Differential secretion of Sap4–6 proteins in *Candida albicans* during hyphae formation

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Secreted aspartyl proteinases (Saps) from *Candida albicans* are encoded by a multi-gene family and are considered to be putative virulence factors for candidiasis. *SAP4–6* mRNAs were first detected during hyphae formation and were assumed to play roles in the development of disseminated candidiasis. Recombinant Sap proteins (Sap2–6) were prepared and specific antibodies were generated against Sap2–6. The presence of Sap4, Sap5 and Sap6, but not Sap2 or Sap3, was demonstrated in culture supernatants of *C. albicans* after induction of hyphae formation. In parallel to hyphae formation, Sap5 (~40 kDa) was detected as early as ~6 h after induction at neutral pH, and Sap4/6 (~43 kDa) were detected after ~24 h when the culture medium became acidic. The differential secretion of Sap5 and Sap4/6 was affected when the culture medium pH was buffered at pH 6.5 or pH 4.5. In addition, intracellular pools of Sap4–6 seem to exist, and protein is not necessary for Sap4–6 induction. This study provides the first evidence that Sap4–6 proteins in *C. albicans* are differentially produced and secreted during hyphae formation.

Keywords: *Candida albicans*, secreted aspartyl proteinase, virulence factor, candidiasis

INTRODUCTION

Candida albicans is an important opportunistic human pathogen, and the number of infections caused by it increased dramatically in the last decade (Chen *et al.*, 1997; Edmond *et al.*, 1999; Edwards, 2000). *C. albicans* can colonize virtually all body sites. It causes superficial or invasive infection and carries high mortality and morbidity, especially in immunocompromised patients (Edwards, 2000; Hung *et al.*, 1996; Chen *et al.*, 2001). Because the modes of management of such patients are limited, with the increased incidence of *C. albicans* infections, the investigation of virulence factors and the search for a new target for development of anti-fungal agents and immunotherapy has become increasingly important.

C. albicans possesses a gene family encoding secreted aspartyl proteinases (Saps) with unusually broad substrate specificities (Cutler, 1991; Hube, 1996; Hube & Naglik, 2001). These enzymes have been linked to the virulence of the fungus since their discovery (Staib, 1965; Kwon-Chung *et al.*, 1985; Cassone *et al.*, 1987; De

Bernardis *et al.*, 1999). The proposed functions of these proteinases during infection include the digestion of host proteins for nutrient supply, evasion of host defences by degrading immunoglobulins and complement proteins, adherence, and degradation of host barriers during invasion (Hube, 1996). Although initially *C. albicans* was believed to express a single *SAP* gene, further studies have identified the existence of at least 9 closely related *SAP* genes (White *et al.*, 1993; Hube *et al.*, 1994; Monod *et al.*, 1994, 1998).

Differences in expression of various *SAP* mRNAs have been investigated *in vitro* and in experimental animal models (Morrow *et al.*, 1992; Hube *et al.*, 1994; White & Agabian, 1995; Borg-von Zepelin *et al.*, 1998; Monod *et al.*, 1998). Experimental infections with various mutants ($\Delta sap1$ to $\Delta sap6$) generated by targeted mutagenesis suggested the importance of different Saps in the virulence of *C. albicans* (Hube *et al.*, 1997; Sanglard *et al.*, 1997; De Bernardis *et al.*, 1999; Kretschmar *et al.*, 1999). These data suggest that the temporal and specific regulation of *SAP* expression might be important for the survival of *C. albicans* in its natural environment and thus in the pathogenesis of this fungus. Yet, because studies on the protein levels are limited, the special role of the individual Sap proteins in infection remains

Abbreviation: Sap, secreted aspartyl proteinase.

unclear (Cutfield *et al.*, 1995; Smolenski *et al.*, 1997; Hube & Naglik, 2001).

Among the various *SAP* gene products, deduced amino acid sequences for Sap4–6, which have 75–89% similarity to each other, form a group distinct from Sap1–3 (Hube *et al.*, 1994; Miyasaki *et al.*, 1994). This sub-family, Sap4–6, was a potential target for intervention because *SAP4–6* mRNAs were first identified during hyphae formation at neutral pH (Hube *et al.*, 1994). Blood and salivary pH are approximately neutral, and hyphae formation is important during tissue invasion (Edwards, 2000); therefore, Sap4–6 proteins were presumed to be important in disseminated candidiasis.

Recently, it has been demonstrated that a $\Delta sap4-6$ triple mutant resulted in attenuated mortality after systemic infection in guinea pigs and mice (Sanglard *et al.*, 1997), and that less tissue damage occurred in a murine peritonitis model (Kretschmar *et al.*, 1999). These results suggest that the *SAP4–6* genes contribute to the development of disseminated infections. Sap4–6 proteins have been detected in *Candida* within macrophages (Borg-von Zepelin *et al.*, 1998); however, production of the extracellular form of Sap4–6 during hyphae formation has never been characterized.

In this study, we generated specific antibodies against each of the Sap2–6 proteins and determined the secretion of Sap4–6 by *C. albicans* during hyphae formation. The effect of culture medium pH on secretion of Sap4–6 during hyphae formation was also examined. Here, we show that production and secretion of Sap4–6 proteins in *C. albicans* are differentially regulated during hyphae formation in a pH dependent manner.

METHODS

Yeast strains and growth conditions. The *C. albicans* wild-type strain SC5314 (Gillum *et al.*, 1984) and the *sap* null mutant strains $\Delta sap1$, $\Delta sap2$, $\Delta sap3$, $\Delta sap4$, $\Delta sap5$, $\Delta sap6$ and $\Delta sap4-6$ (Hube *et al.*, 1997; Sanglard *et al.*, 1997), and $\Delta efg1 \Delta cph1$ (Lo *et al.*, 1997) were used in this study (Table 1). *C. albicans* SC5314, a blood culture isolate from a patient with disseminated candidiasis, was used as the DNA template for cloning individual *SAP* genes and expression of His-tagged fusion proteins in *E. coli*. The following liquid media were used: YPD (1% yeast extract, 2% peptone, 2% glucose) complex medium for general use; YNB-BSA medium for induction of Sap2 protein [0.17%, w/v, yeast nitrogen base (Difco), 2% glucose, 0.2% BSA (Sigma), pH 5.0]; and modified Lee's medium (pH 4.5 and pH 6.5) for induction of Sap4–6 proteins (Lee *et al.*, 1975; Morrow *et al.*, 1992). BSA (Sigma) was added as indicated. *C. albicans* was grown in an orbital incubator at the indicated temperatures.

