither of the mutants expressed *ESS1* mRNA as indicated by Northern analysis (Fig. 2b, right panel). Since *C. neoformans* grows vegetatively as a haploid, our ability to recover mutants in which *ESS1* is disrupted (and partially deleted, see Methods) indicates that the gene is not essential for growth of this organism under standard laboratory conditions.

We next generated reconstituted strains in which a wild-type copy of *ESS1* with its own promoter region was integrated at random in the genome. Such strains served as important controls for characterizing *ess1* mutant phenotypes and for *in vivo* virulence studies. To make reconstituted strains, the Ura$^+$ phenotype was reverted by plating the *ess1*::*URA5* mutants (two mutant isolates) onto 5-FOA-containing medium and selecting for Ura$^-$ cells. This procedure generates *ura5* cells that can be reused for transformation with *URA5*-containing constructs. In our case, the revertants were used as recipients for transformation with a complementing linear DNA fragment containing *ESS1* and a *URA5* selectable marker. The presence of an intact copy of *ESS1* was verified by PCR (Fig. 2b). Several reconstituted strains (*ess1*::*ura5*+*ESS1*) were generated, and the one that we used for most subsequent experiments is called CnPR170 (Table 1).

The reconstituted strain CnPR170 expressed *ESS1* mRNA, but at a much lower level than that of wild-type cells (Fig. 2b, right panel). Other reconstituted strain isolates did not show detectable *ESS1* mRNA (data not shown). The reason for the low expression is not known, but might be due to missing 5′ or 3′ regulatory sequences (or introns) in the construct we used, or to local repressive effects at the site(s) of insertion. The finding that even low levels of *ESS1* expression in CnPR170 can rescue certain *in vitro* and *in vivo* phenotypes (see below) may not be surprising given results in *S. cerevisiae*, which show that Ess1 protein is present in vast excess over the levels required for its essential function (Gemmill *et al.*, 2005).

ess1 mutants show normal growth, morphology and capsule formation

We next compared the *C. neoformans* mutants lacking a functional *ESS1* gene with control strains (wild-type and the reconstituted strain) for general growth properties and the ability to express a standard set of virulence-associated traits. First, we examined the growth rate since slow-growing cells might not be expected to retain virulence in a host organism. The growth rate of *ess1* mutants at three different temperatures appeared to be the same compared to the wild-type and the reconstituted strains (Fig. 3a). This can best be seen by comparing the slopes over the 3–4 log units that represent the linear portion of the growth curves. However, at higher temperatures (30 °C and 37 °C) the *ess1* mutant cells appeared to reach saturation at slightly lower concentrations than did the wild-type or reconstituted strains. Second, cells were examined microscopically for

