Molecular Microbiology (2003) **50**(4), 1309–1318 doi:10.1046/j.1365-2958.2003.03755.x

Candida glabrata STE12 is required for wild-type levels of virulence and nitrogen starvation induced filamentation

Ana-Maria Calcagno,[1] Elaine Bignell,[1] Peter Warn,[2] Michael D. Jones,[1] David W. Denning,[2] Fritz A. Mühlschlegel,[3] Thomas R. Rogers[1] and Ken Haynes[1]*

[1]*Department of Infectious Diseases & Microbiology, Imperial College London, Du Cane Road, London, W12 0NN, UK.*

[2]*School of Medicine, University of Manchester, Manchester, M8 6RB, UK.*

[3]*Research School of Biosciences, University of Kent, Canterbury, Kent CT2 7NJ, UK.*

Summary

The highly conserved fungal Ste12 transcription factor family of proteins play critical roles in the regulation of many cellular processes including mating, cell wall biosynthesis, filamentation and invasive growth. They are also important mediators of fungal virulence. The *Candida glabrata STE12* homologue was cloned. The encoded protein has a single DNA binding homeodomain but lacks both a C_2H_2 zinc finger DNA binding domain and an apparent Dig1/Dig2 regulatory motif. *Candida glabrata STE12* can functionally complement the nitrogen starvation induced filamentation and mating defects of *Saccharomyces cerevisiae ste12* mutants. We also show that *C. glabrata STE12* is required for nitrogen starvation-induced filamentation as *ste12* mutants rarely produce pseudohyphae on nitrogen depeleted media. Finally we describe a novel murine model of *C. glabrata* systemic disease and use this to demonstrate that *C. glabrata ste12* mutants, although still able to cause disease, are attenuated for virulence compared with *STE12* reconstituted strains. *Candida glabrata STE12* is therefore the first virulence factor encoding gene to be described in this increasingly important fungal pathogen.

Introduction

Candida species now account for almost 10% of nosocomial infections and are the fourth most commonly isolated agent causing blood stream infections in the US (Beck-Sague and Jarvis, 1993; Pfaller *et al*., 2000). *Candida albicans* remains the most commonly encountered species in clinical practice but other species now cause significant levels of disease. In particular, *Candida glabrata* which is the second most commonly documented species isolated from up to 20% of all candidiasis patients (Pfaller *et al*., 2001). Infections caused by this haploid fungal pathogen are often difficult to treat as it is naturally resistant to a number of antifungal drugs, especially fluconazole. Furthermore *C. glabrata* candidiasis is associated with a high mortality (Gumbo *et al*., 1999).

Despite this increase in incidence little is known about the molecular basis of *C. glabrata* virulence (Haynes, 2001). Although a *C. glabrata* lectin (Epa1) is required for adhesion to buccal epithelial cells, the *epa1* null mutant is not attenuated in two murine models of candidiasis (Cormack *et al*., 1999). This discrepancy may be due to redundancy within the *EPA1* gene family (De Las Penas *et al*., 2002).

In contrast to *C. glabrata*, a number of attributes have been shown to be required for virulence in *C. albicans*. These include epithelial adhesion, secreted hydrolases and the ability to undergo a reversible morphogenetic switch from yeast to hyphal or pseudohyphal growth forms (Calderone and Fonzi, 2001; Liu, 2001; Navarro-Garcia *et al*., 2001). It is possible that *C. glabrata* shares some of these characteristics. Indeed, recently we have shown that *C. glabrata* possesses a gene family (*GAS1-3*) that share homologies with the *PHR1* and *PHR2* genes from *C. albicans* (Weig *et al*., 2001). The latter are pivotal in cell wall biosynthesis and virulence (De Bernardis *et al*., 1998). *Candida glabrata* can also switch to a pseudohyphal growth form, similar to that seen in diploid *Saccharomyces cerevisiae* strains when starved for nitrogen *in vitro*, however it has not been seen to filament *in vivo* and this trait does not appear to be important in terms of *C. glabrata* disease (Csank and Haynes, 2000; Weig *et al*., 2001).

One of the major regulators of the morphogenetic switch in *S. cerevisiae* is the transcriptional regulator Ste12 which is activated by a mitogen activated protein kinase (MAPK) pathway. Ste12 consists of a DNA binding homeodomain and a Dig1/Dig2 interaction domain that partially regulates function (Yuan and Fields, 1991; Pi

Accepted 1 August, 2003. *For correspondence. E-mail k.haynes@imperial.ac.uk; Tel. (+44) (0)20 8383 1245; Fax (+44) (0)20 8383 3394.

et al., 1997). In haploid *S. cerevisiae* strains Ste12 is known to regulate the response to mating pheromone and the invasive growth phenotype whereas in diploid cells it plays a role in the filamentous growth response to nitrogen starvation (Gustin *et al*., 1998; Roberts *et al*., 2000; Gancedo, 2001). In addition, recent genome-wide location analysis has shown that Ste12 binds to the promoters of a number of genes involved in cell wall biosynthesis (Ren *et al*., 2000). The homologous transcription factor in *C. albicans*, Cph1, is also required for full function of a restricted number of morphogenetic programmes and in combination with a second regulator, Efg1, plays an important role in virulence (Lo *et al*., 1997).

Ste12 homologues have also been described in a number of other fungi. The pathogenic basidiomycete *Cryptococcus neoformans* has two mating type specific *STE12* genes *STE12a* and *STE12α* which play different roles in haploid fruiting mating and the regulation of genes (e.g. *CNLAC1* and the *CAP* family) involved in cryptococcal virulence (Wickes *et al*., 1997; Yue *et al*., 1999; Chang *et al*., 2000; 2001). Similarly the Ste12 homologues in *Aspergillus nidulans* (SteA) and *Candida lusitaniae* (Cls12) are also required for mating (Vallim *et al*., 2000; Young *et al*., 2000), however, neither of these proteins are essential for filamentous growth. Recently a Ste12 homologue (StlA) has been described in the dimorphic fungal pathogen *Penicillium marneffii* (Borneman *et al*., 2001), deletion of which resulted in no detectable phenotypic changes. Ste12-like proteins that play essential roles in virulence have also been described in the plant pathogens *Magnaporthe grisea* (Park *et al*., 2002) and *Colletotrichum lagenariu* (Tsuji *et al*., 2003).

The increasing importance of *C. glabrata* as a pathogen plus the multifunctional nature of Ste12 proteins led us to determine if a homologue was to be found in this fungus and, if so, to investigate what role(s) it played in the many Ste12 regulated processes. Here we describe the cloning of the *C. glabrata STE12* homologue and show that the encoded protein is required for nitrogen starvation induced filamentation and to maintain wild-type virulence in a murine model of candidiasis

Results

C. glabrata STE12 *encodes a member of the fungal Ste12 protein family*

Polymerase chain reaction amplification with primers 1444 and 1447 (Table 1) yielded a 314 bp fragment that had 73% identity with the 5′ end of the *S. cerevisiae STE12* open reading frame (ORF). The PCR product was radiolabelled and used to screen a *C. glabrata* genomic library in YEp24 (kindly provided by Dominique Sanglard). Two independent clones were isolated that contained identical 6.7kb inserts. Sequencing of one insert revealed an uninterrupted 1791 bp ORF, with significant homology to fungal *STE12* genes. In addition, the insert contained the entire *C. glabrata* homologue of *YDR333C* and a 5′ fragment of *YHR085W*. Notably the gene order around *C. glabrata STE12* was not syntenic to that seen in *S. cerevisiae*. These sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession number AJ515385.