Cloning, expression and purification of Sap proteins. Plasmids were constructed according to standard protocols (Sambrook *et al.*, 1989). The following pairs of primers were used to amplify the ORFs of *C. albicans* *SAP2*, *SAP3*, *SAP4*, *SAP5*, *SAP6* genes: *SAP2.1* (5'-CCA TGG ATC AAA AAC TTA ATG TTA TTG TTG AT-3', nt 356–387) and *SAP2.2* (5'-TTC CAC CCC TTC ATC TTA GGT-3', nt 1335–1315); *SAP3.1* (5'-ATA ACA TAT GTT TTT AAA AAA TAT CTT T-3', nt 222–249) and *SAP3.2* (5'-ATC AGG ATC CAG AGG

AGT ACT TCC CCC T-3', nt 1452–1424); *SAP4.1* (5'-ACC ACA TAT GTT CTT ACA AAA TAT CTT GAG-3', nt 131–160) and *SAP4.2* (5'-ACA AGG ATC CTA AAA CCA ATG TCG TCT AAT-3', nt 1416–1387); *SAP5.1* (5'-CAT ATG TTC TTG AAA AAT ATC TTG AG-3', nt 265–290) and *SAP5.2* (5'-TTA ATC TAA AGT CAA AGT TCT TTA-3', nt 1545–1522); *SAP6.1* (5'-CAT ATG TTC TTG AAA AAT ATC TTG AG-3', nt 220–245) and *SAP6.2* (5'-ATC TAA AAC CAA AGT TTT CTA-3', nt 1497–1477). *SAP4.1N* (5'-CTT CCA TAT GAA TAA CGG TTC CTA TTC TCC-3', nt 533–562) and *SAP4.2* primers were used to amplify an N-terminally deleted fragment of the ORF (including the catalytic domain of the aspartyl proteinase) of the *SAP4* gene [*SAP4*(ΔN)]. The pT7Blue T-Vector Kit (Novagen) was used for cloning of PCR products. All PCR products were purified, subcloned and sequenced by the dideoxy chain termination method to confirm their identity (Sanger *et al.*, 1977). For the N-terminal His-Tag fusion proteins, the *Bam*HI–*Sal*I fragment of the pT7Blue T vector containing the *SAP2* ORF was ligated to the expression vector pET30a (Novagen) and the *Nde*I–*Bam*HI fragment of the pT7Blue T vector containing *SAP3–6* was ligated to pET15b (Novagen).

E. coli BL21(DE3) competent cells were transformed with expression plasmids and grown on LB plates containing ampicillin (100 μ g ml⁻¹). For identification of colonies with the highest levels of expression of fusion proteins, transformants were grown for 3 h, followed by induction with IPTG (final concentration 0.5 mM) for 3 h. The total lysates prepared from 1 ml samples of each transformant were first assessed by SDS-PAGE under reducing conditions, followed by Coomassie blue R-250 (Bio-Rad) staining. For large-scale expression, 5 ml overnight culture were used to inoculate 1 l LB broth containing ampicillin and protein expression was induced with 0.5 mM IPTG for 3 h after the OD₆₀₀ reached about 0.6. Cell pellets were suspended in 30 ml phosphate-buffered saline (pH 7.4) containing lysozyme (0.5 mg ml⁻¹) and disrupted by a nitrogen bomb. The lysate was centrifuged after the addition of 6 M urea and 1% Triton X-100, and His-tagged fusion protein was isolated on Ni²⁺-nitrilotriacetic acid resin (Qiagen) as described by the manufacturer. The protein concentration was determined with a BCA Protein Assay kit (Pierce). Protein extracts were analysed by SDS-PAGE followed by Coomassie brilliant blue R-250 staining or silver staining (Pharmacia).

Polyclonal antibody preparation and Western blot analysis.

The His-tagged fusion proteins [Sap2, Sap3, Sap4(ΔN), Sap5 and Sap6], purified from Ni²⁺-nitrilotriacetic acid resin, were further purified by SDS-PAGE separation because trace amounts of proteins of *E. coli* origin were contaminating the eluant samples. Denatured purified proteins were sliced out of the SDS-PAGE gel to be used as antigens to generate polyclonal antibodies in rabbits as described (Harlow & Lane, 1988; Huang *et al.*, 1999). In an attempt to generate Sap4-, Sap5- and Sap6-specific antibodies, the following peptides were designed and synthesized by Genemed Synthesis: Sap4C (AQVKYTSQ-SNIVGIN, aa 413–427), Sap4M (VSVRDQLFANVR, aa 179–190), Sap5N (GPVAVTLHNEAIT, aa 77–89), Sap6C (YTSESNIIVAIN, aa 408–418) and Sap6N (GPVAVKLDNEI-IT, aa 77–89) (Fig. 1). Peptides for Saps 4, 5 and 6 were conjugated to keyhole limpet haemocyanin (Pierce) as described by the manufacturer and used as antigens to generate antibodies. We used an anti-His monoclonal antibody (Clontech; diluted 1:5000) to detect purified His-tagged fusion Sap proteins. For immunoblot analysis, antibodies were diluted in phosphate-buffered saline (pH 7.4) containing 0.1% Tween 20 and 5% dried skimmed milk. Sample proteins were separated

