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Candida glabrata Ste11 is involved in adaptation to hypertonic stress, maintenance of wild-type levels of filamentation and plays a role in virulence

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The conserved family of fungal Ste11 mitogen activated protein kinase/kinases play important roles in several signalling cascades. We have cloned the *STE11* homologue from the fungal pathogen *Candida glabrata*. The *C. glabrata* gene is present in a single copy in the genome, contains a well-conserved catalytic domain typical of the serine–threonine protein kinases and a sterile alpha motif widespread in signalling and nuclear proteins. Hypothetical translation of *C. glabrata STE11* suggests that the protein has 64% identity and 77% similarity at the amino acid level to *Saccharomyces cerevisiae* Ste11. We have shown that *C. glabrata STE11* can complement the mating defect and partially rescue the reduced nitrogen starvation induced filamentation of *S. cerevisiae ste11* mutants. Functional analysis of a *C. glabrata ste11* null mutant demonstrates that Ste11 is required for adaptation to hypertonic stress but is largely dispensable for maintenance of cell wall integrity. It also plays a role in *C. glabrata* nitrogen starvation induced filamentation. Survival analysis revealed that *C. glabrata ste11* mutants, while still able to cause disease, are attenuated for virulence compared to reconstituted, *STE11* cells. These data suggest that *C. glabrata* Ste11, in a similar fashion to the *S. cerevisiae* protein, functions in a number of different signalling modules.

Keywords *Candida glabrata*, filamentation, hypertonic stress, *STE11*, virulence

Introduction

A number of *Candida* species, in particular *Candida albicans* are life threatening opportunistic pathogens of humans [1]. They are able to induce disease in hosts, whose systemic or local immune status is damaged, deteriorating or innately dysfunctional [2]. *Candida glabrata* has been documented as a cause of both superficial infections, including vaginitis, and systemic disease where it is emerging as a significant pathogen [3]. In the US, 45% of *Candida* species isolated from the blood stream are now members of the genus other than *C. albicans,* in particular *C. glabrata* is currently res-ponsible for approximately one in five cases [4]. *C. glabrata* is inherently less susceptible than other *Candida* species to currently available azole antifungals and *C. glabrata* infection is also associated with a high mortality [5].

This emergence of *C. glabrata* as an important cause of candidaemia represents an increasing clinical problem that requires immediate action. Improvement of our understanding of the biology that determines *C. glabrata* pathogenicity should facilitate the tackling of this problem. It seems reasonable to hypothesize that a key attribute for many fungal pathogens, including *C. glabrata*, is an ability to sense and respond

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to changes in the external environment. A central component of a number of signal transduction pathways that sense environmental changes in fungi is a mitogen activated protein kinase (MAPK) cascade module [6]. These modules are highly conserved in eukaryotic organisms and comprise three core protein kinases (i.e. a MAPK, a MAPK kinase [MAPKK] and a MAPKK kinase [MAPKKK]) [7]. In *Saccharomyces cerevisiae*, five MAPK cascades have been associated with biological responses to specific external stimuli [6]. The mating pathway is activated by pheromones and induces cell-cycle arrest and morphological changes required for mating [8]. The invasive growth pathway is activated by nitrogen starvation and induces filamentous growth and invasion into agar [9]. The putative STE vegetative growth pathway helps to mediate cell wall integrity in response to osmotic stress [10,11]. The high osmolarity glycerol (HOG) pathway increases intracellular glycerol levels in response to hypertonic stress [12]. The cell integrity pathway acts to maintain cell wall integrity and is activated by hypotonic stress, heat shock and nutrient depletion [13]. The first four of these signalling pathways share a common MAPKKK (i.e. Ste11).

Homologues of *S. cerevisiae STE11* have been described in a number of other fungal species. These include *Aspergillus nidulans SteC* (accession number CAD44493), *C. albicans STE11* (*Candida*DB [http://genolist.pasteur.fr/CandidaDB] accession number CA2018), *Cryptococcus neoformans STE11α* [14], *Neurospora crassa nrc-1* [15], *Schizosaccharomyces pombe byr2/ste8* [16] and *Ustilago maydis ubc4* [17]. This Ste11 family of proteins play important roles in mediating the regulation of various aspects of morphogenesis, sporulation, conjugation, mating and pathogenicity.

We have cloned the *C. glabrata STE11* homologue in order to begin an analysis of MAPK signalling in this opportunistic pathogenic yeast. Here, we show that inactivation of *C. glabrata STE11* results in defective adaptation to hypertonic stress and reduced ability to filament in response to nitrogen starvation. In addition *C. glabrata ste11* mutants are mildly attenuated in a murine model of candidiasis.

Materials and methods

Strains and growth media

All the strains used in this study are listed in Table 1. Fungal cells were routinely cultured in YAPD (2% w/v peptone, 2% w/v glucose, 1% w/v yeast extract, 0.01% w/v adenine) or SD (0.17% w/v yeast nitrogen base without amino acids; Difco, Detroit, MI, USA); 2% w/v glucose and appropriate drop out mix (Clontech, Basingstoke, UK) at 30 or 37°C. For solid media 2% w/v agar was added prior to autoclaving. Synthetic low ammonia (10 μmol/l) dextrose (SLAD) media for nitrogen starvation experiments was prepared as previously described [18]. Other additives were filter sterilized and added at the appropriate concentration to media after autoclaving. *Escherichia coli* XL1-blue (Stratagene, La Jolla, CA, USA) was used as the host strain for all plasmid manipulations.