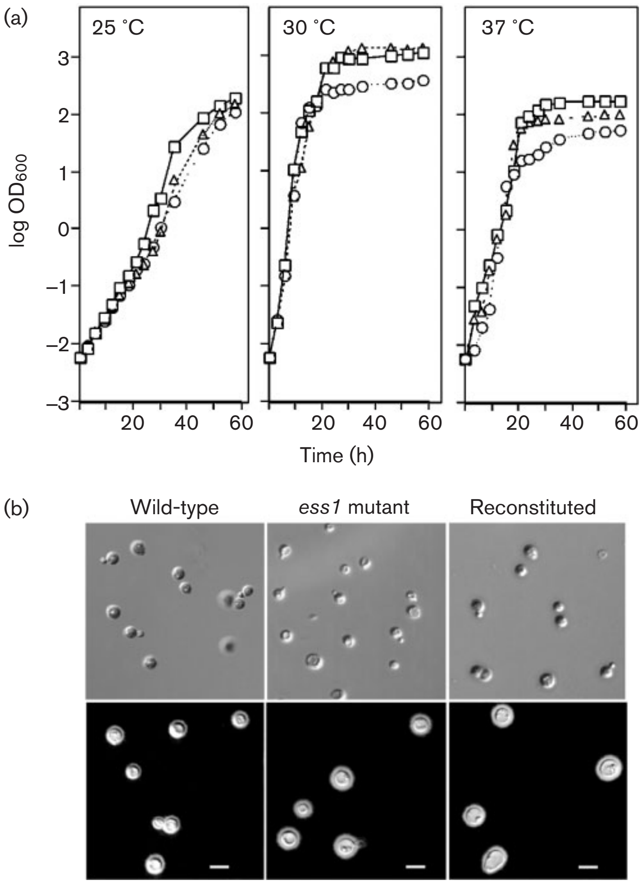

Fig. 3. *C. neoformans ess1* mutants show normal growth, morphology and capsule formation. (a) Growth curves of the wild-type (JEC21, □), *ess1* mutant (CnPR68, ○) and *ess1*::*ura5*+*ESS1* reconstituted strain (CnPR170, △) in liquid YPD medium at 25 °C, 30 °C and 37 °C. (b, upper panels) The wild-type, *ess1* mutant and reconstituted strain were grown in YPD medium at 25 °C to mid-exponential phase before being photographed at ×400 magnification. (b, lower panels) The wild-type, *ess1* mutant and reconstituted strain were inoculated into limited-iron media with EDTA and bathophenanthroline disulphonic acid, and cultured at 30 °C, 250 r.p.m. for 7 days. The cells were stained with India Ink and photographed at ×400 magnification. Bars, 10 μm.

possible defects in overall morphology. No obvious defects could be discerned; cells still appeared normal in size and shape (Fig. 3b, upper panels). Third, we assayed the ability of *ess1* mutants to undergo capsule formation, a key differentiation step required for virulence *in vivo*. Cells were induced to form capsules by growth on standard inducing medium and then stained with India ink. No defects in capsule formation were observed; the size of the capsules appeared to be the same between mutant and control strains (Fig. 3b, lower panels). Finally, we tested for the ability of *ess1* mutants to undergo mating and haploid fruiting, and to generate pheromone-induced conjugation tubes. No changes compared to control strains were observed (data not shown). Thus, *ess1* mutant cells behaved normally for several basic properties of growth and differentiation.

ess1 mutants show defects in melanin production and urease activity

In contrast to the results described above, disruption of *ESS1* results in obvious defects in two important virulence-associated factors. As shown in Fig. 4(a) (upper panel), production of the pigment melanin is greatly reduced (or delayed) in the *ess1* mutant as compared to the wild-type. That the defect is due to loss of *ESS1* function is demonstrated by the fact that melanin production is at least partially restored in the reconstituted strain (CnPR170). Since melanin production is important for virulence, probably due to its role in helping cells resist oxygen-radical-induced killing by host macrophages (Liu *et al.*, 1999), these results were the first hint that *ess1* mutants might have a reduced virulence *in vivo*.

Melanin production depends on the metalloenzyme laccase, which is encoded by the gene *LAC1*. We therefore examined *LAC1* expression in *ess1* mutant cells. Surprisingly we found that *LAC1* RNA levels appear to go up about threefold (relative to actin) in *ess1* mutants vs wild-type cells. (Fig. 4c, upper panel). This result suggests that the defect in melanin production is due either to post-transcriptional defects in laccase activity (see below), or to defects in transcription of other genes (e.g. *LAC2*) also involved in melanin production (Pukkila-Worley *et al.*, 2005).

Production of the enzyme urease, which is known to be required for virulence in animal models (Cox *et al.*, 2000), was also reduced in *ess1* mutant cells. A spot-test assay for urease activity showed that the *ess1* mutant strains produce a much smaller zone of substrate utilization than do the wild-type or the reconstituted strain (Fig. 4b). Consistent with this decrease in activity, expression of the *URE1* gene, which encodes urease, is reduced slightly (about twofold) in *ess1* mutants vs wild-type cells (Fig. 4c, lower panel). As expected, *URE1* levels were partially restored in the reconstituted strain.