The *C. glabrata STE12* ORF is predicted to encode a protein of 597 amino acids with a molecular mass of 67.62 kDa. The homology to other members of the fungal Ste12 family is mainly found in the DNA binding homeobox domain, having 66%, 64% and 42% identity over the 219, 233 and 403 N-terminal amino acids of the Ste12 homologues of *C. albicans*, *S. cerevisiae* and *Kluveromyces lactis* respectively. It contains the homeodomain found in all Ste12 homologues described to date but lacks the two C_2H_2 zinc fingers found only in StlA (*P. marneffei*), SteA (*A. nidulans*) and both cryptococcal

Table 1. Sequences of primers used in this study.

Primer name	Primer sequence (5′ to 3′)
1444	AAYTGGCARGARAAYCA
1447	AACCARAARAAIACYTTYTGYTTYTT
CgSTE12-890	AAGCTTCAGGAGGCATTGCTA
CgSTE12-890SalI	NNNNGTCGACAAGCTTCAGGAGGCATTGCTA
CgSTE12-890SphI	NNNNGCATGCAAGCTTCAGGAGGCATTGCTA
CgSTE12-473SalI	NNNNGTCGACCAATGCCAGGGTTCTGAGTCAGG
CgSTE12-16	CTGTAATATCTACACCATGAGCC
CgSTE12-16SpeI	NNNNACTAGTCTGTAATATCTACACCATGAGCC
CgSTE12+720	GAAGAGATATCATTTGCCTC
CgSTE12+720SpeI	NNNNACTAGTGAAGAGATATCATTTGCCTC
CgSTE12+2030	GAACCCGCAAGAACTAGATA
CgSTE12+2030SphI	NNNNGCATGCGAACCCGCAAGAACTAGATA
CgTIP1F	CTAGTCCACGCTCAAAATGC
CgTIP1R	TTCATAGCAACATAGCGGCAGC
CgCIS3F	TGTTTAACAGTCAACCAAAGAG
CgCIS3R	ACGATGGTACTTTGCAATTGAC

Restriction enzyme recognition sequences are underlined.

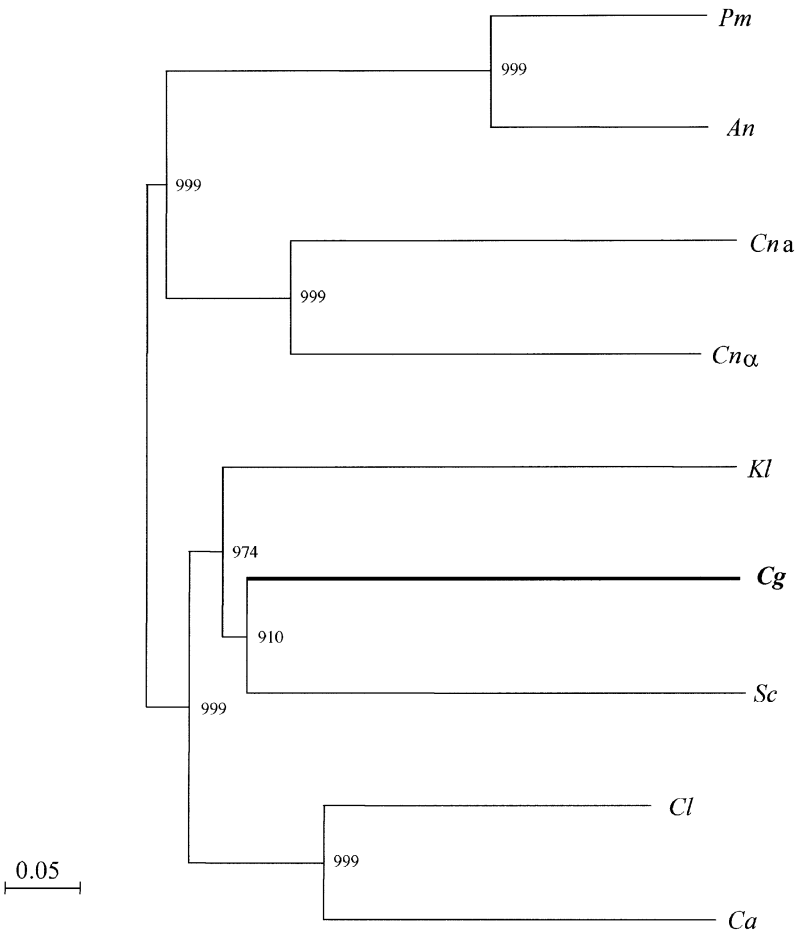

Fig. 1. *Candida glabrata STE12* encodes a member of the Ste12 family of fungal transcription factors. Unrooted, bootstrapped phylogentic tree of fungal Ste12 sequences demonstrating that *C. glabrata* Ste12 is most closely related to the *S. cerevisiae* and *K. lactis* proteins (An, *A. nidulans*; Ca, *C. albicans*; Cl, *C. lusitaniae*; Cn, *C. neoformans*; Kl, *K. lactis*; Pm, *P. marneffei*; Sc. *S. cerevisiae*). Figures on the tree represent bootstrap confidence levels for each branch.

proteins. *Candida glabrata* Ste12 also lacks the Dig1/Dig2 consensus binding site (DFPLDYF). It is most closely related to the *S. cerevisiae* and *K. lactis* proteins (Fig. 1).

Computational analysis of the *C. glabrata* Ste12 promoter with MatInspector (Quandt *et al*., 1995) revealed no matches with *S. cerevisiae* pheromone or filamentation response elements (Bardwell *et al*., 1994; Madhani and Fink, 1997). However, possible binding sites for Gcn4 (−390, −397, −551, −558) and Nit2 (−386, −679, −883) were identified suggesting that *C. glabrata* Ste12 may be regulated by amino acid and/or nitrogen starvation.

C. glabrata *Ste12 is a partial functional homologue of* S. cerevisiae *Ste12*

Transformation of *S. cerevisiae* L5627, a homozygous diploid *ste12* null mutant, with the *C. glabrata STE12* containing plasmid pKH310 resulted in rescue of the filamentation defect seen in this strain (Fig. 2A). In addition *C. glabrata STE12* was able to complement the mating defect of the haploid *S. cerevisiae ste12* null mutant HLY635 (Fig. 2B). However, *C. glabrata STE12* was unable to complement the defective growth phenotype of *S. cerevisiae ste12* mutants on calcofluor white and *S. cerevisiae* HLS105 acquired a slight SDS sensitive growth defect (data not shown). These data demonstrate that *C. glabrata STE12*, in addition to having sequence homology with *STE12*, encodes a protein that is at least a partial functional homologue of Ste12.