Cloning of C. glabrata STE11

Degenerate primers FD-11 (YTNGARTAYGTNCCN GGNGG) and RD-11 (ACNACYTCNGGNSHCAT

Table 1 Fungal strains used in this study

Strain	Genotype	Reference
Candida glabrata ATCC 2001	Wild type	ATCC*
C. glabrata ΔHT6	*his3::URA3 Δura3 Δtrp1*	19
C. glabrata AM3	*his3::URA3 Δura3 Δtrp1 ste11::HIS3*	this study
C. glabrata AM3p	*his3::URA3 Δura3 Δtrp1 ste11::HIS3* pCgACT-14 (*TRP1*)	this study
C. glabrata AM5	*his3::URA3 Δura3 Δtrp1 ste11::HIS3* pAMC76 (*STE11 TRP1*)	this study
Saccharomyces cerevisiae L5366	*ura3/ura3*	FLC†
S. cerevisiae L5625	*ura3/ura3 ste11/ste11*	FLC†
S. cerevisiae AM10	*ura3/ura3* YEp24 (*URA3*)	this study
S. cerevisiae AM11	*ura3/ura3 ste11/ste11* YEp24 (*URA3*)	this study
S. cerevisiae AM12	*ura3/ura3 ste11/ste11* pAMC44 (*CgSTE11 URA3*)‡	this study
S. cerevisiae Y15271	*MATα his3 leu2 lys2 ura3 ste11::kanMx4*	Euroscarf§
S. cerevisiae AM13	*MATα his3 leu2 lys2 ura3 ste11::kanMx4* YEp24 (*URA3*)	this study
S. cerevisiae AM14	*MATα his3 leu2 lys2 ura3 ste11::kanMx4* pAMC44 (*CgSTE11 URA3*)‡	this study
S. cerevisiae X4003-Ba	*MAT***a** *leu2 ade1 his4 met2 ura3 trp5 gal1*	AFGLC¶

*American Type Culture Collection; †Fink Laboratory Collection; ‡*Cg* is prefixed to differentiate the *C. glabrata* gene from the *S. cerevisiae* gene; §European *S. cerevisiae* archives for functional analysis; ¶Aberdeen Fungal Group Laboratory Collection.

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CC) were designed against alignments of the conserved kinase domain of the Ste11 proteins from *S. cerevisiae*, *C. albicans, N. crassa and S. pombe.* All PCR reactions were performed on a PCR Engine (MJ Research, Watertown, MA, USA) in 1 × PCR buffer (MBI, Vilnius, Lithuania) containing 1 U Taq polymerase and 100 ng *C. glabrata* ATCC 2001 genomic DNA. PCR conditions were 2 min at 94°C, then 25 cycles of 60 s at 94°C, 60 s at 45°C and 2 min at 72°C followed by a final extension step of 7 min at 72°C. The resulting PCR products were cloned into pGEM T-Easy (Promega, Southampton, UK) and sequenced. A 380-bp fragment showed significant sequence homology to fungal *STE11* genes.

A *C. glabrata* ATCC 2001 genomic DNA library constructed in YEp24 was a gift from Dominique Sanglard (CHUV, Lausanne, Switzerland). The 380-bp *STE11* PCR product was labelled with ^{32}P using Ready-to-Go beads (Amersham-Pharmacia, Chalfont, UK) and used to probe replicate filters of this library. Three positive colonies were identified that contained apparently identical YEp24 plasmids. The entire 6.0 kb insert of one of these plasmids (pAMC44) was sequenced on both strands at the Imperial College London sequencing facility using synthetic primers.

Disruption of C. glabrata STE11

In order to disrupt *STE11*, a 3.4 kb *Dra*I fragment from pAMC44 (−733 to +2615, with respect to the *STE11* start codon) containing the entire *C. glabrata STE11* gene was subcloned into the *Sma*I site of pBluescript SKII+ to give the plasmid pAMC71. This was digested with *Bpi*I and *Eco*721 to remove 2321 bp including the entire *STE11* ORF (−148 to +2173). The digested plasmid was blunt ended and a *C. glabrata HIS3* cassette was cloned in as previously described [19]. The resulting plasmid, pAMC60, was used as template for PCR with the primer pair FL4-STE11 (AGATATACTAGCTACGACCAGA, −633 to −655) and RL4-STE11 (ACAGATAGACAATTGAC GCC, +2563 to +2583) to generate a linear *STE11* gene replacement cassette. This cassette was transformed into *C. glabrata* ΔHT6, using a lithium acetate protocol [19]. Histidine prototrophs were selected. Homologous integration and gene replacement were confirmed by Southern analysis and PCR (Fig. 1). A representative strain was selected and designated *C. glabrata* AM3 (*his3::URA3 Δura3 Δtrp1 ste11::HIS3*). This strain was made prototrophic by transformation with pCgACT-14 [20]. The resultant strain was designated *C. glabrata* AM3p.