Table 1. *C. albicans* strains used in this study

Strain type and no.	Genotype	Reference
Clinical isolate SC5314	URA3/URA3	Gillum <i>et al.</i> (1984)
<i>sap1</i> (M5/BH24-15-1)	<i>ura3::imm434/ura3::imm434</i> <i>sap1::hisG/sap1::hisG::URA3::hisG</i>	Hube <i>et al.</i> (1997)
<i>sap2</i> (M12/BH52-1-17)	<i>ura3::imm434/ura3::imm434</i> <i>sap2::hisG/sap2::hisG::URA3::hisG</i>	Hube <i>et al.</i> (1997)
<i>sap3</i> (M21/BH1-1-1)	<i>ura3::imm434/ura3::imm434</i> <i>sap3::hisG/sap3::hisG::URA3::hisG</i>	Hube <i>et al.</i> (1997)
<i>sap4</i> (DSY436)	<i>ura3::imm434/ura3::imm434</i> <i>sap4::hisG/sap4::hisG::URA3::hisG</i>	Sanglard <i>et al.</i> (1997)
<i>sap5</i> (DSY452)	<i>ura3::imm434/ura3::imm434</i> <i>sap5::hisG/sap5::hisG::URA3::hisG</i>	Sanglard <i>et al.</i> (1997)
<i>sap6</i> (DSY346)	<i>ura3::imm434/ura3::imm434</i> <i>sap6::hisG/sap6::hisG::URA3::hisG</i>	Sanglard <i>et al.</i> (1997)
<i>sap4–6</i> (DSY459)	<i>ura3::imm434/ura3::imm434</i> <i>sap6::hisG/sap6::hisG sap4::hisG/</i> <i>sap4::hisG sap5::hisG/</i> <i>sap5::hisG::URA3::hisG</i>	Sanglard <i>et al.</i> (1997)
<i>efg1 cph1</i>	<i>ura3::imm434/ura3::imm434 efg1::hisG/</i> <i>efg1::hisG cph1::hisG/</i> <i>cph1::hisG::URA3::hisG</i>	Lo <i>et al.</i> (1997)

Fig. 1. Diagrams of recombinant Sap2–6 proteins and synthetic peptides used to generate antibodies. The deduced amino acid sequences of Sap2–6 include a signal sequence (black box), a propeptide (grey box) and a mature protein (white box). GenBank accession numbers of the SAP genes are M83663 (SAP2), L22358 (SAP3), L25388 (SAP4), Z30191 (SAP5) and Z30192 (SAP6). The positions of the amino acids of recombinant proteins and synthetic peptides are numbered as indicated.

by SDS-PAGE, transferred onto Immobilon-P membrane (Millipore) and incubated with the primary specific antibodies at room temperature for 60 min, followed by horseradish

peroxidase-conjugated goat anti-rabbit IgG (diluted 1:5000). Bound antibodies were detected with the ECL system (Amersham) according to the manufacturer's instructions.

Table 2. Sensitivity and specificity of polyclonal antibodies against recombinant fusion proteins and peptides determined by immunoblotting

Recombinant proteins (10, 20, 50, 100 and 200 ng) were separated by SDS-PAGE and dilutions (as indicated) of antiserum were used for determining the relative sensitivity and specificity of each antiserum. A fixed Western blot protocol was followed, including the dilution of secondary antibody and exposure time. Experiments reported here were repeated at least twice and in most cases additional times, with essentially the same results. Data presented are the detection of specific amounts of recombinant proteins by ECL as described in Methods. The dilution of primary antibody is indicated in parentheses. Data represented are the minimal amount of recombinant proteins necessary to be detected by a distinct dilution of antibodies.

Antibody	Sap2	Sap3	Sap4(Δ N)	Sap5	Sap6
Anti-Sap2 protein	10 ng (1:7500)	100 ng (1:5000)	100 ng (1:5000)	100 ng (1:5000)	100 ng (1:5000)
Anti-Sap3 protein	100 ng (1:2000)	20 ng (1:2000)	100 ng (1:2000)	—	—
Anti-Sap4(Δ N) protein	100 ng (1:5000)	100 ng (1:5000)	10 ng (1:5000)	100 ng (1:2000)	100 ng (1:2000)
Anti-Sap4C peptide	—	—	100 ng (1:500)	200 ng (1:500)	200 ng (1:500)
Anti-Sap4M peptide	—	—	50 ng (1:2000)	—	—
Anti-Sap5 protein	—	—	100 ng (1:1000)	10 ng (1:1000)	100 ng (1:1000)
Anti-Sap5N peptide	—	—	—	10 ng (1:2000)	—
Anti-Sap6 protein	—	—	10 ng (1:2000)	10 ng (1:2000)	10 ng (1:2000)
Anti-Sap6N peptide	—	—	—	—	100 ng (1:1000)
Anti-Sap6C peptide	—	—	50 ng (1:1000)	50 ng (1:1000)	50 ng (1:1000)

Fig. 2. Specificity of antibodies against recombinant Sap proteins. (A) ~100 ng of the indicated recombinant Sap were subjected to SDS-PAGE in 12.5% gels and visualized by silver staining. Positions of protein standards (kDa) are shown on the left. Proteins were transferred to Immobilon-P and reacted with anti-Sap2 (B), anti-Sap3 (C), anti-Sap4(Δ N) (D), anti-Sap4M (E), anti-Sap5N (F), anti-Sap6 (G) or anti-Sap6N antibodies (H), followed by detection with the ECL system. The asterisk in (A) marks contaminant protein of *E. coli* origin, which was co-purified from the Ni²⁺-nitrilotriacetic acid resin.

Fig. 3. Identification of Sap4–6 proteins in *C. albicans* after 24 h induction of hyphae formation. Wild-type strain SC5314 and *sap* mutants of *C. albicans* were grown in modified Lee's medium (pH 4.5) for 48 h at 25 °C. Cells were then transferred to modified Lee's medium (pH 6.5) containing 0.2% BSA at 37 °C to induce hyphae formation. Supernatants (30 µl) were collected from individual cell cultures (2 ml) at 24 h, subjected to SDS-PAGE in 12.5% gels, and proteins visualized by Coomassie brilliant blue R-250 staining (A). Proteins were transferred to Immobilon-P and reacted with anti-Sap5N (B), anti-Sap4M (C), anti-Sap6 (D), anti-Sap2 (E), and anti-Sap3 (F) antibodies, followed by detection with the ECL system. Positions of protein standards (kDa) are on the left.

Induction of hyphae formation. For hyphae growth, *C. albicans* were incubated in modified Lee's medium (pH 4.5) (Lee *et al.*, 1975; Morrow *et al.*, 1992) at 25 °C for 48 h

(stationary phase), transferred to pre-warmed modified Lee's medium (pH 6.5) containing 0.2% BSA (final cell density approximately 5×10^6 cells ml⁻¹), then incubated at 37 °C for 48–72 h. Growth was measured at OD₆₀₀ and the pH values of the culture media were determined at the indicated times. Hyphae formation was visualized by phase-contrast microscopy and percentage of hyphae formation was calculated by counting of ~200 cells. For kinetic study, proteins prepared from culture media obtained from wild-type and mutant strains before and after hyphae induction were evaluated. To determine the expression of the intracellular Sap4–6, we prepared *Candida* total lysates by use of liquid nitrogen, followed by addition of lysis buffer as previously described (Lee *et al.*, 1997).

