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Quantitative Expression of *Candida albicans* Aspartyl Proteinase Genes *SAP7*, *SAP8*, *SAP9*, *SAP10* in Human Serum *in vitro*

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Abstract

The different members of the secreted aspartyl proteinase (Sap) family of the human pathogenic yeast *Candida albicans* are proposed to play different roles during infection and are differentially expressed at various body sites. In recent reports, expression analysis has focused on the genes *SAP1-6*, while the expression pattern of *SAP7-10* was less well studied. We analyzed the *SAP7-SAP10* expression profile of *C. albicans* under human serum influence that may be elucidated in the course of blood infection in humans and how this *in vitro* expression profile is associated with hyphal formation. The phenotypes of strains were examined under scanning electron microscopy. Quantitative RT-PCR ($2^{-\Delta\Delta C}{}_{T}$) was used to monitor *SAP* expression of *C. albicans* wild type cells and mutants lacking *SAP9* and/or *SAP10*. Of the four analyzed *SAP* genes, only *SAP7* was detectably induced in the double mutant and in the wild type strains in the model that mimics bloodstream infections. On the other hand, in the wild types (isolate 83 and CAF2-1), *SAP7* was expressed 0.8- or 0.4-fold less than *SAP10*, respectively. Our findings suggest that Sap7 may respond to the challenge of the human blood environment. Furthermore, the results support the notion that compensatory upregulation of *SAP7* and *SAP8* in the *Δsap9*/*Δsap10* mutant occurs in these conditions. *SAP7-10* expression was strain-specific. Our findings point to a link between morphogenesis and expression of *SAP9* in serum, where these conditions induce both hyphae and *SAP9*, but temporal gene expression patterns might be controlled by other factors.

Key words: *Candida albicans*, aspartic proteinases expression, morphogenesis, blood human serum

Introduction

Candida albicans possesses a number of virulence attributes and among them widely studied are aspartyl proteinases (Saps) that can digest epithelial cell surface components and thereby provide an entrance into or between host cells (Dalle *et al.*, 2010). Although plenty of data dealing with the Saps involved in the infection process have been extensively studied (Naglik *et al.*, 2008; Rehaume *et al.*, 2008; Schelenz, 2008) the comparative analysis of Saps expression in morphologies induced under human serum influence at human body temperature has not been described in depth. Given the role of *SAP7*, *SAP8* and *SAP9-10* in the growth of *C. albicans* in serum (Lermann and Morschhäuser, 2008), we asked whether those isoenzymes are expressed in *C. albicans* hyphal forms induced under human serum influence *in vitro*. In addition, we analyzed whether any differences exist in the expression level between these subfamilies. In contrast to all other members of the Sap family, the Sap9-10 proteases monitored under *in vitro* and *in vivo* conditions are independent of pH and morphotype (Hornbach *et al.*, 2009; Schild *et al.*, 2011). Furthermore, according to Schild *et al.* (2011) it seems apparent that the host associated lifestyle of *C. albicans* requires regulatory proteolytic digestion at different niches with different pH values. Moreover, the latter authors demonstrated that Sap9 and Sap10 influence on cell wall functions by proteolytic cleavage of cell wall proteins which are significant for morphogenesis (Schild *et al.*, 2011). Thus, in the current study *SAP9* and *SAP10* expression analysis was undertaken in hyphal forms under serum influence at pH 7.1–7.4. An earlier study (Naglik *et al.*, 2003) showed that *SAP7* was found to be induced in response to infection of reconstituted human vaginal epithelium model as well as in isolates from patients. Furthermore, Taylor *et al.* (2005) observed that induction of *SAP7* correlates with virulence in an intravenous infection model of candidiasis in mice. Many differences in the

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SAP gene expression profiles observed in various studies remain to be explained and more thoroughly characterized. Therefore, the aim of the study was to determine the relative levels of *SAP7*, *SAP8*, *SAP9* and *SAP10* transcripts in *C. albicans* strains *in vitro* at neutral pH at 18 h-growth in human blood serum (an artificial model of bloodstream infections). That may provide evidence for a role of these proteinases in *C. albicans* survive and escape of disseminated infection. Moreover, we determined whether any correlation might exist between overexpression of the *SAP7-10* genes and true hyphae formation.