Reconstitution of C. glabrata STE11

To reconstitute *STE11* in *C. glabrata* AM3 the entire *STE11* open reading frame was released from pAMC44 with *Dra*I. The resultant 3.4 kb *STE11* fragment was cloned into *Sac*I digested blunt ended pCgACT-14 [20], yielding pAMC76. *C. glabrata* AM3 was transformed with pAMC76. Trytophan prototrophs were selected. Reconstitution of *STE11* was confirmed by diagnostic PCR with the primer pair FL4-STE11 and RL4-STE11 (data not shown). A representative *STE11* reconstituted strain *C. glabrata* AM5 (*his3::URA3 Δura3 Δtrp1 ste11::HIS3* pAMC76 [*STE*11, *TRP1*]) was selected for further analysis.

Complementation of S. cerevisiae ste11 *mutants*

The diploid *S. cerevisiae ste11/ste11* mutant L5625 and the haploid *ste11* mutant Y15271 were both transformed with pAMC44. Uracil prototrophs were selected and episomal uptake of *C. glabrata STE11* was confirmed by PCR with the primer pair FL4-STE11 and RL4-STE11 (data not shown). Representative transformants from each background were selected for further analysis and designated *S. cerevisiae* AM12 (*ura3/ura3 ste11/ste11* pAMC44 [*CgSTE11 URA3*]) and *S. cerevisiae* AM14 (*MATα his3 leu2 lys2 ura3 ste11::kanMx4* pAMC44 [*CgSTE11 URA3*]). *Cg* is prefixed to *STE11* here to avoid confusion between the *C. glabrata* and *S. cerevisiae* genes.

S. cerevisiae L5625 was also transformed with YE p24 to give the prototrophic *ste11/ste11* strain AM11. In addition *S. cerevisiae* L5366 (*ura3/ura3*) was transformed with YEp24 to give a prototrophic wild-type strain AM10.

Mating assays

To determine whether or not *C. glabrata* Ste11 could complement the mating defect of the haploid *S. cerevisiae ste11* mutant Y15271 we performed a series of patch matings with the *MAT***a** strain X4003-5Ba (*MAT***a** *leu2 ade1 his4 met2 ura3 trp5 gal1*). This strain was crossed with Y15271, AM13 (*MATα his3 leu2 lys2 ura3 ste11::kanMx4* YEp24) and AM14. After 48 h at 30°C on YAPD portions of each patch were transferred to SC without histidine and incubated for a further 48 h at 30°C.

Model of systemic candidiasis

To compare the virulence of the wild-type strain *C. glabrata* ATCC 2001 and the *ste11* mutant *C. glabrata* AM3p we used a murine model of systemic candidiasis that we have recently developed [21].

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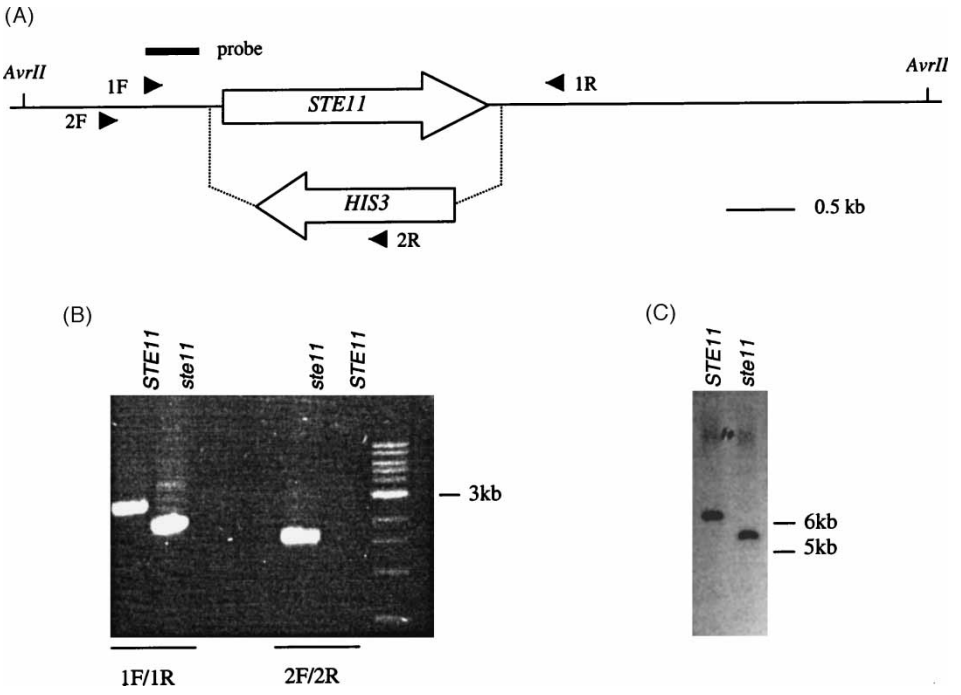