In addition to direct transcriptional effects on *LAC1* and *URE1* genes, mutation of *ESS1* might affect laccase and urease enzyme activity indirectly, for example, by affecting expression or function of genes such as *VPH1*, which encodes an intracellular vesicular proton pump. Both

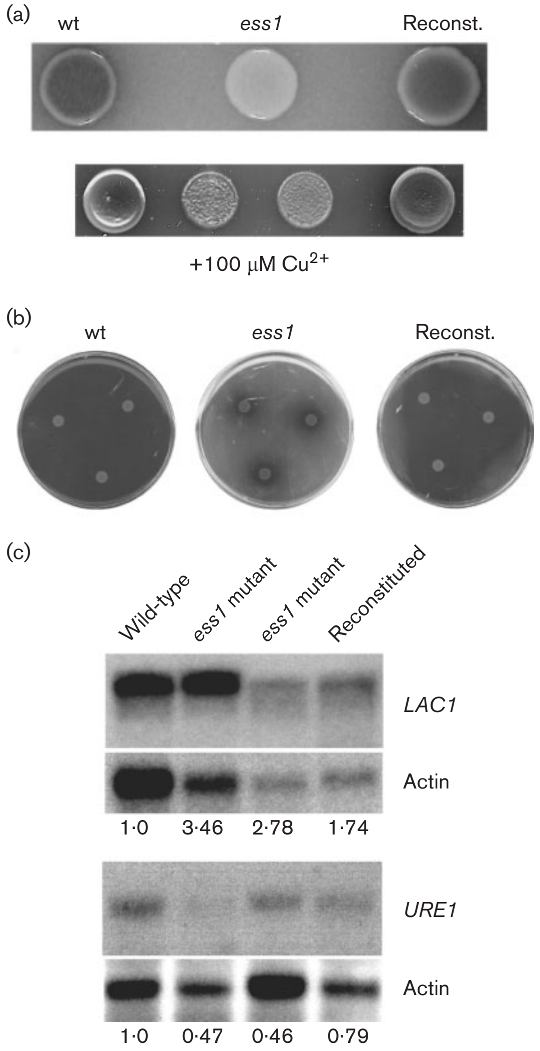

Fig. 4. *C. neoformans* Ess1 protein is required for melanin and urease biosynthesis. (a, b) The wild-type (JEC21), *ess1* mutant (CnPR68) and *ess1::ura5+ESS1* reconstituted (CnPR170) strains were grown as indicated. (a) For 4 days at 30 °C on Niger seed medium to assay melanin production (upper panel) or 5 days at 30 °C on Niger seed medium with 100 μM copper sulphate (lower panel). (b) For 2 days at 30 °C on urea agar to assay urease production. (c) Northern analysis of *LAC1* and *URE1* expression in wild-type, *ess1* mutant and reconstituted strains. Cells were grown in asparagine (*LAC1*) or YPD (*URE1*) liquid medium at 30 °C to mid-exponential phase (Ikeda & Jacobson, 1992). The amount of total RNA used per lane was 21 μg (*LAC1*) or 25 μg (*URE1*). Probes used are indicated. The numbers indicate the fold change of *LAC1* or *URE3* vs wild-type, using an actin probe as a control. Values were based on mean density of signal determined using NIH IMAGE software.

laccase and urease are metalloenzymes whose activities are greatly reduced in *vph1* mutants (Erickson *et al.*, 2001). The *vph1* defect can be overcome by the addition of excess copper, which, for example, restores laccase activity allowing melanin production (Zhu *et al.*, 2003). Addition of copper sulphate did not seem to restore melanin production to *ess1* mutants (Fig. 4a, lower panel), indicating that the defect is not due to post-transcriptional defects in laccase metallation. The results suggest that *VPH1* may not be affected in *ess1* mutants, consistent with our finding that *ess1* mutants generate normal-looking capsules, whereas no capsules are formed in *vph1* mutants. Thus, some other defect(s) must occur in the pathway leading to melanin production.

These results and those shown in Fig. 3 show that some, but not all, of the standard virulence factors in *C. neoformans* are affected by disruption of the *ESS1* gene. Note that the levels of melanin and urease production are not completely restored in the reconstituted strain. This was not surprising given that the *ESS1* mRNA levels in the reconstituted strain were much lower than in the wild-type (Fig. 2b).