RESULTS

Specificity and sensitivity of polyclonal antibodies against Sap proteins

His-tagged fusion proteins of full-length Sap2, Sap3, Sap5 and Sap6 were expressed in *E. coli* and purified as described in Methods. N-terminally deleted Sap4, Sap4(ΔN), was cloned and expressed because *E. coli* transformants carrying full-length Sap4 grew very poorly (data not shown). Purified recombinant His-tagged Sap proteins and synthetic peptides for specific Saps (Fig. 1) were used to immunize rabbits and produce specific polyclonal antibodies.

Table 2 shows that most antibodies can detect ~10 ng of their specific recombinant Sap antigens; however, antibodies against synthetic peptides were less sensitive. Fig. 2 shows that antibodies against Sap2 recombinant protein, Sap4M peptide, Sap5N peptide and Sap6N peptide reacted specifically with their own recombinant protein. Antibodies against recombinant Sap3, Sap6 and an N-terminally deleted fragment of Sap4 protein [Sap4(ΔN)] proteins, as well as Sap4C and Sap6C peptides, had significant cross-reactivity with other members of the Sap subfamily, highlighting the difficulty of generating specific and sensitive antibodies against each Sap.

Differential secretion of Sap4–6 proteins during hyphae formation

Proteins prepared from culture supernatants of wild-type strain SC5314 and *sap* mutants, induced for hyphae formation, were used to characterize the specificity of these antibodies and to study the differential expression of Sap4–6 proteins in *C. albicans* (Fig. 3). A single band (~40 kDa) was detected by anti-Sap5N in the wild-type strain, and in all strains except those lacking the *SAP5* gene (Fig. 3B). No immunoreactivity was detected after incubation of anti-Sap5N antibodies, which were pre-depleted with purified recombinant Sap5 (data not shown). We inferred that the ~40 kDa band was Sap5 protein. In addition to the 40 kDa band, a second band (~43 kDa) was identified by anti-Sap4M in *Δsap6* strains (Fig. 3C). Furthermore, the upper band was denser in the *Δsap6* mutant than that in the *Δsap4* mutant or the wild-type strain, where we used anti-Sap4M or anti-Sap6 antibodies (Fig. 3C, D). We inferred

that the ~43 kDa band was Sap4 protein (in the *Δsap6*) or Sap6 protein (in the *Δsap4* mutant), or a mixture of both proteins. There was no detectable Sap2 or Sap3 under the same conditions (Fig. 3E, F). Anti-Sap6N and anti-Sap4M were specific for their own recombinant proteins; however, the sensitivity of these antibodies is not good enough for detection of secreted Sap4–6 in culture supernatants (data not shown). Although anti-Sap6 cross-reacted with Sap4 and Sap5 (Fig. 3D, *Δsap6* mutant), its high sensitivity was chosen for detecting Sap4–6 in the following studies.

To determine the differential expression of Sap4–6 in the induction of early hyphae formation, we took supernatants from wild-type strain and *sap* mutant cultures at the indicated times, and the immunoreactivity of Sap4–6 was detected by use of anti-Sap6 antibody (Fig. 4). Hyphae formed within 4 h of induction in wild-type strain, *Δsap4*, *Δsap5* and *Δsap6*, and the representative (wild-type) hyphae formation is shown in Fig. 4C. The percentages of hyphae formation in wild-type and mutants (*Δsap4*, *Δsap5*, *Δsap6* and *Δsap4–6*) showed no significant difference, and >95% of cells formed hyphae within 6 h of induction (Fig. 4D). Sap5 (lower band in Fig. 4B) was first detected in supernatant from the wild-type culture after ~8 h of induction, whereas it was detected after ~4 h induction from the *Δsap4* and *Δsap6* mutants (Fig. 4B). However, Sap4/6 (upper band) was faintly detected after 24 h induction in the *Δsap4* and *Δsap6* mutants. Figs 3 and 4 together demonstrate that the upper band is Sap4 in the *Δsap6* mutant, Sap6 in the *Δsap4* mutant or a mixture of both in the wild-type strain. The denser bands in the *Δsap6* mutant may represent up-regulation of Sap4 and Sap5. Sap4–6 proteins were also detected during hyphae formation induced by temperature/pH shift in modified Lee's medium without BSA (Buffo *et al.*, 1984) (data not shown).

To determine whether Sap4/6 protein was produced in the intracellular form, but was not secreted into the culture medium after 24 h hyphae induction, we took cell lysates from wild-type strain and *sap* mutant cultures at 24 and 48 h, and the immunoreactivity of Sap4–6 was detected (Fig. 5). The small GTPase Arf1p was used as internal control in this study (Lee *et al.*, 1997). Sap5 was clearly detected in all cell lysates except in *Δsap5*; however, Sap4/6 was hardly detected in the wild-type and *sap* mutants after 24 h induction (Fig. 5). Consistent with the result shown in Fig. 4, Sap4 could clearly be detected in both cell lysate and culture supernatant after 48 h induction in the *Δsap6* mutant.

Effects of medium pH on the expression of Sap4–6 proteins

Hyphae formation was induced in modified Lee's medium at neutral pH, and the pH of the medium fell gradually to 3.0–4.0 after 18–24 h (Fig. 6A, wild-type), which was approximately the time that Sap4/6 was detected in the medium (Fig. 6C). Under the same growth conditions, expression of Sap5 was detected at

~4 h (*Δsap4* or *Δsap6* mutant) or ~8 h (in wild-type) (Fig. 4B), and reached a higher level after ~48 h of hyphae induction (Fig. 6C). The drop in the medium pH was also observed in the *Δsap4*, *Δsap5* and *Δsap6* cultures. To investigate whether a pH change in the culture medium could affect the expression of Sap4–6, different *C. albicans* strains were first grown in modified Lee's medium (pH 4.5) at 25 °C for 48 h, then transferred to buffered modified Lee's medium (pH 6.5) containing 0.2% BSA at 37 °C for induction of hyphae formation. The pH of the culture medium was buffered with sodium citrate to pH 6.5 or pH 4.5, at a final concentration of 50 mM. Hyphae formation of all tested strains was visualized by use of phase-contrast microscopy, and representative cells with hyphae formation at the indicated times are shown in Fig. 7. In the unbuffered control and buffered pH 6.5 cultures, hyphae formation was observed at ~2 h induction, approached ~90% after 3–4 h (Fig. 7), and >95% at 6–48 h (data not shown). Compared to the expression in the unbuffered control culture, the expression of Sap5 was the same or only slightly lower in a buffered pH 6.5 medium in the wild-type and in the *Δsap4* and *Δsap6* mutants. The upper band was detected as early as ~12 h in the wild-type and the *Δsap4* and *Δsap5* mutants, but was hardly detected even at 48 h in the *Δsap6* mutant (Fig. 6C). The results suggested that the upper band detected at pH 6.5 at 12 h was Sap6. In the buffered pH 4.5 culture, most hyphae were formed as pseudohyphae at ~2 h induction and approached ~80% after ~6 h induction (Fig. 7). Interestingly, Sap5 was detected at significant levels only in *Δsap6*, and Sap4/6 could still be detected at low levels in the wild-type and *Δsap4* mutant in buffered pH 4.5 cultures (Fig. 6C).