Fig. 1 Disruption of *Candida glabrata STE11*. Schematic representation of the wild type *STE11* genomic locus showing position of diagnostic PCR primers (1F/1R and 2F/2R), *Avr*II recognition sequences and site of *HIS3* insertion in AM3 (A). *ste11* diagnostic PCRs (B). Genomic DNA from *C. glabrata* ΔHT6 (*STE11*) or AM3 (*ste*11) was amplified with the primer pairs 1F/1R and 2F/2R (see below for description). The expected ~3.2 kb fragment was amplified with primers 1F/1R from *C. glabrata* ΔHT6 and an ~800 bp smaller fragment was amplified from AM3, due to replacement of 2.3 kb of *STE11* with 1.5 kb of the *HIS3* cassette. No PCR product was obtained when DNA from *C. glabrata* ΔHT6 was amplified with the primers 2F/2R. A 1.7 kb fragment was obtained with AM3 DNA demonstrating homologous integration of the disruption cassette at the *STE11* genomic locus and inactivation of *STE11* in *C. glabrata* AM3. Primer 1F (AGATATACTAGCTA CGACCAGA) and 1R (ACAGATAGACAATTGACGCC) are contained on the *STE11* disruption cassette and amplify a fragment from −655 to +2583 with respect to the *STE11* start codon. Primer 2F (AGGATCTGATCTTGACAGAGA) is external to the disruption cassette (−849). Primer 2R (CGGGATCATCTCTGTGGATAGC) is on the *HIS3* marker. Confirmation of *STE11* disruption by Southern analysis (C). Total genomic DNA from *C. glabrata* ΔHT6 (*STE11*) or AM3 (*ste11*) was digested to completion with *Avr*II, separated on an agarose gel and transferred to nylon. *Avr*II recognition sequences are located at −1601 and +5089. The membrane was probed with a ^{32}P-labelled fragment of the *STE11* upstream region (see A), washed to high stringency and visualized with X-OMAT film (Kodak). The anticipated 6.7 kb *Avr*II fragment was seen in *C. glabrata* ΔHT6 and an ~800 bp smaller fragment was labelled in AM3 DNA. These data confirm the disruption of *STE11* in *C. glabrata* AM3.

Briefly, groups of 15 cyclophosphamide (200–250 mg/kg) immunosuppressed CD1 mice (25–28 g) mice were infected via the lateral tail vein with equivalent doses of the *C. glabrata STE11* reconstituted strain AM5 (6.2×10^7) or the *C. glabrata ste11* mutant AM3p (6.1×10^7). Mice were killed when the pre-determined end-point of 20% weight loss was reached. Survival curves were compared using Kaplan–Meier log rank analysis.

Results

C. glabrata STE11 *encodes a member of the fungal Ste11 protein family*

PCR amplification with degenerate primers FD-11 and RD-11 yielded two amplicons of 850 and 380 bp, respectively. Sequencing of these two products revealed that the 380 bp band had substantial similarity to a number of fungal *STE11* genes. Use of this PCR product to screen a *C. glabrata* genomic library in YEp24 yielded three independent clones each containing an apparently identical 6 kb insert. Sequence analysis of one of these clones (pAMC44) revealed the entire *C. glabrata STE11* open reading frame, plus regulatory sequences, with substantial sequence similarity to fungal *STE11* genes. We have designated this open reading frame *C. glabrata STE11*. The DNA sequence has been assigned the accession number AY603493.

The *C. glabrata STE11* gene contains a putative uninterrupted open reading frame of 2031 bp, with a conserved TATA element in the 5′ untranslated region

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60 bp upstream of the proposed initiation codon and a possible poly-adenylation signal sequence at position +2056 to +2062. The translated sequence is predicted to encode a protein of 676 amino acids with a molecular weight of 76.2 kDa. *C. glabrata* Ste11 has substantial amino acid sequence similarity to other members of the Ste11 protein family including *A. nidulans* SteC (33%), *C. albicans* Ste11 (35%), *C. neoformans* Ste11α (52%), *N. crassa* nrc-1 (34%), *S. cerevisiae* Ste11 (64%) and *S. pombe byr2* (38%). This sequence homology is particularly striking in the highly conserved catalytic domain (Fig. 2) typical of the serine-threonine protein kinase family [22]. The *C. glabrata* Ste11 putative catalytic domain has the conserved boundaries and 11 subdomains characteristic of the family [23] (Fig. 2). The N-terminus of the protein contains a sterile alpha motif (SAM) widespread in signalling and nuclear proteins [24].