C. glabrata *Ste12 is required for nitrogen starvation induced filamentation*

We have previously demonstrated that *C. glabrata* produces pseudohyphae, similar to those of diploid *S. cerevisiae* strains, in response to nitrogen starvation (Csank and Haynes, 2000). We therefore sought to determine if *C. glabrata* Ste12 was required for this filamentation. The *ste12* null mutant *C. glabrata* HLS112T rarely produces filaments in response to nitrogen starvation whereas the parental strain *C. glabrata* ATCC 2001 and the *C. glabrata STE12* reconstituted strain HLS113 produce large numbers of filaments. However, the reconstituted strain was unable to produce filaments to the same extent as the

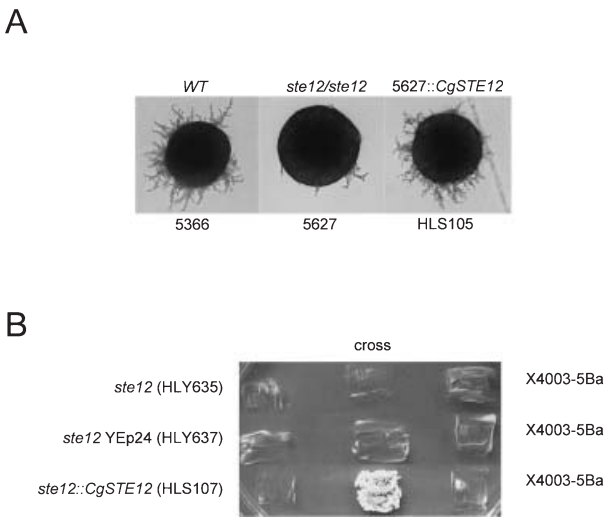

Fig. 2. *Candida glabrata STE12* complements the filamentation and mating defects of *S. cerevisiae ste12* mutants.
A. Diploid wild-type (5366), *ste12/ste12* (5627) and *C. glabrata STE12* complemented *ste12/ste12* (HLS105) *S. cerevisiae* strains were cultured on SLAD medium for 7 days at 30°C. Representative 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.
B. Haploid *S. cerevisiae* HLY635 (*MATα ste12*), HLY637 (*MATα ste12* YEp24) and HLS107 (*MATα ste12 CgSTE12*) were patch mated with the *MATa* strain X4003–5Ba. After 48 h culture on YPAD 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.

wild-type parental strain, possibly as a result of the episomal complementation (Fig. 3).

C. glabrata STE12 *is not required for cell wall integrity*

We constructed a mutant, *C. glabrata* HLS108, with a doxycycline regulatable *C. glabrata STE12* allele and demonstrated that *STE12* expression in this strain was suppressed by the presence of 10 μg ml^{-1} doxycycline (Fig. 4A). Total RNA was extracted from *C. glabrata* HLS108 grown in the presence and absence of doxycycline, and converted to cDNA. The two cDNA populations were used to probe *S. cerevisiae* GF100 gene filters (Research Genetics). Visual comparison of the hybridization patterns obtained revealed apparent differences in binding to at least five ORFs, i.e. *TIP1*, *CIS3*, *PAU1*, *PAU6* and *YJL051W*. In all cases expression of *C. glabrata* Ste12 resulted in de-repression of gene expression. We attempted to confirm these data by Northern analysis. Polymerase chain reaction primers were designed using available *C. glabrata* genomic information (*TIP1* and *CIS3*) or by analogy to the *S. cerevisiae* genes (*PAU1*, *PAU6* and *YJL051W*). The resultant PCR products were sequenced and used to probe Northern blots of total RNA extracted from *C. glabrata* HLS108 cultured in the presence or absence of doxycycline. This analysis revealed that *C. glabrata CIS3* expression was de-repressed in the absence of *C. glabrata* Ste12 whereas *TIP1* expression appeared to be only partially dependent on Ste12 (Fig. 4B). Neither *TIP1* nor *CIS3* expression was induced in *C. glabrata* ATCC 2001 by the presence of doxycycline (data not shown). We were unable to design primers that

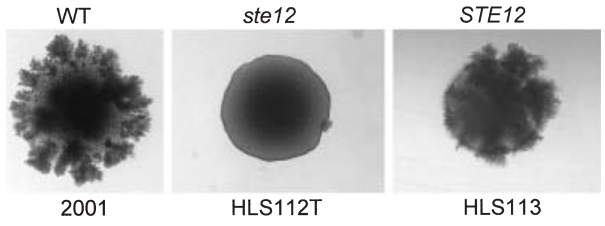

Fig. 3. *Candida glabrata STE12* is required for nitrogen starvation induced filamentation. Wild-type ATCC 2001, *ste12* (HLS112T) and reconstituted *STE12* (HLS113) *C. glabrata* strains were cultured on SLAD medium for 10 days at 37°C. Representative colonies were viewed with a Leitz Laborlux 12 microscope using a 10× objective. Images were captured with a Nikon CoolPix digital camera and imported into Microsoft Word.

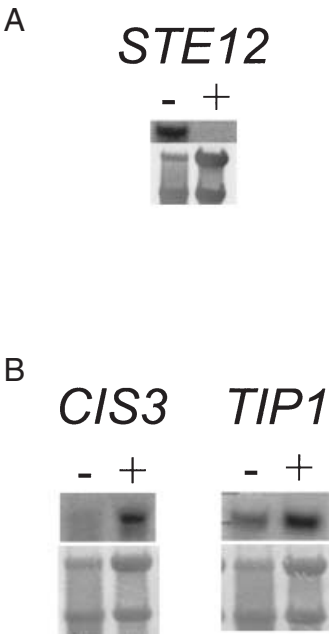

Fig. 4. The absence of *C. glabrata* Ste12 de-represses *CIS3* and *TIP1* expression. *C. glabrata* HLS108 was cultured overnight in YPAD, diluted 1:20 in fresh YPAD and cultured for a further 2 h in the presence (+) or absence (–) of 10 μg ml^{-1} doxycycline. The upper panels show the Northern analysis and the lower panels total RNA stained with methylene blue.
A. Doxycyline represses *STE12* expression in *C. glabrata* HLS108. Total RNA was transferred onto a nylon membrane and probed with a *C. glabrata STE12* (–16/+720) PCR product.
B. *C. glabrata CIS3* and to a lesser extent *TIP1* expression is de-repressed by the absence of Ste12. Blots were probed with PCR products generated by CgCIS3F/CgCIS3R and CgTIP1F/CgTIP1R respectively.