Effects of Saps on the degradation of BSA during hyphae formation

Comparing the residual amount of BSA in culture medium at different time intervals (Fig. 6B), degradation of BSA was noticed after ~48 h induction in the *Δsap6* mutant and was correlated with the detection of higher expression of Sap4 in the culture supernatants. In buffered medium, no degradation of BSA was observed after 48 h induction. Despite the relatively stable level of expression of Sap5 in the *Δsap6* mutant during 12–48 h (with or without control of the medium pH), there was no significant degradation of BSA as shown on SDS-PAGE, suggesting that other Saps, but not Sap5, might be involved in the degradation of BSA. To determine further whether specific Saps play a role in the degradation of BSA during hyphae formation, wild-type strain SC5314, *Δsap1*, *Δsap2*, *Δsap3*, *Δsap4*, *Δsap5*, *Δsap6*, *Δsap4–6* and *Δefg1 Δcph1* (defective for hyphae formation; Lo *et al.*, 1997) mutants were grown and hyphae formation was induced. Fig. 8 shows that, compared with the *Δsap6* mutant, degradation of BSA was also evident, to a lesser extent, in the wild-type and *Δsap4–6* mutants after ~48 h induction. Interestingly, there were no detectable levels of Sap4–6 proteins after induction in the *Δefg1 Δcph1* double mutant.

Fig. 4. Differential secretion of Sap4–6 proteins during hyphae formation. Wild-type strain SC5314 and *sap* mutants of *C. albicans* were grown in modified Lee's medium (pH 4.5) for 48 h at 25 °C. Cells were then transferred to modified Lee's medium (pH 6.5) with BSA at 37 °C to induce hyphae formation. Supernatants (30 µl) were collected from individual cell cultures (2 ml) at the indicated times, subjected to SDS-PAGE in 12.5% gels and proteins visualized by Coomassie brilliant blue R-250 staining (A). Proteins were transferred to Immobilon-P and reacted with anti-Sap6 antibody (B), followed by detection with the ECL system. Hyphae formation in wild-type strain was visualized by use of phase-contrast microscopy at the indicated times (C). The percentages of hyphae formation in wild-type (◆), Δsap4 (■), Δsap5 (▲), Δsap6 (○) and Δsap4–6 (□) mutants were determined at the indicated times (D).

Fig. 5. Production of intracellular Sap4–6 proteins during hyphae formation. Cell lysates (A) and supernatants (B) were prepared from wild-type strain and *sap* mutant cultures after 24 h or 48 h hyphae induction as described in Methods. Sap4–6 was reacted with specific antibodies as indicated, followed by detection with the ECL system. The small GTPase Arf1p was used as the internal control.

DISCUSSION

Sap4–6 production and characterization

The present study was the first to demonstrate detectable levels of Sap5 (~40 kDa) and Sap4/6 (~43 kDa) in culture supernatants of *C. albicans* during hyphae formation in Lee's medium. Our data agree with the previous finding that the relative molecular mass of Sap5, expressed and secreted in the *Pichia pastoris* system, is lower than those of Sap4 and Sap6 (Borg-von Zepelin *et al.*, 1998). The difference in the relative molecular mass of Sap4–6 might be due to potential glycosylation sites in Sap4 and Sap6, but not Sap5.

We attempted to generate antibodies with higher sensitivity and specificity against Sap4–6; however, only anti-Sap4M, anti-Sap5N and anti-Sap6N have characteristics that can specifically detect their recombinant forms. Borg-von Zepelin *et al.* (1998), using antibodies generated against recombinant Sap proteinases prepared in the *P. pastoris* system, also failed to distinguish Sap4 and Sap6. Although recombinant Sap1–6 proteins prepared from the *P. pastoris* (Borg-von Zepelin *et al.*, 1998) and the *Escherichia coli* expression systems (Koelsch *et al.*, 2000) have been purified and charac-

Fig. 6. The pH of the culture medium affects expression of Sap4–6 proteins. Wild-type strain SC5314 and the $\Delta sap4$, $\Delta sap5$ and $\Delta sap6$ mutants were grown in modified Lee's medium (pH 4.5) at 25 °C for 48 h. Cells were then transferred to modified Lee's medium (pH 6.5) with 0.2% BSA, with or without sodium citrate buffer [pH 6.5 (○) or 4.5 (▽)], at 37 °C to induce hyphae formation. ●, Control. The pH of the culture media was measured at the indicated times (A). Supernatants (30 μ l) were collected from individual cell cultures (2 ml) at the indicated times, subjected to SDS-PAGE in 12.5% gels and proteins visualized by Coomassie brilliant blue R-250 staining (representative $\Delta sap6$ in B). Proteins were transferred to Immobilon-P and reacted with anti-Sap6 (C), followed by detection with the ECL system.

terized, only 'native' secreted Sap2p from the culture supernatant of *C. albicans* has been purified and characterized.

A differential expression of individual *SAP* mRNAs has been shown *in vitro* and in animal models (Morrow *et al.*, 1992; Hube *et al.*, 1994; White & Agabian, 1995; Borg-von Zepelin *et al.*, 1998; Monod *et al.*, 1998). The temporal and specific regulation of *SAP* gene expression might be important for the survival of *C. albicans* in its natural environment and thus in the pathogenesis of this fungus. However, it has been shown recently that the correlation between mRNA and protein levels was insufficient to predict protein expression levels from quantitative mRNA data (Gygi *et al.*, 1999). For some genes, where the mRNA levels were the same, the protein levels varied by more than 20-fold. However, invariant steady-state levels of certain proteins were observed with respective mRNA transcript levels that

varied by as much as 30-fold. Because studies on the secreted form of Sap protein levels are limited, the special role of the individual Sap proteins in infection remains unclear (Cutfield *et al.*, 1995; Smolenski *et al.*, 1997; Hube & Naglik, 2001).