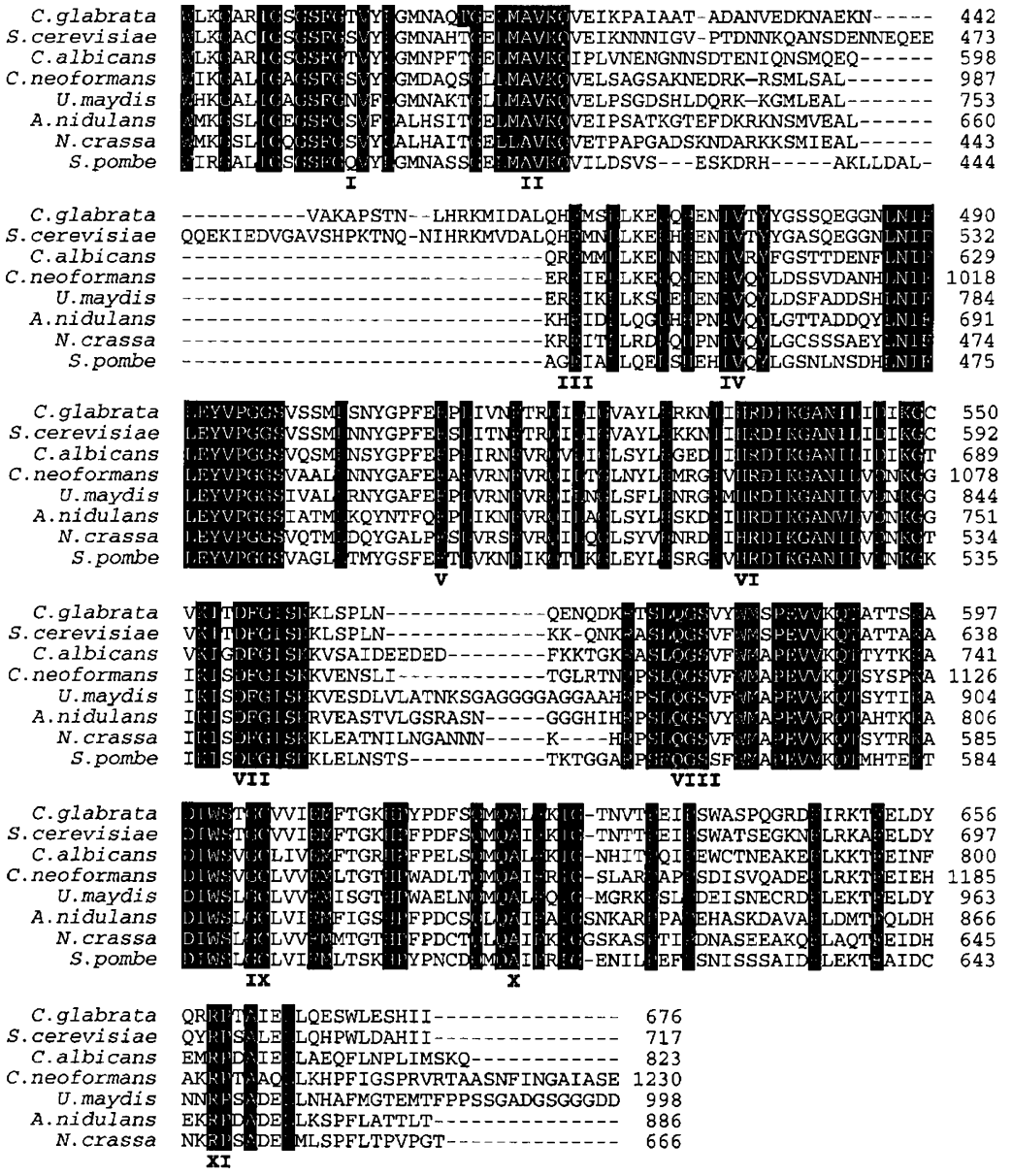

Fig. 2 *Candida glabrata STE11* encodes a member of the Ste11 family of MAP kinase kinase kinases. Alignment of the putative kinase domain of *C. glabrata* Ste11 with Ste11 proteins from *Saccharomyces cerevisiae* (Ste11), *Candida albicans* (Ste11), *Cryptococcus neoformans* (Ste11α), *Ustilago maydis* (ubc4), *Aspergillus nidulans* (SteC), *Neurospora crassa* (nrc-1) and *Schizosaccharomyces pombe* (byr2). The Roman numerals beneath the amino acid sequence indicate the 11 subdomains characteristic of the Ste11 family while the Arabic numerals to the sides of the sequence indicate relative amino acid numbers.

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Southern analysis revealed that the *STE11* gene is present in a single copy in the *C. glabrata* genome (data not shown). In addition, interrogation of the *C. glabrata* proteome (http://cbi.labri.fr/Genolevures/C_glabrata.php) revealed a single Ste11 homologue (CAGL CDS1172.1).

C. glabrata STE11 *is a partial functional homologue of* S. cerevisiae STE11

The ability of the product of *C. glabrata STE11* to complement the nitrogen starvation-induced filamentation defect of diploid *S. cerevisiae ste11/ste11* mutants was investigated. The extent of filamentation was gauged semi-quantitatively using the criteria developed by O'Rourke and Herskowitz [25]. The *S. cerevisiae ste11/ste11* diploid strain L5625 had 66% ($n=200$) robustly filamenting colonies when cultured on SLAD for 96 h at 30°C; the remaining 34% exhibited weak filamentation. Wild-type AM10 colonies showed 100% ($n=200$) with profuse filamentation. Transformation of *S. cerevisiae* L5625 with the *C. glabrata STE11* containing plasmid pAMC44 resulted in partial complementation of the nitrogen starvation-induced filamentation defect seen in this strain, as 93% ($n=200$) of colonies exhibited profuse filamentation. *C. glabrata* Ste11 was also able to reverse the mating defect of the haploid *S. cerevisiae ste11* null mutant Y15271 (Fig. 3). *C. glabrata STE11* was unable to complement the SDS and calcofluor white defective growth phenotypes of *S. cerevisiae* L5625 (data not shown). Taken together these data demonstrate that *C. glabrata STE11*, in addition to having sequence homology to *STE11*, encodes a protein that can complement some, but not all, of the phenotypes associated with loss of *STE11* in *S. cerevisiae*. *C. glabrata STE11* therefore encodes a partial functional homologue of *S. cerevisiae* Ste11.