Experimental

Materials and Methods

Strains and growth conditions. The *Candida* strains used in this study are listed in Table I. The stock culture of examined strains was stored on ceramic beads (Microbank™, Pro-Lab Diagnostics, Canada) at –70°C. Routine culturing of strains for growth in YEPD medium as well as for morphogenesis development in human serum was conducted as described previously (Staniszewska *et al.*, 2012). The usefulness of fresh human serum in order to mimic blood serum infections and to investigate the gene expression pattern of *C. albicans* during such infections incubated under semi-aerobic conditions at 37°C was described by Fradin *et al.* (2003; 2005).

Quantitative RT-PCR. Total RNA was isolated from cells according to the protocol by Amberg *et al.* (2005). RNA concentrations were determined by measuring absorbance at 260 nm (Nano Drop 2000, Thermo SCIENTIFIC, USA). First-strand cDNAs were synthesized from total RNA, using two-step qRT-PCR analysis (Sigma, USA) and following the recommendations of the manufacturer. Real-time RT-PCR was used to determine the quantitative levels of *SAP7-10* mRNA transcripts in RNA samples using Rotor Gene 6000 (RCorbett, Qiagen, Germany). Quantitative reverse transcription-PCR assay was performed following the protocol described previously by Naglik *et al.* (2008) using QuantiTect Probe PCR Kit (Qiagen, Germany) according to the manufacturer's instructions. TaqMan primer and probe (5' FAM, 3' TAMRA) sets were used as described previously Naglik *et al.* (2008). The $2^{-\Delta\Delta C}{}_{T}$ method was used to analyze the relative changes in gene expression from quantitative RT-PCR experiment (Livak and Schmittgen, 2001). The C_T values were provided from RT-PCR instrumentation and were imported into a spreadsheet Microsoft Excel 2010. The data were analyzed using Eq. (Livak and Schmittgen, 2001), where ΔC_T = Avg. sap C_T – Avg. β-actin C_T and $\Delta\Delta C_T = \Delta CT - \Delta CT$ parental strain.

Scanning electron microscopy study. To examine cell morphology, samples were prepared as described previously by Oliviera *et al.* (2010). Briefly, morphotypes of each strain were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 18 h at a low temperature (5–6°C). Then, the samples were carefully washed with 0.1 M phosphate buffer (pH 7.2). Post-fixation was carried out for 2 h at room temperature with 2% osmium tetroxide. Initial dehydration was accomplished by placing specimens in the following series of ethanol gradients: 50% and 70% (two times for 10 min), 95% (two times for 5 min) and 100% (two times for 1 min), respectively. Then, samples were dehydrated with acetone (two times for 30 sec.) until dried by the critical point method in liquid CO_2. Subsequently, the specimens of the wild type strain were coated with gold in a vacuum evaporator and examined with a scanning electron microscope (SEM Quanta-200, FEI, Czech Republic). In the case of the mutant strains, after post-fixation morphotypes were dehydrated two times for 10 min with graded ethanol (50% and 75%) followed by dehydration with acetone (two times for 30 sec). The dried specimens were coated with osmium tetroxide and observation was done under scanning electron microscope Hitachi S-5500 (Japan). Images were assembled with Photoshop (Adobe Photoshop CS3 Extended). Cell dimensions were determined by using bars.

Table I
Strains used in the study

Strain	Parent	Genotype	Reference
SC5314		Wild strain	Gillum *et al.* (1984)
83		Clinical isolate	Staniszewska (2009)
CAI4[pCIp10]	SC5314	*Δura3::imm434/Δura3::imm434* + pCIp10 (Integration)	Fonzi and Irwin (1993)
CAF2-1	SC5314	*Δura3::imm434/URA3*	Fonzi and Irwin (1993)
Δ*sap9*	CAI4[pCIp10]	*Δsap9::hisG/Δsap9::hisG* + pCIp10 (Integration)	Schild *et al.* (2011)
Δ*sap9/10*	CAI4[pCIp10]	*Δsap10::hisG/Δsap10::hisG Δsap9::hisG/Δsap9::hisG* + pCIp10 (Integration)	Schild *et al.* (2011)
Δ*sap10*	CAI4[pCIp10]	*Δsap10::hisG/Δsap10::hisG* + pCIp10 (Integration)	Schild *et al.* (2011)