Differential secretion of Sap5 and Sap4/6

Our differential expression studies showed that Sap5 was produced and secreted earlier than Sap4/6 during hyphae formation (Fig. 4). Although it seems to be impossible to compare an *in vitro* expression study using defined induction medium and a study with cells from infected tissue, our findings agree with a recent study of *in vivo* expression of *SAP* genes in animal models, which showed that *SAP5* was the first *SAP* gene induced after intraperitoneal infection or haematogenous dissemination (Staib *et al.*, 2000). *SAP5* expression at this stage of the infection did not correlate with the presence of

Fig. 7. The pH of the culture medium affects hyphae formation. Wild-type strain SC5314 and the $\Delta sap4$, $\Delta sap5$ and $\Delta sap6$ mutants were grown and hyphae formation induced as described in Fig. 6. Hyphae formation was visualized by use of phase-contrast microscopy at the indicated times. Hyphae and pseudohyphae formation (%) of wild-type strain SC5314 is shown in (A). ♦, Control; ■, pH 6.5; ▲, pH 4.5. After 6 h hyphae induction, phase-contrast images of hyphae and pseudohyphae formation of wild-type and $\Delta sap5$ strains at different culture pH are shown in panel B as indicated. Parts of (B) are composite images.

germ tubes or hyphae, and *SAP6* gene activation was detected only when *C. albicans* hyphae also were observed in the infected tissue (Staib *et al.*, 2000). Furthermore, in the $\Delta efg1 \Delta cph1$ double mutant, which is an avirulent mutant locked in the yeast form (Lo *et al.*, 1997), there were no detectable levels of Sap4–6 proteins after induction. These data suggest that the expression of Sap5 may be induced by host signals that also result in hyphae formation. However, our data indicated that hyphae formation can be induced in wild-type, $\Delta sap4$, $\Delta sap5$ and $\Delta sap6$ mutants, and Sap4–6 proteins were hardly detected even at 48 h in the $\Delta sap5$ mutant, suggesting that production of Sap5 as well as Sap4/6 was not required for hyphae formation (Figs 6C and 7B). *SAP4–6* mRNAs were first detected during serum-induced hyphal induction (Hube *et al.*, 1994; White & Agabian, 1995). Our data also showed that Sap4–6 proteins could not be detected in the culture supernatants until hyphae formation occurred and Sap4–6

could not be detected in the $\Delta efg1 \Delta cph1$ double mutant, suggesting that hyphae formation might be essential for Sap4–6 expression. With regard to the time of maximal Sap production during hyphal induction and the existence of intracellular pools of Sap4–6, our findings were consistent with previous studies (Hube *et al.*, 1994; White & Agabian, 1995) and suggested that maximal Sap protein production can be later than the maximal mRNA level.

In the $\Delta efg1 \Delta cph1$ double mutant, which is defective for hyphae formation (Lo *et al.*, 1997), there were no detectable levels of Sap4–6 proteins after induction (Fig. 8). However, a recent study demonstrated that wild-type expression levels of Efg1 are not sufficient for regular hyphae development, expression of *SAP4–6* genes and evasion from macrophage in the absence of CaTec1, a TEA/ATTS transcription factor in *C. albicans* (Schweizer *et al.*, 2000). Besides, the *catec1* null mutant

Fig. 8. Effect of Saps on the degradation of BSA during hyphae formation. Wild-type strain SC5314 and the $\Delta sap1$, $\Delta sap2$, $\Delta sap3$, $\Delta sap4$, $\Delta sap5$, $\Delta sap6$, $\Delta sap4-6$ and $\Delta efg1 \Delta cph1$ mutants were grown and hyphae formation was induced as described in Fig. 6. (A) Supernatants (30 μ l) were collected from individual cell cultures (2 ml) at the indicated times, subjected to SDS-PAGE in 12.5% gels and proteins visualized by Coomassie brilliant blue R-250 staining. (B) Proteins were transferred to Immobilon-P and reacted with anti-Sap6, followed by detection with the ECL system.

is almost avirulent in a systemic model of candidiasis, despite the formation of hyphae. It seems that Sap4–6 proteins are the downstream effector of these either coordinately regulated or independent signalling pathways (Schweizer *et al.*, 2000; Felk *et al.*, 2002).

White & Agabian (1995) showed that, of the three *SAP4–6* genes, expression of *SAP6* mRNA is the highest during hyphae induction at neutral pH. However, our data indicated that production of Sap5 was highest during hyphae induction at pH 6.5. Interestingly, up-regulation of Sap4/6 occurred in the $\Delta sap5$ mutant at pH 6.5 (Fig. 6C, pH 6.5 panel). In the buffered pH 6.5 culture, secretion of Sap4/6 in wild-type, $\Delta sap4$ and $\Delta sap5$ mutants is earlier than that in unbuffered culture. Moreover, Sap5 was detectable in the $\Delta sap6$ mutant at a low level in buffered pH 4.5 medium (Fig. 6). The culture pH affected not only the expression of Sap4–6 proteins, but also the induction of hyphae and pseudo-hyphae formation. De Bernardis *et al.* (1998) suggested that gene expression and virulence of *C. albicans* are controlled by the pH of the host microniche. Our data also suggest that the low pH values influence hyphae formation and consequently may change the expression pattern of *SAP4–6* genes. Therefore, changes in the pH may affect the expression pattern only indirectly.

It was shown that there was no *SAP4* mRNA when *SAP6* mRNA was first detected in culture medium in *in vitro* cell cultures, or in animal models (White & Agabian, 1995; Schaller *et al.*, 1998, 1999; Staib *et al.*, 2000), except after haematogenous dissemination to the kid-

neys (Staib *et al.*, 2000). Although differential expression of Sap4/6 can be detected when the medium pH is unbuffered or buffered at 6.5 in the wild-type strain and in *sap* mutants (Fig. 5C), it is difficult to determine which is expressed first. Until specific antibodies against Sap4 or Sap6 are generated, all of these findings can provide only indirect evidence that Sap6 and Sap4 might not be expressed under the same conditions, and/or play a different role in *C. albicans*. A combination of Sap4/6-specific antibodies and specific mRNA detection may also provide evidence as to which Sap is expressed under which conditions.