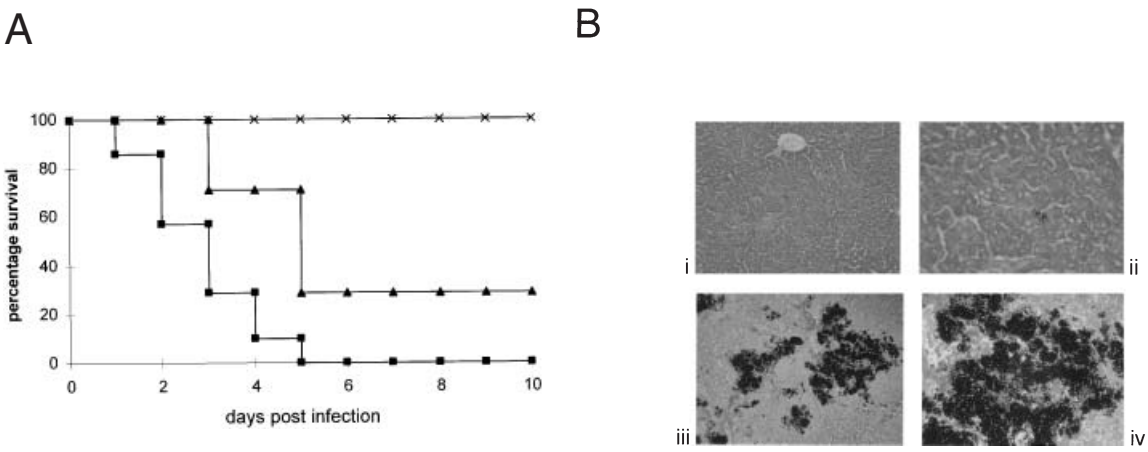

Fig. 5. *Candida glabrata* causes systemic disease in a murine model of candidiasis.
A. Groups of seven mice were immunosuppressed with 200 mg kg^{-1} cyclophosphamide and inoculated i.v. with (x) 1×10^7 (▲) 7×10^7 and (■) 2×10^8 wild-type *C. glabrata* ATCC 2001 blastospores. Mice were sacrificed when predetermined end-points (as described in the text), e.g. 20% weight loss, were reached.
B. Representative liver sections from mice infected with 7×10^7 (i, ii) or 2×10^8 (iii, iv) *C. glabrata* ATCC 2001 wild-type cells. Sections were stained with Grocott and Light Green. The resultant stained slides were viewed with a Leitz Laborlux 12 microscope using a 10× (i, iii) or 25× (ii, iv) objective. Images were captured with a Nikon CoolPix digital camera and imported into Microsoft Word. The differences in tissue colour represent varying intensities of Grocott/Light green staining.

were capable of amplifying the *C. glabrata* homologues of *PAU1*, *PAU6* and *YJL051W* and therefore the *C. glabrata* Ste12 regulatory status of these genes could not be determined. We were also unable to confirm their Ste12 dependant regulation by probing with heterologous *S. cerevisiae* probes.

As four of the putative *C. glabrata* Ste12 regulated ORFs (*TIP1*, *CIS3*, *PAU1* and *PAU6*) encode proteins that have known or putative cell wall functions we decided to investigate the role of *C. glabrata STE12* in maintenance of cell wall integrity. *Candida glabrata* HLS112T demonstrated no growth defects on media containing SDS, caffeine or calcofluor white (data not shown).

Murine model of C. glabrata *systemic candidiasis*

We initially attempted an immunosuppressive regimen where cyclophosphamide (200 mg kg^{-1}) was administered the day before infection with *C. glabrata*. Using this regimen we were unable to initiate infection with *C. glabrata* ATCC 2001. However disease was established when immunosuppression was started three days before infection. Administration of increasing numbers of *C. glabrata* cells resulted in more severe disease (Fig. 5A). A dose of 2×10^8 cells reproducibly resulted in a 100% fatal infection after 5 days. Histological examination of major organs (brain, heart, kidney, liver, lung and spleen) demonstrated the presence of large numbers of *C. glabrata* cells, occurring as large microcolonies (Fig. 5B). In all cases there was little or no evidence of necrosis or inflammation around the sites of *C. glabrata* microcolonies. Large numbers of *C. glabrata* cells were recoverable from all organs in mice that died after 3 days (from 7×10^7 g^{-1} in the lung to 9.4×10^8 g^{-1} in the spleen). Conversely culture of blood, obtained from the lateral tail vein, revealed only limited candidaemia (<10 cells ml^{-1}). When a lower dose of 7×10^7 was administered mortality fell to 70% by day 10 (Fig. 5A). In addition histology revealed a much lower tissue burden. Infrequent small microcolonies or individual blastospores only were seen (Fig. 5B). Again no necrosis or inflammation was evident. All mice inoculated with 1×10^7 cells survived for the entire course of the experiment (Fig. 5A).

C. glabrata *Ste12 is required to maintain wild-type levels of virulence*

Groups of 15 immunosuppressed mice were inoculated with equivalent doses of the *ste12* mutant *C. glabrata* HLS112T (9.1×10^7) or the reconstituted *STE12* strain HLS113 (8.8×10^7). Kaplan Meier Log Rank analysis of the survival curves shown in Fig. 6 demonstrate that *C. glabrata ste12* cells are attenuated compared with their *STE12* reconstituted siblings ($P = 0.004$). Median survival times for animals infected with the *STE12* reconstituted strain was three days compared with five days for mice inoculated with *C. glabrata ste12* cells. These data demonstrate that *C. glabrata STE12* is required to maintain wild type levels of virulence and indicates that the encoded protein plays a role in the pathogenesis of candidiasis caused by this fungus.

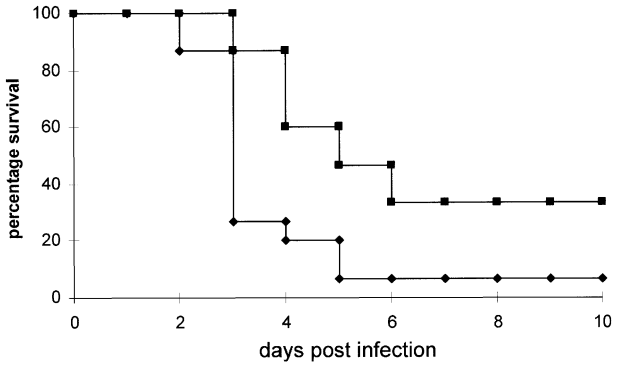

Fig. 6. *Candida glabrata STE12* is required to maintain wild-type levels of virulence. Groups of 15 mice were immunosuppressed with 200 mg kg^{-1} cyclophosphamide and inoculated with the *C. glabrata ste12* mutant (■) HLS112T (9.1×10^7) or the *STE12* reconstituted strain (◆) HLS113 (8.8×10^7). Mice were sacrificed when predetermined end-points (as described in the text), e.g. 20% weight loss, were reached. Kaplan Meier Log Rank analysis of the survival curves demonstrate that *ste12* cells are significantly attenuated ($P = 0.004$) compared to *STE12* reconstituted strains, and have an extended median survival time of 5 days compared to 3 days.