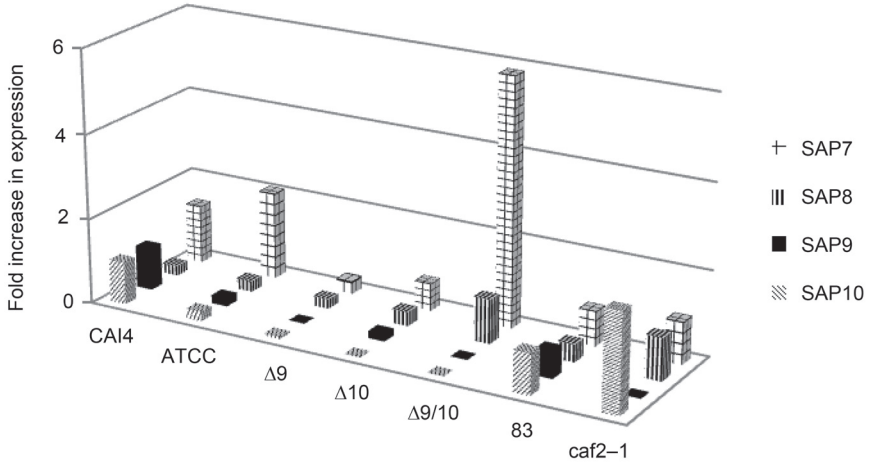

Fig. 1. Expression of the *SAP* genes.

The expression of *SAP7*, *SAP8*, *SAP9* and *SAP10* was quantified by qRT-PCR from cells in stationary growth phase in human serum at 37°C. *ACT1* transcript was used as internal control and the multifold increase over the value of the wild type at 37°C is shown for each strain

Results

Quality control and quantitative expression of *SAP7-SAP10* genes in human blood serum. *SAP7*, *SAP8*, *SAP9-10* and *ACT1* TaqMan primer/probe sets were calibrated and they demonstrated similar efficiency in titration experiments using *C. albicans* SC5314 genomic DNA (9.7–52.3 ng) in serial $\log_{10}$ dilutions (data not shown). The expression levels of *SAP7-SAP10* genes were monitored after a 18 h period after inoculation of human serum with the *C. albicans* strains (Fig. 1). All *SAP* genes tested were detected in human serum, albeit at very low levels, except *SAP7*, which was expressed at relatively high levels compared to the remaining genes tested (Table II). Contrariwise, the wild type strain 83 and CAF2-1 showed slightly enhanced *SAP10* expression (at least 1.2- and 2.3-fold) compared to *SAP7* respectively. We therefore examined the expression of *SAP7-10* genes under serum influence by comparing the expression profile among particular mutants. Considering *SAP7*, in the double mutant *Δsap9*/*Δsap10* the expression level of *SAP7* was significantly higher (20-fold compared to *Δsap9* or 8.4-fold compared to *Δsap10*) and so was that of *SAP8* (5.0- or 3.3-fold for *Δsap9* and *Δsap10* respectively). As a result of the comparison of the expression pattern of wild type strains it was shown that in the reference strain SC5314 *SAP7* was expressed 1.5-fold compared to CAI4. Contrariwise, the wild type CAI4 displayed around 1.6-fold higher expression of *SAP9* than strain 83. We demonstrated that CAF2-1 has a 2.3-fold change of *SAP10* gene expression compared with either CAI4 or strain 83. Contrariwise, the wild type SC5314 showed 5-fold lower expression (compared to strain 83 and CAI4) and 11.5-fold lower expression than CAF2-1. Comparison of the expression patterns of the protease mutants and the wild types demonstrated high diversity. The expression levels of *SAP7*, *SAP8* and *SAP10* in the wild type CAF2-1 were around 3.3- and 5.0- and 46-fold higher than in mutant the *Δsap9* respectively. Moreover, in the mutant *Δsap9 SAP10* was underexpressed 20-fold *vs* the parental strain CAI4. In view of *SAP10,* the mutant *Δsap9* displayed expression from 4.0- to 21-fold lower than the wild types. Similarly, deletion of *SAP9* or *SAP10* appeared to have a minor influence on expression of either *SAP8* or *SAP9* (comparable to the wild types). Thus, *Δsap10* as well as SC5314 displayed comparable expression of *SAP9* ($2^{\Delta\Delta C}{}_{T} = 0.3$ or 0.2 respectively).