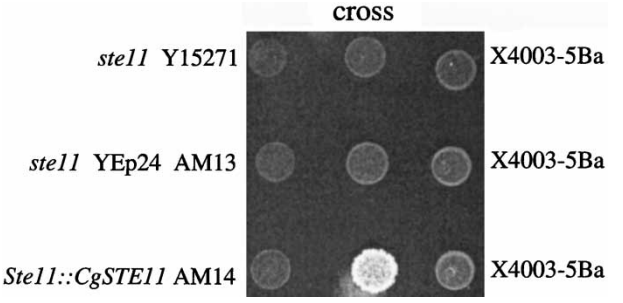

Fig. 3 *Candida glabrata* Ste11 complements the mating defect of *S. cerevisiae ste11* mutants. Haploid *S. cerevisiae* Y15271 (*MATα ste11*), AM13 (*MATα ste11* YEp24) and AM14 (*MATα ste11* pAMC44 (*CgSTE11 URA3*)) were patch mated with the *MAT***a** strain X4003-5Ba. After 48 h culture on YAPD the patches were transferred to SC without histidine and cultured for a further 48 h at 30°C. The left and right columns represent the parental strains as indicated and the centre column the products of mating.

C. glabrata STE11 *is partially required for nitrogen starvation induced filamentation*

We have previously demonstrated that *C. glabrata* produces pseudohyphae, similar to diploid *S. cerevisiae* strains, in response to nitrogen starvation and that the transcription factor Ste12 *is required for this response [18,21]. We therefore sought to determine whether or not C. glabrata* Ste11 is also required for nitrogen-starvation-induced filamentation. The *ste11* null mutant *C. glabrata* AM3p had a partial defect in filamentation on nitrogen starvation medium (Fig. 4). The filamentation response is restored to wild-type levels by reconstitution of *C. glabrata STE11* (Fig. 4),

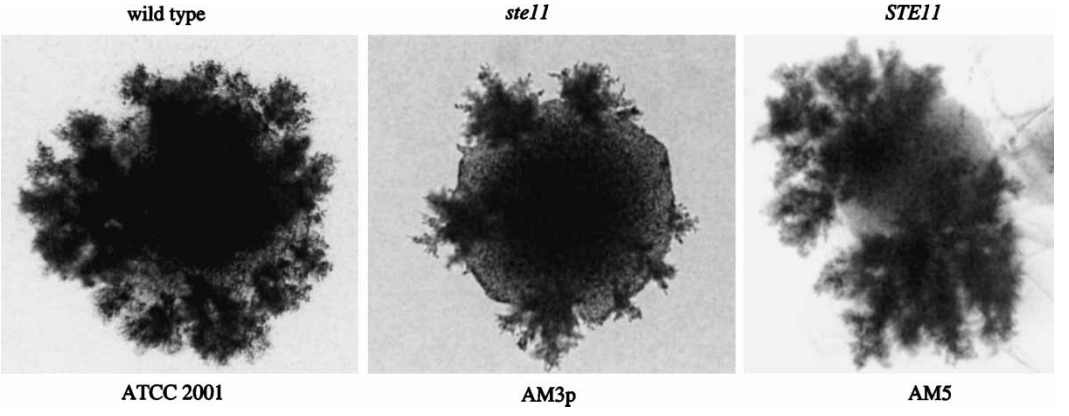

Fig. 4 *Candida glabrata STE11* is only partially required for nitrogen starvation induced filamentation in *C. glabrata.* Wild-type (ATCC 2001), *ste11* (AM3p) and reconstituted *STE11* (AM5) *C. glabrata* strains were cultured on SLAD medium for 8 days at 37°C. Representative colonies were viewed with a Leitz Laborlux 12 microscope using a ×10 objective. Images were captured with a Nikon CoolPix digital camera and imported into Microsoft Word.

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indicating that the observed phenotype was due to *STE11* deletion.

C. glabrata STE11 *is required for adaptation to hypertonic stress but dispensable for maintenance of cell wall integrity*

The *C. glabrata ste11* strain AM3p is unable to grow under the tested hypertonic stress conditions (i.e. 1 mol/l sodium chloride or 1.5 mol/l sorbitol) but is largely insensitive to agents that interfere with cell wall integrity, showing only slightly reduced growth in the presence of SDS (Fig. 5). All these phenotypes could be restored to wild type levels by reintroduction of *C. glabrata STE11*, demonstrating that the observed phenotypes are due to *STE11* deletion and suggesting that *C. glabrata* Ste11 is involved in adaptation to hypertonic stress but is largely dispensable for the maintenance of cell wall integrity.