Discussion

Ste12 proteins are conserved in many fungi, regulating processes involved in mating, filamentation, substrate invasion, cell wall integrity and virulence (Liu *et al*., 1994; Singh *et al*., 1994; Lo *et al*., 1997; Wickes *et al*., 1997; Chang *et al*., 2000; 2001; Vallim *et al*., 2000; Young *et al*., 2000; Borneman *et al*., 2001). We have demonstrated that *C. glabrata* has a Ste12 homologue. There are two major subclasses within the Ste12 family of proteins, those with both homeodomain and C_2H_2 zinc finger DNA binding domains (i.e. SteA, CnSte12a, CnSte12α and StlA) and those that only have the homeodomain (i.e. Cph1, Cls12, KlSte12 and Ste12). *Candida glabrata* Ste12 falls into the latter category. Unusually for this group of Ste12 proteins *C. glabrata* Ste12 lacks an apparent consensus binding motif for the regulatory proteins Dig1 and Dig2 (Pi *et al*., 1997). These two *S. cerevisiae* proteins bind to Ste12 in their unphosphorylated state and repress its function. Upon activation of the pheromone/filamentation MAPK cascade these proteins are phosphorylated resulting in their disassociation from Ste12 and its activation (Olson *et al*., 2000). The lack of a Dig1/2 binding motif on *C. glabrata* Ste12 suggests a different regulatory model. One possibility is that *C. glabrata* Ste12 is directly activated by a MAPK cascade. We are currently investigating this hypothesis.

The complementation of the mating defect of *ste12 S. cerevisiae* strains by *C. glabrata STE12* is an interesting observation. This demonstrates that *C. glabrata* Ste12 can activate transcription from *S. cerevisiae* promoters containing pheromone response elements resulting in mating. This data taken together with our cloning of other *C. glabrata STE* genes, plus the identification of a large number of genes with no known roles outside of sexual development during a genome sequence scan (Wong *et al*., 2003) and the characterization of three mating type-like loci (Srikantha *et al*., 2003), suggest that at least a cryptic mating pathway exists in this apparently asexual organism. Recently a sexual cycle has been demonstrated in the diploid species *C. albicans* (Hull *et al*., 2000; Magee and Magee, 2000). The frequency of this mating was initially reported to be very low. However, more recent evidence has shown that phase variation is an important stimulus of mating (Miller and Johnson, 2002). *Candida albicans* opaque phase cells mate approximately 10^6 times more efficiently than white cells, in which initial mating experiments were performed. These data demonstrate the need to define conditions under which mating can proceed. It is possible that the conditions that would allow *C. glabrata* to mate have not yet been defined.

Our Northern analysis data suggests that *C. glabrata* Ste12 plays an important role in *C. glabrata* cell wall biology as its absence results in derepression of *TIP1* and *CIS3* expression. Tip1 and Cis3 are structural components of the *S. cerevisiae* cell wall (http://www.yeastgenome.org). *Saccharomyces cerevisiae* Ste12 also plays a major role in maintaining cell wall integrity as it can be targeted to the promoters of a set of genes involved in cell wall biology by interaction with another DNA binding protein Mcm1 (Kuo *et al*., 1997). However, *C. glabrata ste12* null mutants are not sensitive to growth on SDS. This suggests that the cell wall integrity pathway of *C. glabrata* is distinct from that in *S. cerevisiae*.

Candida albicans cph1/cph1 mutants do not filament in response to certain stimuli and are slightly attenuated whereas *cph1/cph1 efg1/efg1* mutants cannot form filaments *in vitro* and are severely attenuated (Lo *et al*., 1997). The attenuation has been causally linked to defective filamentation. However, recent work has shown that these mutants can, and do, produce filamentous forms *in vivo* (Riggle *et al*., 1999). In contrast *C. glabrata* has not been shown to form filaments *in vivo* even though filamentation occurs in response to nitrogen starvation *in vitro* (Csank and Haynes, 2000). We have extended our previous findings and demonstrated that this filamentation is dependent on *C. glabrata* Ste12, i.e. *ste12* null mutants in contrast to wild-type cells only rarely produce filaments when starved for nitrogen. We have also shown that *C. glabrata ste12* null mutants are not as virulent as wild-type or *STE12* reconstituted strains. As filamentation is not thought to be important in *C. glabrata* virulence it is possible, even likely, that other *C. glabrata* Ste12 dependent processes mediate disease initiation and progression in the mammalian host. Recent genome-wide analysis of *S. cerevisiae* Ste12 DNA binding has

Table 2. Fungal strains used in this study.

Strain	Genotype	Reference
C. glabrata ATCC 2001	type strain	ATCC[a]
C. glabrata ΔH1	*his3::URA3 Δura3*	Weig, 2001
C. glabrata ΔHT6	*his3::URA3 Δura3 Δtrp1*	Weig, 2001
C. glabrata ACG4	*his3, trp1, PScHOP1::tetR::GAL4AD::TRP1*	Hanic-Joyce,1998
C. glabrata HLS108	*his3, trp1, PScHOP1::tetR::GAL4AD::TRP1 tetOpSTE12 HIS3*	this study
C. glabrata HLS112	*his3::URA3 Δura3 Δtrp1 Δste12::HIS3*	this study
C. glabrata HLS112T	*his3::URA3 Δura3 Δtrp1 Δste12::HIS3* [pCgACT-14]	this study
C. glabrata HLS113	*his3::URA3 Δura3 Δtrp1 Δste12::HIS3* pKH269 [*STE12 TRP1*]	this study
S. cerevisiae L5633	*ura3/ura3*	Mösch, 1997
S. cerevisiae L5627	*ura3/ura3 ste12/ste12*	Mösch, 1997
S. cerevisiae HLY635	*MATα ste12::LEU2 ura3 leu2 his3*	FLC[b]
S. cerevisiae HLY637	*MATα ste12::LEU2 ura3 leu2 his3 YEp24*	FLC[b]
S. cerevisiae X4003–5Ba	*MATa leu2 ade1 his4 met2 ura3 trp5 gal1*	AFGLC[c]
S. cerevisiae HLS105	*ura3/ura3 ste12/ste12* pKH310 [*CgSTE12 URA3*]	this study
S. cerevisiae HLS107	*MATα ste12::LEU2 ura3 leu2 his3* pKH310 [*CgSTE12 URA3*]	this study

a. American Type Culture Collection.
b. Fink laboratory collection.
c. Aberdeen Fungal Group laboratory collection.

revealed a role for this transcription factor in the regulation of many aspects of *S. cerevisiae* biology, e.g. transport, environmental sensing and vacuolar trafficking which in pathogenic fungi may act to mediate *in vivo* survival and growth (Ren *et al.*, 2000). These data support the hypothesis that many Ste12 dependent processes, in addition to the regulation of filamentous growth, may play a significant role in virulence and that perhaps the causal link ascribed to the relationship between reduced ability to filament and an attenuated phenotype needs to be re-evaluated.