ESS1 is required for virulence

We tested if *ESS1* is required for virulence of *C. neoformans* in a murine model of cryptococcosis. Each BALB/c animal was infected by lateral tail vein injection with 10^6 *C. neoformans* cells. The mean survival of these mice with the wild-type serotype D strain JEC21 was 56 days and all injected mice were moribund or dead after 72 days. In contrast, all mice injected with the *ess1* mutant strain were still viable with no signs of sickness even after 100 days, at which time the experiment was ended. These results indicate that the virulence of the *ess1* mutant strain is severely attenuated compared with the wild-type (Kaplan–Meier analysis log-rank, $P<0{\cdot}001$). Consistent with the idea that the loss of virulence is due to the loss of *ESS1* function, virulence was largely restored in the reconstituted strain (*ess1*::*ura5*+*ESS1*) compared with the mutant ($P<0{\cdot}001$). Results showed that the mean survival of infection of the mice injected with the reconstituted strain was 74 days, and that all mice were moribund or dead after 80 days (Fig. 5). In summary, the results indicate that the *C. neoformans* *ESS1* gene is required for virulence in a murine model for cryptococcosis.

As expected, *C. neoformans* cells were detected in brain tissue obtained post-mortem from mice infected with the wild-type or reconstituted strain. Surprisingly, brain tissue from mice infected with the *ess1* mutant strain still contained *C. neoformans* cells, although qualitatively they appeared to be in lower concentrations compared to brain tissue from mice infected with the wild-type or reconstituted strains (data not shown). These cells were white (no melanin production) and carried the mutant *ess1*::*URA5* allele as detected by PCR, confirming that the cells were genetically similar to the injected cells. Thus, although some *ess1* mutant cells persist in brain, they do not seem to cause overt disease.

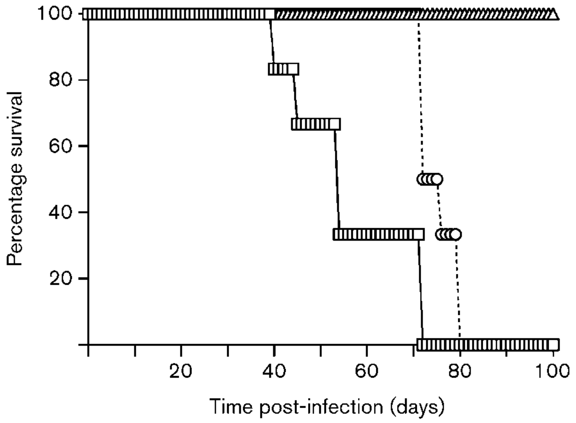

Fig. 5. *C. neoformans* Ess1 protein is required for virulence in *C. neoformans*. Six male BALB/c mice per strain were infected by lateral tail vein injection with 100 μl cells (10^7 ml^{-1}) of the wild-type (JEC21), the *ess1* mutant (CnPR68) and the *ess1*::*ura5*+*ESS1* reconstituted strain (CnPR170). Mice were monitored once daily and those that were moribund or showed apparent discomfort were sacrificed by CO_2 inhalation. The number of surviving mice was plotted against time. □, Wild-type; ○, *ess1* mutant; △, reconstituted.

Ess1 and cyclophilins may have some overlapping functions

Previous work in *S. cerevisiae* had shown that overexpression of cyclophilin A suppresses *ess1* mutations, and that under some conditions *ess1* mutants are hypersensitive to the effects of the CsA, an inhibitor of cyclophilin A (Arévalo-Rodríguez *et al.*, 2000; Wu *et al.*, 2000). *C. neoformans* is known to have two cyclophilin A homologues (Wang *et al.*, 2001). We therefore tested whether CsA affected the growth of *C. neoformans ess1* mutant cells. As shown in Fig. 6(a), CsA strongly inhibited the growth of *ess1* mutant cells as compared to wild-type control cells. At least some of the inhibitory effects of CsA were reversed by adding back a wild-type copy of the *ESS1* gene, as in the reconstituted strain. We obtained similar results at 28 °C (data not shown). In this plate assay, *ess1* mutant cells grew slightly slower than the wild-type or the reconstituted strain, even without the addition of drug. However, the magnitude of this difference does not fully account for the CsA inhibitory effect. Note that this experiment was conducted at 25 °C because calcineurin (a target of cyclophilins) becomes essential at 30–37 °C, and therefore CsA inhibits cell growth even in wild-type cells at elevated temperature (Odom *et al.*, 1997).