STE11 *is required for wild type levels of* C. glabrata *virulence*

We have developed a model of murine candidiasis [21]. Briefly CD1 mice were immunosuppressed with 200–250 mg/kg cyclophosphamide, then infected with equivalent doses of *C. glabrata* cells by intravenous injection into the lateral tail vein. We used this model to compare the virulence of *C. glabrata ste11* (AM3p) mutants and *STE11* (AM5) reconstituted cells. *C. glabrata ste11* cells were statistically significantly less able ($P = 0.045$; Kaplan–Meier log rank analysis) to cause disease than reconstituted *STE11* cells (Fig. 6). These data suggest that while still able to cause disease *ste11* mutants are mildly attenuated for virulence compared to *STE11* reconstituted strains.

Discussion

Ste11 proteins are conserved in many fungi. They are involved in the regulation of many cellular processes including mating, filamentation, adaptation to hypertonic stress, cell wall integrity, morphogenesis and virulence [6]. In *S. cerevisiae* Ste11 is a component of three relatively well defined signalling pathways that mediate mating, the hypertonic stress response and filamentous growth [6] and the less well characterized *STE* vegetative growth pathway which functions to promote cell wall integrity [10,11]. We have demonstrated that *C. glabrata* has a Ste11 homologue that can substitute for *S. cerevisiae* Ste11 in the yeast mating pathway, mediates adaptation to hypertonic stress and

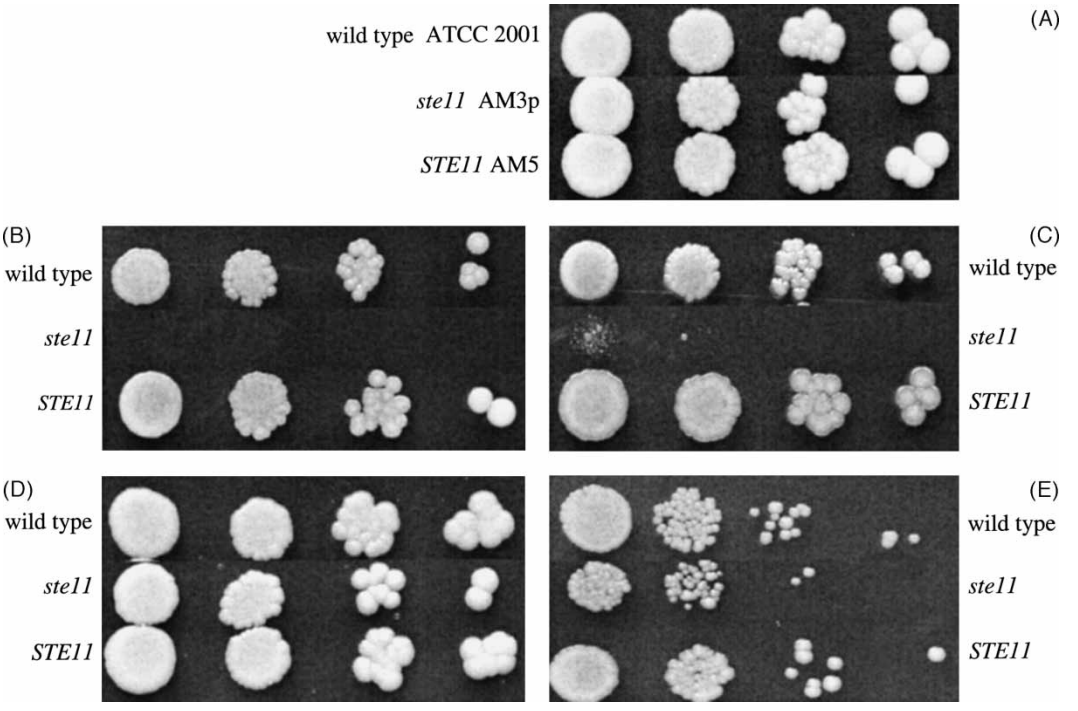

Fig. 5 Ste11 plays an important role in adaptation of *Candida glabrata* to hypertonic stress but is largely dispensable for maintenance of cell wall integrity. *C. glabrata* wild type (ATCC 2001), *ste11* (AM3p) and reconstituted *STE11* strains (AM5) were cultured on YAPD (A), YAPD plus 1 mol/l sodium chloride (B), YAPD plus 1.5 mol/l sorbitol (C), YAPD plus 1% (w/v) calcofluor white (D) and YAPD plus 0.15% (w/v) SDS (E) at 37°C for 48 h.

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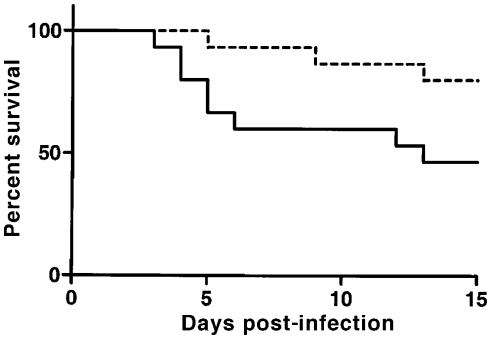

Fig. 6 *Candida glabrata ste11* mutants are mildly attenuated. Groups of 15 immunosuppressed CD1 mice were inoculated with equivalent doses (see text) of either the *C. glabrata STE11* reconstituted strain AM5 (solid line) or the *ste11* null mutant AM3p (hatched line). Mice were killed when the pre-determined end-point of 20% weight loss was reached. Kaplan–Meier log rank analysis revealed that the survival curves were significantly different ($P = 0.045$) for the two groups.

plays a role in nitrogen starvation-induced filamentation. Thus our data support the view that *C. glabrata* Ste11, in a similar fashion to *S. cerevisiae* Ste11 functions in a number of different signalling pathways.