Our data suggest that the *C. glabrata* and *S. cerevisiae* Ste12 proteins play different roles in these two fungi. This hypothesis is supported by our observations of the different phenotypes of *C. glabrata* and *S. cerevisiae ste12* mutants. Furthermore *C. glabrata* Ste12 while able to fulfil a number of the functions of the *S. cerevisiae* protein, e.g. complementation of the diploid filamentation defect and haploid sterility, it is unable to complement all phenotypes, e.g. the calcofluor white sensitive growth defect. It is possible that analysis of other biological differences between *C. glabrata* and *S. cerevisiae* will reveal insights into why the former is a much more common cause of infection than the latter.

Experimental procedures

Strains and growth media

All strains used in this study are listed in Table 2. 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), 2%(w/v) glucose and appropriate drop out mix (Clontech, Basingstoke), at 30°C or 37°C. For solid media 2%(w/v) agar was added prior to autoclaving. SLAD media was prepared as previously described (Csank and Haynes, 2000). Other additives were filter sterilised and added at the appropriate concentration to media after autoclaving.

Cloning of C. glabrata STE12

Degenerate PCR primers (1444 and 1447) designed to alignments of Ste12 proteins from *S. cerevisiae, C. albicans* and *K. lactis* were a generous gift from Mike Lorenz (Whitehead Institute, Boston, USA). All primers used in this study are listed in Table 1. Polymerase chain reaction amplification was performed on a PCR Engine (MJ Research, Watertown, MA, USA) in 1× PCR buffer (MBI, Vilnius, Lithuania) containing 1 U of Taq polymerase and 100 ng of *C. glabrata* ATCC 2001 genomic DNA. Polymerase chain reaction conditions were 5 min at 94°C, then 25 cycles of 30 s at 94°C, 30 s at 50°C and 2 min at 72°C followed by a final extension step of 10 min at 72°C. The resulting PCR product was cloned into pGEM T-Easy (Promega, Southampton) and sequenced. The 314 bp fragment showed significant sequence homology to fungal *STE12* genes.

A *C. glabrata* genomic DNA library constructed in YEp24 was a kind gift from Dominique Sanglard (CHUV, Lausanne, Switzerland). The 314 bp *C. glabrata STE12* PCR product was labelled with ^{32}P using Ready-to-Go beads (Amersham-Pharmacia, Chalfont) and used to probe replicate filters of this library. Two positive colonies were identified that contained the same YEp24 plasmid (pCgSTE12). The entire 6.7kb insert of one of these plasmids was sequenced on both strands at the Imperial College London sequencing facility using synthetic primers.

Disruption of C. glabrata STE12

Polymerase chain reaction primers CgSTE12–890SphI and CgSTE12 + 2030SphI were used to amplify a 2920 bp fragment from pCgSTE12. Each of these primers contained embedded *Sph*I sites. The PCR product was cloned into pGEM T-Easy. The resultant plasmid (pKH313) was digested

with *Xba*I and *Kpn*I to remove 2027 bp from −505 to +1522, with respect to the *C. glabrata STE12* ATG start codon. The *C. glabrata STE12* stop codon is at +1791. The plasmid backbone was blunt ended and a *C. glabrata HIS3* cassette was cloned in as previously described (Weig *et al*., 2001). This disruption cassette was amplified with the primer pair CgSTE12-890 and CgSTE12 + 2030 (without embedded *Sph*I sites) and transformed into *C. glabrata* ΔHT6 (*his3::URA3 Δura3 Δtrp1*) using a lithium acetate protocol. Histidine prototrophs were selected. Homologous integration was confirmed by Southern analysis and gene disruption by PCR using the primer pair CgSTE12-6 and CgSTE12 + 720. A representative strain *C. glabrata* HLS112 (*his3::URA3 Δura3 Δtrp1 Δste12::HIS3*) was selected for further analysis. For the infection and phenotypic analysis this strain was made prototrophic for tryptophan by transformation with pCgACT-14 (Kitada *et al*., 1996) and designated *C. glabrata* HLS112T.

Reconstitution of C. glabrata STE12

Plasmid pKH269 was constructed by digesting pKH313 with *Sph*I and cloning the purified 2920 bp *C. glabrata STE12* fragment into *Sph*I digested dephophorylated pCgACT-14 (Kitada *et al*., 1996). To reconstitute *C. glabrata STE12* we transformed *C. glabrata* HLS112 with pKH269. Tryptophan prototrophs were selected. Reconstitution of *C. glabrata STE12* was confirmed by PCR with the primer pair CgSTE12-16 and CgSTE12 + 720 plus Northern analysis. A representative *C. glabrata STE12* reconstituted transformant *C. glabrata* HLS113 (*his3::URA3 Δura3 Δtrp1 Δste12::HIS3* pKH269 [*STE12 TRP1*]) was selected for further analysis.

Complementation of S. cerevisiae ste12 *mutants*

The 2920 bp *C. glabrata STE12 Sph*I fragment was removed from pKH313 and cloned into the *Sph*I site of YEp24. The resultant plasmid pKH310 was used to transform the diploid *S. cerevisiae ste12* mutant L5627 (*ura3/ura3 ste12/ste12*) (Mosch and Fink, 1997). Uracil prototrophs were selected and episomal uptake of *C. glabrata STE12* was confirmed by PCR with the primer pair CgSTE12-16 and CgSTE12 + 720. A representative transformant *S. cerevisiae* HLS105 (*ura3/ura3 ste12/ste12* pKH310 [*STE12 URA3*]) was selected. In addition the haploid *S. cerevisiae ste12* mutant HLY635 (*MATα ste12::LEU2 ura3 leu2 his3*) was transformed with pKH310. A representative transformant *S. cerevisiae* HLS107 (*MATα ste12::LEU2 ura3 leu2 his3* pKH310 [*CgSTE12 URA3*]) was selected.

Mating assays

To determine if *C. glabrata* Ste12 could complement the mating defect of the haploid *S. cerevisiae ste12* mutant HLY635 we performed a series of patch matings with the *MATa* strain X4003–5Ba (*MATa leu2 ade1 his4 met2 ura3 trp5 gal1*). This strain was crossed with HLY635 (*MATα ste12::LEU2 ura3 leu2 his3*), HLY637 (*MATα ste12::LEU2 ura3 leu2 his3* YEp24) and HLS107. 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.

Construction of a doxycycline regulatable C. glabrata STE12 *allele*

Two PCR products were generated by amplification with the primer pair CgSTE12–890SalI/CgSTE12–473SalI and CgSTE12–16SpeI/CgSTE12 + 720SpeI, respectively, and cloned into pGEM T-Easy. The −16/+720 fragment was released by *Sal*I digestion and cloned into *Sal*I cut p99CGH (Nakayama *et al*., 1998). The −890/−473 fragment was released from pGEM T-Easy by *Spe*I digestion and cloned into the *Spe*I site of the resultant plasmid. The promoter replacement cassette was generated by amplification with the primer pair CgSTE12-890/CgSTE12 + 720 (there were no embedded *Sal*I or *Spe*I restriction sites in these primers) and transformed into *C. glabrata* ACG4 (Nakayama *et al*., 1998). Histidine prototrophs were selected and doxycycline repressible *C. glabrata STE12* expression was confirmed by Northern analysis. DNA sequencing was performed to establish that no mutation had occurred in the *C. glabrata STE12* locus of these strains. A representative strain was selected and designated *C. glabrata* HLS108 (*his3 trp1 PScHOP1::tetR::GAL4AD::TRP1 tetO*p *STE12 HIS3*).