In control experiments, no such growth inhibitory effects were observed using the FK506 analogue (FK520), indicating that the effect was specific for cyclophilins

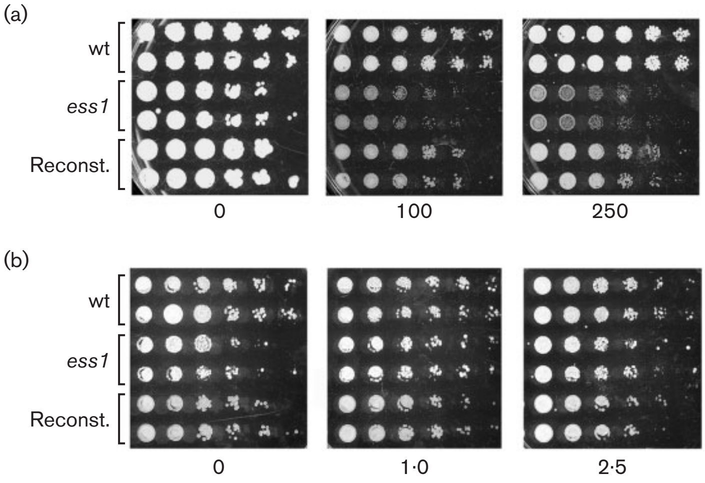

Fig. 6. *ess1* mutants are hypersensitive to CsA. Plate assays using the PPIase inhibitors CsA (a) or FK520 (b) were done at the concentrations (μg ml^{-1}) indicated below each panel. The wild-type (JEC21), *ess1* mutant (CnPR68) and *ess1::ura5+ESS1* reconstituted (CnPR170) strains were grown to mid-exponential phase, and serial dilutions (1 : 5) starting at 0·2 OD_{600} were spotted onto YPD agar plus inhibitor (CsA or FK520) and incubated at 25 °C for 3·5 days.

(Fig. 6b). The experiments shown were also done at 25 °C, since calcineurin is also a target of FKBPs. To confirm that the FK520 was indeed active in these cells, growth was also tested at 32 °C, 34 °C and 37 °C. At these temperatures, FK520 severely inhibited the growth of all three strains even at lower drug concentrations (e.g. 0·25 μg ml^{-1}), but this inhibition was not specific to *ess1* mutant cells (data not shown). Thus, our results indicate that *ess1* mutants are hypersensitive to inhibitors of cyclophilins, but not of FKBPs, suggesting that cyclophilins might augment some growth-related function of Ess1 in *C. neoformans*, similar to what was observed in *S. cerevisiae* (Arévalo-Rodríguez *et al.*, 2000). Genetic studies will be needed to further confirm these results.

DISCUSSION

In this study, we isolated and characterized the *C. neoformans ESS1* gene. This is believed to be the first study in which a parvulin-class PPIase has been described in this organism. Cyclophilin- and FKBP-class PPIases have been previously described (Cruz *et al.*, 1999, 2001; Wang *et al.*, 2001). We showed that *ESS1* is expressed in vegetatively growing cells but is not required for growth. Instead it appears to be required for differentiated functions and for virulence *in vivo*. Together with these previous reports, our results indicate that PPIases play a crucial role in specialized functions of *C. neoformans* and that at least two of the many PPIases identified by the sequencing of the *C. neoformans* genome are required for virulence of this organism (Cpa1 and Ess1).

The mechanism by which the Ess1 protein contributes to virulence in *C. neoformans* is not yet known. Our findings showed that mice infected with the *ess1* mutant continued to carry at least some viable *C. neoformans* cells, despite having no clinical signs of illness. These results are intriguing and indicate that *ess1* mutant cells can still colonize the brain, at least to some degree, but are unable to cause disease over the time-frame of our experiments (100 days). Thus, we suggest that *ess1* mutant cells have defects in differentiated functions related to disease progression, but this idea will require further investigation to confirm.