BSA degradation in Lee's medium

Fig. 6(B, C) shows that expression of Sap4/6 was associated with significant degradation of BSA at 48 h hyphae induction, when the culture reached pH 4.0. In the same growth culture, Sap2 and Sap3 were not detected. Our result agrees with that of Hube *et al.* (1994), who previously reported that the *SAP2* gene was not expressed during serum-induced hyphae formation. BSA or serum is an important component in the induction of Sap2 (Homma *et al.*, 1993; White & Agabian, 1995) and *SAP4–6* expression (Hube *et al.*, 1994). This may be important for *Candida* to survive in hosts because it would enable the yeast to use host proteins as a nitrogen source. In addition, *Candida* expresses virulence factor(s) to overcome the host immune defence after exposure to intravascular component(s) (Hube & Naglik, 2001). In this study, during

hyphae formation the medium pH fell gradually, and there was no degradation of BSA at 24 h induction, despite the presence of a significant amount of Sap5. Although expression of Sap4 was observed when BSA was degraded in the culture medium at ~48 h of induction (Fig. 6B, C), there was still significant degradation of BSA in $\Delta sap4-6$ (Fig. 8), suggesting that other Saps, but not Sap4–6, might be involved in the degradation of BSA. Another scenario is that Sap4–6 are the main enzymes involved in BSA degradation during hyphae formation, but that other Sap enzymes are up-regulated in the $\Delta sap4-6$ mutant and have a compensatory function.

Conclusion

Many members of the SAP gene family have been identified. This raises the question whether they exist in redundancy or play a special role under different environmental or physiological conditions (Hube *et al.*, 1997; Sanglard *et al.*, 1997; De Bernardis *et al.*, 1999; Kretschmar *et al.*, 1999; Naglik *et al.*, 1999; Staib *et al.*, 2000; Felk *et al.*, 2002). The present study was the first to demonstrate clearly that secretion of Sap5 in *C. albicans* was induced during hyphae formation, followed by secretion of Sap4/6 under acidic conditions, which occurred in parallel to the degradation of BSA. These results are consistent with the *in vivo* expression patterns (Staib *et al.*, 2000) and suggest that Sap5 might play a key role in the initial phase of *Candida* infection.

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REFERENCES

- Borg-von Zepelin, M., Beggah, S., Boggian, K., Sanglard, D. & Monod, M. (1998). The expression of the secreted aspartyl proteinases Sap4 to Sap6 from *Candida albicans* in murine macrophages. *Mol Microbiol* **28**, 543–554.
- Buffo, J., Herman, M. A. & Soll, D. R. (1984). A characterization of pH-regulated dimorphism in *Candida albicans*. *Mycopathologia* **85**, 21–30.
- Cassone, A., De Bernardis, F., Mondello, F., Ceddia, T. & Agatensi, L. (1987). Evidence for a correlation between proteinase secretion and vulvovaginal candidosis. *J Infect Dis* **156**, 777–783.
- Chen, Y. C., Chang, S. C., Sun, C. C., Yang, L. S., Hsieh, W. C. & Luh, K. T. (1997). Secular trends in the epidemiology of nosocomial fungal infections at a teaching hospital in Taiwan, 1981 to 1993. *Infect Control Hosp Epidemiol* **18**, 369–375.
- Chen, Y. C., Lin, S. F., Liu, C. J., Jiang, D. D. S., Yang, P. C. & Chang, S. C. (2001). Risk factors of ICU mortality in critically ill patients. *J Formos Med Assoc* **100**, 656–661.
- Cutler, J. E. (1991). Putative virulence factors of *Candida albicans*. *Annu Rev Microbiol* **45**, 187–218.
- Cutfield, S. M., Dodson, E. J., Anderson, B. F., Moody, P. C. E., Marshall, C. J., Sullivan, P. A. & Cutfield, J. F. (1995). The crystal structure of a major secreted aspartic proteinase from *Candida albicans* in complexes with two inhibitors. *Structure* **3**, 1261–1271.
- De Bernardis, F., Muhlschlegel, F. A., Cassone, A. & 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 Bernardis, F., Arancia, S., Morelli, L., Hube, B., Sanglard, D., Schafer, W. & Cassone, A. (1999). Evidence that members of the secretory aspartyl proteinase gene family, in particular SAP2, are virulence factors for *Candida vaginitis*. *J Infect Dis* **179**, 201–208.
- Edmond, M. B., Wallace, S. E., McClish, D. K., Pfaller, M. A., Jones, R. N. & Wenzel, R. P. (1999). Nosocomial bloodstream infections in United States hospitals: a three-year analysis. *Clin Infect Dis* **29**, 239–244.
- Edwards, J. E., Jr (2000). *Candida* species. In *Mandell, Douglas and Bennett's Principles and Practice of Infectious Diseases*, 5th edn, p. 2656–2673. Edited by G. L. Mandell, J. E. Bennett and R. Dolin. New York and London: Churchill Livingstone.
- Felk, A., Kretschmar, M., Albrecht, A. & 7 other authors (2002). *Candida albicans* hyphal formation and the expression of the Efg1-regulated proteinases Sap4 to Sap6 are required for the invasion of parenchymal organs. *Infect Immun* **70**, 3689–3700.
- Gillum, A. M., Tsay, E. Y. & Kirsch, D. R. (1984). Isolation of the *Candida albicans* gene for orotidine-5'-phosphate decarboxylase by complementation of *S. cerevisiae* *ura3* and *E. coli* *pyrF* mutations. *Mol Gen Genet* **198**, 179–182.
- Gygi, S. P., Rochon, Y., Franza, B. R. & Aebersold, R. (1999). Correlation between protein and mRNA abundance in yeast. *Mol Cell Biol* **19**, 1720–1730.
- Harlow, E. & Lane, D. (1988). *Antibodies: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Homma, M., Chibana, H. & Tanaka, K. (1993). Induction of extracellular proteinase in *Candida albicans*. *J Gen Microbiol* **139**, 1187–1193.
- Huang, C.-F., Buu, L.-M., Yu, W.-L. & Lee, F.-J. S. (1999). Characterization of a novel ADP-ribosylation factor-like protein (yARL3) from *Saccharomyces cerevisiae*. *J Biol Chem* **274**, 3819–3827.
- Hube, B. (1996). *Candida albicans* secreted aspartyl proteinases. *Curr Top Med Mycol* **7**, 55–69.
- Hube, B. & Naglik, J. (2001). *Candida albicans* proteinases: resolving the mystery of a gene family. *Microbiology* **147**, 1997–2005.
- Hube, B., Monod, M., Schofield, D. A., Brown, A. J. & Gow, N. A. (1994). Expression of seven members of the gene family encoding secretory aspartyl proteinases in *Candida albicans*. *Mol Microbiol* **14**, 87–99.
- Hube, B., Sanglard, D., Odds, F. C., Hess, D., Monod, M., Schafer, W., Brown, A. J. & Gow, N. A. (1997). Disruption of each of the secreted aspartyl proteinase genes SAP1, SAP2, and SAP3 of *Candida albicans* attenuates virulence. *Infect Immun* **65**, 3529–3538.
- Hung, C. C., Chen, Y. C., Chang, S. C., Luh, K. T. & Hsieh, W. C. (1996). Nosocomial candidemia in a university hospital in Taiwan. *J Formos Med Assoc* **95**, 19–28.
- Koelsch, G., Tang, J., Loy, J. A., Monod, M., Jackson, K., Foundling, S. I. & Lin, X. (2000). Enzymic characteristics of secreted aspartic proteases of *Candida albicans*. *Biochim Biophys Acta* **1480**, 117–131.