Nucleic acid manipulations

DNA isolation, RNA preparation, Northern and Southern analysis were essentially as previously described (Weig *et al*., 2001).

Saccharomyces cerevisiae Yeast Index Gene Filters (GF100, Research Genetics, Huntsville, AL, USA), which contain PCR products corresponding to each annoated *S. cerevisiae* ORF on two nylon membranes, were probed with either 500 ng of genomic DNA (labelled using Ready-to-Go beads) or the cDNA generated from 5 μg of total RNA that was reverse transcribed, radiolabelled and hybridized following the manufacturers instructions.

Model of systemic candidiasis

All mice included in this study were part of ongoing studies performed under UK Home Office project licence PPL 70/5361. Experiments were performed at dedicated facilities at Imperial College London. Male CD1 mice, 4–5 weeks old and weighing between 18 and 20 g were purchased from Charles River UK (Margate, Kent). The mice were virus-free and were allowed free access to food and water. Each cage was inspected at least twice daily.

When the mice had reached a weight of 25–28 g cyclophosphamide (Sigma-Aldrich Poole, Dorset) was administered ip to all animals at a dose of 200 mg kg^{-1} to induce immunosuppression. A state of profound neutropenia was achieved three days after administration of the drug. Cyclophosphamide was re-administered every fourth day to maintain neutropenia. Initial dose LD90 experiments were performed in groups of seven mice. Animals were immunosuppressed with 200 mg kg^{-1} cyclophosphamide and inoculated with varying doses of *C. glabrata* ATCC 2001 (1×10^7, 7×10^7 and 2×10^8) using IV injection into the lateral tail vein of 0.15 ml. Mice were sacrificed when predetermined endpoints, as described in the project licence were reached, e.g. 20% weight loss, ascites where the burden exceeds 10% of

baseline bodyweight, a moribund state, lethargy or failure to respond to gentle stimuli. Infection of non-immunosuppressed mice with 2×10^8 *C. glabrata* ATCC 2001 cells resulted in 100% survival for the duration of the experiment (data not shown).

For the infection experiments groups of 15 mice were immunosuppressed with 200 mg kg^{-1} cyclophosphamide and then inoculated with blastospores of *C. glabrata* HLS112T (9.1×10^7) or HLS113 (8.8×10^7) using IV injection into the lateral tail vein. All dilutions were perfomed in saline and mice were inoculated with a volume of 200 µl. Mice were sacrificed when the previously described predetermined end-points, were reached. Survival curves were compared using Kaplan-Meier Log Rank analysis.

Acknowledgements

We would like to thank Al Brown, Kunio Kitada, Mike Lorenz and Dominique Sanglard for strains and reagents, Sabine Eckert for critical reading of the manuscript, Ken Wolfe and Derek Sullivan for helpful discussions. We are particularly grateful to Bernard Dujon and Christophe Hennequin for allowing us access to prepublication *C. glabrata* genome sequence data. Financial support was provided by the MRC (K.H., T.R.), BBSRC (K.H., T.R.), Fungal Research Trust (A.M.C.), CGD Research Trust (E.B., K.H., T.R.) and DFG (F.A.M.).

References

Bardwell, L., Cook, J.G., Inouye, C.J., and Thorner, J. (1994) Signal propagation and regulation in the mating pheromone response pathway of the yeast *Saccharomyces cerevisiae*. *Dev Biol* **166:** 363–379.

Beck-Sague, C., and Jarvis, W.R. (1993) Secular trends in the epidemiology of nosocomial fungal infections in the United States, 1980–90. National Nosocomial Infections Surveillance System. *J Infect Dis* **167:** 1247–1251.

Borneman, A.R., Hynes, M.J., and Andrianopoulos, A. (2001) An STE12 homolog from the asexual, dimorphic fungus *Penicillium marneffei* complements the defect in sexual development of an *Aspergillus nidulans steA* mutant. *Genetics* **157:** 1003–1014.

Calderone, R.A., and Fonzi, W.A. (2001) Virulence factors of *Candida albicans*. *Trends Microbiol* **9:** 327–335.

Chang, Y.C., Wickes, B.L., Miller, G.F., Penoyer, L.A., and Kwon-Chung, K.J. (2000) *Cryptococcus neoformans* STE12alpha regulates virulence but is not essential for mating. *J Exp Med* **191:** 871–882.

Chang, Y.C., Penoyer, L.A., and Kwon-Chung, K.J. (2001) The second STE12 homologue of *Cryptococcus neoformans* is MATa-specific and plays an important role in virulence. *Proc Natl Acad Sci USA* **98:** 3258–3263.

Cormack, B.P., Ghori, N., and Falkow, S. (1999) An adhesin of the yeast pathogen *Candida glabrata* mediating adherence to human epithelial cells. *Science* **285:** 578–582.

Csank, C., and Haynes, K. (2000) *Candida glabrata* displays pseudohyphal growth. *FEMS Microbiol Lett* **189:** 115–120.

De Bernardis, F., Muhlschlegel, F.A., Cassone, A., and Fonzi, W.A. (1998) The pH of the host niche controls gene expression in and virulence of *Candida albicans*. *Infect Immun* **66:** 3317–3325.

De Las Penas, A., Pan, S., and Cormack, B.P. (2002) A family of cell wall proteins implicated in virulence of the pathogenic yeast *Candida glabrata*. Tampa, Florida, USA: 6th American Society for Microbiology Conference on Candida and Candidiasis.

Gancedo, J.M. (2001) Control of pseudohyphae formation in *Saccharomyces cerevisiae*. *FEMS Microbiol Rev* **25:** 107–123.

Gumbo, T., Isada, C.M., Hall, G., Karafa, M.T., and Gordon, S.M. (1999) *Candida glabrata* fungemia. Clinical features of 139 patients. *Medicine (Baltimore)* **78:** 220–227.

Gustin, M.C., Albertyn, J., Alexander, M., and Davenport, K. (1998) MAP kinase pathways in the yeast Saccharomyces cerevisiae. *Microbiol Mol Biol Rev* **62:** 1264–1300.

Hanic-Joyce, P.J., and Joyce, P.B. (1998) A high copy number *ADE2*-bearing plasmid for transformation of *Candida glabrata*. *Gene* **211:** 395–400.

Haynes, K. (2001) Virulence in *Candida* species. *Trends Microbiol* **9:** 591–596.

Hull, C.M., Raisner, R.M., and Johnson, A.D. (2000) Evidence for mating of the 'asexual' yeast *Candida albicans* in a mammalian host. *Science* **289:** 307–310.

Kitada, K., Yamaguchi, E., and Arisawa, M. (1996) Isolation of a *Candida glabrata* centromere and its use in construction of plasmid vectors. *Gene* **175:** 105–108.