The inability of *C. glabrata ste11* mutants to grow in the presence of high concentrations of sorbitol or sodium chloride suggests that Ste11 is involved in a pathway analogous to the HOG MAPK cascade that mediates the hypertonic stress response in *S. cerevisiae*. Activation of the *S. cerevisiae* HOG pathway can be triggered by three different branches [26]. The first involves Sln1, Ypd1, Ssk1, and the Ssk2/Ssk22 kinases, while the second involves Sho1, a putative membrane osmosensor that is able to activate Pbs2 via Ste11 [27]. The third involves Msb2 and is partially redundant with the Sho1 branch [26]. The signals are thought to converge at the Pbs2 level, which phosphorylates and activates the stress-activated kinase Hog1. In *S. cerevisiae* the *ste11 ssk2 ssk22* triple mutant cannot grow at high osmolarity. In this strain all the pathways that converge on Pbs2 are interrupted [27]. Interestingly in *C. glabrata* deletion of *STE11* alone was sufficient to prevent growth at high osmolarity. This result suggests that the hypertonic stress response in *C. glabrata* is different from that in *S. cerevisiae*, with possibly only one pathway triggering the HOG response. We have obtained the sequence of *C. glabrata HOG1* (B. Dujon and C. Hennequin, unpublished data) and the encoded protein has 86% similarity to *S. cerevisiae* Hog1. We are currently attempting to characterize the phosphorylation state of Hog1 in wild type and *C. glabrata ste11* null mutants in order to determine if this hypothesis is correct.

S. cerevisiae cell wall integrity is maintained in part by the *STE* vegetative pathway which is thought to sense changes in the integrity of the cell wall in a Och1/Sho1 dependent manner resulting in the transmission of a signal, via Ste11, that activates the transcription factor Ste12 and effects subsequent transcription of genes encoding proteins required for maintenance of cell wall integrity [10,11]. The integrity of the cell wall can be determined in straightforward plate assays using SDS or calcofluor white [28,29]. We have shown that *C. glabrata ste11* mutants exhibit essentially wild type growth in the presence of either of these agents. In contrast *S. cerevisiae ste11* mutants are unable to grow in the presence of either SDS or calcofluor white and *C. glabrata STE11* is unable to rescue these defective growth phenotypes. These data suggest that *C. glabrata* cell wall integrity is maintained in a different manner to that in *S. cerevisiae*.

We have previously demonstrated that *C. glabrata* cells produce filaments in response to nitrogen starvation [18]. This filamentation response is lost in *ste12* mutants [21]. But *C. glabrata ste11* null mutants can still produce filaments, albeit in reduced numbers, in response to nitrogen starvation. These data strongly support the view that the nitrogen starvation signal in *C. glabrata* is transduced not only via Ste11 but also an independent, as yet uncharacterized, pathway both of which activate the transcription factor Ste12.

Our data lead us to conclude that *C. glabrata* Ste11 is involved in various signal transduction pathways similar to the situation in *S. cerevisiae*. In *S. cerevisiae* insulation between these pathways is maintained in a number of ways including protein–protein interactions [30]. It is possible that *C. glabrata* Ste11 may be insulated in a similar way. The N-terminus of *C. glabrata* Ste11 has an obvious SAM domain that mediates such connections [24]. In *S. cerevisiae* at least three proteins (Ste50, Ste5 and Ste20] interact with the N-terminus of Ste11 [31]. Interaction of Ste50 with Ste11 is required for modulation of Ste11 function during mating, filamentous growth and the Sho1-dependent response to hyperosmolarity [32,33]. Ste5 is a scaffold protein that interacts with the N-terminal negative regulatory domain of Ste11 [34]. It is possible that the role of Ste5 may be not just to facilitate interactions between protein kinases of the MAPK cascade but also to directly regulate kinase activity. Ste20 directly phosphorylates Ste11 *in vivo* and *in vitro* on serine 302 and/or serine 306 plus threonine 307 [35]. These amino acids are conserved in the N-terminal regulatory domain of *C. glabrata* Ste11 at serines 258 and 262 plus threonine 263 respectively. They are also conserved in other organisms [7,35]. We are currently

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constructing tagged alleles of *C. glabrata STE11* in order to conduct immunoprecipitation and phosphorylation studies with a view to determining how Ste11 function is regulated in this species.

The differences in the responses of *C. glabrata* and *S. cerevisiae* to external stimuli that trigger MAPK cascades suggest that although some of the components of these pathways have been conserved between the two yeast species they function in different ways. The elucidation of these differences may yield valuable information on how *C. glabrata* has evolved to survive in mammalian hosts and allowed it to emerge as an opportunist pathogen.

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