- Kretschmar, M., Hube, B., Bertsch, T., Sanglard, D., Merker, R., Schroder, M., Hof, H. & Nichterlein, T. (1999). Germ tubes and proteinase activity contribute to virulence of *Candida albicans* in murine peritonitis. *Infect Immun* **67**, 6637–6642.
- Kwon-Chung, K. J., Lehman, D., Good, C. & Magee, P. T. (1985). Genetic evidence for role of extracellular proteinase in virulence of *Candida albicans*. *Infect Immun* **49**, 571–575.
- Lee, K. L., Buckley, H. R. & Campbell, C. C. (1975). An amino acid liquid synthetic medium for the development of mycelial and yeast forms of *Candida albicans*. *Sabouraudia* **13**, 148–153.
- Lee, F.-J. S., Huang, C.-F., Yu, W.-L., Buu, L.-M., Lin, C.-Y., Huang, M.-C., Moss, J. & Vaughan, M. (1997). Characterization of an ADP-ribosylation factor-like 1 protein in *Saccharomyces cerevisiae*. *J Biol Chem* **272**, 30998–31005.
- Lo, H. J., Kohler, J. R., DiDomenico, B., Loeberberg, D., Cacciapuoti, A. & Fink, G. R. (1997). Nonfilamentous *C. albicans* mutants are avirulent. *Cell* **90**, 939–949.
- Miyasaki, S. H., White, T. C. & Agabian, N. (1994). A fourth secreted aspartyl proteinase gene (*SAP4*) and a *CARE2* repetitive element are located upstream of the *SAP1* gene in *Candida albicans*. *J Bacteriol* **176**, 1702–1710.
- Monod, M., Togni, G., Hube, B. & Sanglard, D. (1994). Multiplicity of genes encoding secreted aspartic proteinases in *Candida* species. *Mol Microbiol* **13**, 357–368.
- Monod, M., Hube, B., Hess, D. & Sanglard, D. (1998). Differential regulation of *SAP8* and *SAP9*, which encode two new members of the secreted aspartic proteinase family in *Candida albicans*. *Microbiology* **144**, 2731–2737.
- Morrow, B., Srikantha, T. & Soll, D. R. (1992). Transcription of the gene for a pepsinogen, *PEP1*, is regulated by white-opaque switching in *Candida albicans*. *Mol Cell Biol* **12**, 2997–3005.
- Naglik, J. R., Newport, G., White, T. C., Fernandes-Naglik, L. L., Greenspan, J. S., Greenspan, D., Sweet, S. P., Challacombe, S. J. & Agabian, N. (1999). In vivo analysis of secreted aspartyl proteinase expression in human oral candidiasis. *Infect Immun* **67**, 2482–2490.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**, 5463–5467.
- Sanglard, D., Hube, B., Monod, M., Odds, F. C. & Gow, N. A. (1997). A triple deletion of the secreted aspartyl proteinase genes *SAP4*, *SAP5*, and *SAP6* of *Candida albicans* causes attenuated virulence. *Infect Immun* **65**, 3539–3546.
- Schaller, M., Schafer, W., Korting, H. C. & Hube, B. (1998). Differential expression of secreted aspartyl proteinases in a model of human oral candidosis and in patient samples from the oral cavity. *Mol Microbiol* **29**, 605–615.
- Schaller, M., Korting, H. C., Schafer, W., Bastert, J., Chen, W. & Hube, B. (1999). Secreted aspartic proteinase (Sap) activity contributes to tissue damage in a model of human oral candidosis. *Mol Microbiol* **34**, 169–180.
- Schweizer, A., Rupp, S., Taylor, B. N., Rollinghoff, M. & Schroppel, K. (2000). The TEA/ATTS transcription factor CaTec1p regulates hyphal development and virulence in *Candida albicans*. *Mol Microbiol* **38**, 435–445.
- Smolenski, G., Sullivan, P. A., Cutfield, S. M. & Cutfield, J. F. (1997). Analysis of secreted aspartic proteinases from *Candida albicans*: purification and characterization of individual Sap1, Sap2 and Sap3 isoenzymes. *Microbiology* **143**, 349–356.
- Staib, F. (1965). Serum-proteins as nitrogen source for yeastlike fungi. *Sabouraudia* **4**, 187–193.
- Staib, P., Kretschmar, M., Nichterlein, T., Kohler, G. & Morschhauser, J. (2000). Expression of virulence genes in *Candida albicans*. *Adv Exp Med Biol* **485**, 167–176.
- White, T. C. & Agabian, N. (1995). *Candida albicans* secreted aspartyl proteinases: isoenzyme pattern is determined by cell type, and levels are determined by environmental factors. *J Bacteriol* **177**, 5215–5221.
- White, T. C., Miyasaki, S. H. & Agabian, N. (1993). Three distinct secreted aspartyl proteinases in *Candida albicans*. *J Bacteriol* **175**, 6126–6133.

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