Kuo, M.H., Nadeau, E.T., and Grayhack, E.J. (1997) Multiple phosphorylated forms of the *Saccharomyces cerevisiae* Mcm1 protein include an isoform induced in response to high salt concentrations. *Mol Cell Biol* **17:** 819–832.

Liu, H. (2001) Transcriptional control of dimorphism in *Candida albicans*. *Curr Opin Microbiol* **4:** 728–735.

Liu, H., Kohler, J., and Fink, G.R. (1994) Suppression of hyphal formation in *Candida albicans* by mutation of a STE12 homolog. *Science* **266:** 1723–1726.

Lo, H.J., Kohler, J.R., DiDomenico, B., Loebenberg, D., Cacciapuoti, A., and Fink, G.R. (1997) Nonfilamentous *C. albicans* mutants are avirulent. *Cell* **90:** 939–949.

Madhani, H.D., and Fink, G.R. (1997) Combinatorial control required for the specificity of yeast MAPK signaling. *Science* **275:** 1314–1317.

Magee, B.B., and Magee, P.T. (2000) Induction of mating in *Candida albicans* by construction of MTLa and MTLalpha strains. *Science* **289:** 310–313.

Miller, M.G., and Johnson, A.D. (2002) White-opaque switching in *Candida albicans* is controlled by mating-type locus homeodomain proteins and allows efficient mating. *Cell* **110:** 293–302.

Mosch, H.U., and Fink, G.R. (1997) Dissection of filamentous growth by transposon mutagenesis in *Saccharomyces cerevisiae*. *Genetics* **145:** 671–684.

Nakayama, H., Izuta, M., Nagahashi, S., Sihta, E.Y., Sato, Y., Yamazaki, T., *et al.* (1998) A controllable gene-expression system for the pathogenic fungus *Candida glabrata*. *Microbiology* **144:** 2407–2415.

Navarro-Garcia, F., Sanchez, M., Nombela, C., and Pla, J. (2001) Virulence genes in the pathogenic yeast *Candida albicans*. *FEMS Microbiol Rev* **25:** 245–268.

Olson, K.A., Nelson, C., Tai, G., Hung, W., Yong, C., Astell, C., and Sadowski, I. (2000) Two regulators of Ste12p inhibit

pheromone-responsive transcription by separate mechanisms. *Mol Cell Biol* **20:** 4199–4209.

Park, G., Xue, C., Zheng, L., Lam, S., and Xu, J.R. (2002) MST12 regulates infectious growth but not appressorium formation in the rice blast fungus *Magnaporthe grisea*. *Mol Plant Microbe Interact* **15:** 183–192.

Pfaller, M.A., Diekema, D.J., Jones, R.N., Sader, H.S., Fluit, A.C., Hollis, R.J., and Messer, S.A. (2001) International surveillance of bloodstream infections due to *Candida* species: frequency of occurrence and *in vitro* susceptibilities to fluconazole, ravuconazole, and voriconazole of isolates collected from 1997 through 1999 in the SENTRY Antimicrobial Surveillance Program. *J Clin Microbiol* **39:** 3254–3259.

Pfaller, M.A., Jones, R.N., Doern, G.V., Sader, H.S., Messer, S.A., Houston, A., *et al.* (2000) Bloodstream infections due to *Candida* species: SENTRY antimicrobial surveillance program in North America and Latin America, 1997–98. *Antimicrob Agents Chemother* **44:** 747–751.

Pi, H., Chien, C.T., and Fields, S. (1997) Transcriptional activation upon pheromone stimulation mediated by a small domain of *Saccharomyces cerevisiae* Ste12p. *Mol Cell Biol* **17:** 6410–6418.

Quandt, K., Frech, K., Karas, H., Wingender, E., and Werner, T. (1995) MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. *Nucleic Acids Res* **23:** 4878–4884.

Ren, B., Robert, F., Wyrick, J.J., Aparicio, O., Jennings, E.G., Simon, I., *et al.* (2000) Genome-wide location and function of DNA binding proteins. *Science* **290:** 2306–2309.

Riggle, P.J., Andrutis, K.A., Chen, X., Tzipori, S.R., and Kumamoto, C.A. (1999) Invasive lesions containing filamentous forms produced by a *Candida albicans* mutant that is defective in filamentous growth in culture. *Infect Immun* **67:** 3649–3652.

Roberts, C.J., Nelson, B., Marton, M.J., Stoughton, R., Meyer, M.R., Bennett, H.A., *et al.* (2000) Signaling and circuitry of multiple MAPK pathways revealed by a matrix of global gene expression profiles. *Science* **287:** 873–880.

Singh, P., Ganesan, K., Malathi, K., Ghosh, D., and Datta, A. (1994) ACPR, a STE12 homologue from *Candida albicans*, is a strong inducer of pseudohyphae in Saccharomyces cerevisiae haploids and diploids. *Biochem Biophys Res Commun* **205:** 1079–1085.

Srikantha, T., Lachke, S.A., and Soll, D.R. (2003) Three mating type-like loci in *Candida glabrata*. *Euk Cell* **2:** 328–340.

Tsuji, G., Fujii, S., Tsuge, S., Shiraishi, T., and Kubo, Y. (2003) The *Colletotrichum lagenariu* Ste12-like gene CST1 is essential for appressorium penetration. *Mol Plant Microbe Interact* **16:** 315–325.

Vallim, M.A., Miller, K.Y., and Miller, B.L. (2000) Aspergillus SteA (sterile12-like) is a homeodomain-C2/H2-Zn+2 finger transcription factor required for sexual reproduction. *Mol Microbiol* **36:** 290–301.

Weig, M., Haynes, K., Rogers, T.R., Kurzai, O., Frosch, M., and Mühlschlegel, F.A. (2001) A GAS-like gene family in the pathogenic fungus *Candida glabrata*. *Microbiology* **147:** 2007–2019.

Wickes, B.L., Edman, U., and Edman, J.C. (1997) The Cryptococcus neoformans STE12alpha gene: a putative *Saccharomyces cerevisiae* STE12 homologue that is mating type specific. *Mol Microbiol* **26:** 951–960.

Wong, S., Fares, M.A., Zimmerman, W., Butler, G., and Wolfe, K.H. (2003) Evidence from comparative genomics for a complete sexual cycle in the 'asexual' pathogenic yeast *Candida glabrata*. *Genome Biol* **4:** R10.

Young, L.Y., Lorenz, M.C., and Heitman, J. (2000) A STE12 homolog is required for mating but dispensable for filamentation in *Candida lusitaniae*. *Genetics* **155:** 17–29.

Yuan, Y.L., and Fields, S. (1991) Properties of the DNA-binding domain of the *Saccharomyces cerevisiae* STE12 protein. *Mol Cell Biol* **11:** 5910–5918.

Yue, C., Cavallo, L.M., Alspaugh, J.A., Wang, P., Cox, G.M., Perfect, J.R., and Heitman, J. (1999) The STE12alpha homolog is required for haploid filamentation but largely dispensable for mating and virulence in *Cryptococcus neoformans*. *Genetics* **153:** 1601–1615.