The exact function of *C. neoformans* Ess1 is not known. However, studies in the model organism *S. cerevisiae* would suggest that the molecular nature of the *ess1* defect is likely to involve gene-specific changes in transcription regulation. In *S. cerevisiae*, Ess1 binds to and regulates the function of RNA polymerase II (reviewed by Shaw, 2002; Arévalo-Rodríguez *et al.*, 2004). It seems plausible, therefore, that expression of certain virulence-associated genes in *C. neoformans* might be affected by disruption of *ESS1*. Indeed, expression of *URE1*, which encodes an enzyme required for synthesis of urease, was reduced (albeit only twofold) in *ess1* mutant cells (Fig. 4c). In contrast, expression of *LAC1*, which is required for melanin production, showed an unexpected increase in *ess1* mutants. We note that it is possible that expression of the actin gene, which was used as a control, might also vary in *ess1* mutants. A more complete study of gene expression of virulence factors affected by *ess1* deletion could be accomplished by microarray analysis using the *C. neoformans* strains generated in this study.

ESS1 was first identified as a gene essential for growth in *S. cerevisiae* (Hanes *et al.*, 1989). However, in addition to its non-essentiality in *C. neoformans*, *ESS1* homologues are not essential in several other organisms (reviewed by Arévalo-Rodríguez *et al.*, 2004), including fungi such as *Sch. pombe* (Huang *et al.*, 2001). While functional studies of *ESS1*/*PIN1* have been undertaken only in a limited number of organisms, phylogenetic analysis using this small dataset (Fig. 1b) reveals that within the ascomycetes group, fungi are divided into two subgroups, one in which Ess1 is essential and one in which it is not essential. Since *C. neoformans* is a basidiomycete and is outside this group, perhaps it should not have been surprising that *ESS1* is not essential in this organism.

One explanation for the fact that *ESS1* is essential in some organisms but not others is that under some circumstances, cyclophilin A can substitute for Ess1 (Arévalo-Rodríguez *et al.*, 2000; Wu *et al.*, 2000). These and other studies indicate that Ess1 and cyclophilin A exhibit crosstalk, i.e. that they possess partially overlapping functions (Fujimori *et al.*, 2001). *C. neoformans* is very unusual in that it has two distinct cyclophilin A homologues, Cpa1 and Cpa2 (Wang *et al.*, 2001). It is possible, therefore, that in *ess1* mutant strains, the two cyclophilin A homologues might compensate for many, although not all, of the functions of Ess1 lost by gene disruption. Such an overlap in function might explain why *ESS1* is not essential for growth in *C. neoformans*, despite playing an important role in virulence. Consistent with this idea, *ess1* mutant cells were more sensitive than wild-type cells to the cyclophilin inhibitor CsA.

Interestingly, both *ess1* and *cpa1 cpa2* double mutants are defective in melanin synthesis, and in virulence in a mouse model (this study; Wang *et al.*, 2001). These similarities, and the fact that both Ess1 and cyclophilin A homologues have been implicated in transcription (Arévalo-Rodríguez *et al.*, 2000, 2004; Wu *et al.*, 2000), support the idea of functional overlap. However, there are important differences. For example, unlike *ess1* mutants, *cpa1 cpa2* double mutants are also defective in capsule formation and mating, and are resistant to the effects of CsA (Wang *et al.*, 2001). Thus, while some pathway overlap probably occurs, Ess1 and cyclophilins clearly have distinct functions *in vivo*. Further genetic analysis would be useful to investigate these differences.

Based on studies using another human fungal pathogen, *Candida albicans*, we proposed that parvulin-class PPIases such as Ess1 might be valuable targets for antifungal drug development (Devasahayam *et al.*, 2002). Indeed, *ESS1* appears to be important for virulence of *Ca. albicans* (G. Devasahayam, V. Chaturvedi & S. D. Hanes, unpublished). The present study demonstrates that *ESS1* is also required for virulence of *C. neoformans* in at least one animal model system. It seems reasonable to predict that inhibitors of *ESS1* might attenuate the virulence of *C. neoformans* in humans. Since Pin1, the vertebrate Ess1 homologue, does not appear to be essential in mammals (Fujimori *et al.*, 1999), targeting this family of PPIases might be feasible for human therapeutic applications.

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