When comparing the three genes (*SAP7 vs SAP8 vs SAP9*) in the mutant *Δsap10* at 18 h-growth in human

Table II
Fold mRNA expression of *SAP7* to *SAP10* relative to *ACT1* and normalized over the value of the wild type strains' expression

Gene	Fold mRNA expression[a] in *C. albicans* strains						
	CAI4	SC5314	*Δsap9*	*Δsap10*	*Δsap9*/*Δsap10*	83	CAF2-1
SAP7	1.4	2.1	0.3	0.7	5.9	0.8	1.0[b]
SAP8	0.2	0.3	0.2	0.3	1.0	0.4	1.0[b]
SAP9	1.0[b]	0.2	ND	0.2	ND	0.6	ND
SAP10	1.0[b]	0.2	0.05	ND	ND	1.0	2.3

[a] – Values are average of triplicate readings; [b] – Parental strain; ND – not determined

serum, one can see a clear trend in underexpression of these genes compared to the wild types, with the exception of *SAP8* upregulation (1.5-fold) compared to CAI4. Furthermore, *SAP7* and *SAP8* were expressed 1.4-fold and 3.3-fold higher in the wild type CAF2-1 compared to *Δsap10* respectively. Likewise, in the parental strain CAI4 *SAP9* displayed 5.0-fold overexpression *vs Δsap10*. The double mutant *Δsap9*/*Δsap10* showed an increased expression of *SAP7* (2.8-fold compared to the wild type SC5314). Furthermore, Fig. 1. and Table II showed that *SAP7* was clearly the most highly expressed gene at around 5.9-fold (with reference to CAF2-1) in the *Δsap9*/*Δsap10* mutant. Consequently, *SAP8* displayed a significant overexpression in the *Δsap9*/*Δsap10* mutant compared to the wild types SC5314 and CAI4 (in the range 3.3 and 5.0-fold, respectively). While in the double mutant *Δsap9*/*Δsap10* the expression level of *SAP8* was comparable to CAF2-1.

Hypha-forming capability in *Candida albicans* strains. In order to characterize the phenotype of *Δsap*, we examined the ability to undergo the dimorphic transition. For this purpose, cells were grown in liquid undiluted human serum and incubated at 37°C, cell morphology was then observed microscopically after 18-h incubation. To test this possibility, we examined which genes could prevent from the morphogenesis defect of *Δsap9*/*rsap10* in human serum. As shown in Fig. 2. the mutants *Δsap9* and *Δsap9*/*Δsap10* exhibited decreased ability to filament compared to the wild type cells. Although, the latter mutants are able to form filaments, they fail to filament abundantly in these preferable conditions probably because the absence of *SAP9* represses the dimorphic transition in *C. albicans*. Contrariwise, the Δ*sap10* mutant formed true hyphae when grown under these conditions as revealed by microscopic examination. The deletion of genes *SAP8* (data not shown) and *SAP10* had no effect on morphogenesis process.

Discussion

Our results demonstrated that from the tested *SAP7* to *SAP10*, only *SAP7* mRNA level is significantly altered at the later stage of bloodstream infections in different genetic backgrounds (the wild types and the *Δsap* mutants). Comparing the expression of *SAP9* and *SAP10*, our assay suggested that the single mutants lacking either of these proteins do not show a significant expression increase of the other. We found that the expression levels of *SAP7-10* were downregulated in all the single mutants compared with the wild types. One possible explanation for this observation may be that the single mutants have a reduced ability to survive in blood serum. Contrariwise, the double mutant *Δsap9*/*Δsap10* displayed overexpression of *SAP7* and *SAP8* compared to the wild types. These results support the notion that compensatory upregulation of *SAP7* and *SAP8* in the *Δsap9*/*Δsap10* mutant occurred. However, the mechanism underlying how and when *SAP9* and *SAP10* regulate other genes as well as each other remains to be elucidated. Interestingly, lack of Sap10 had only a minor influence on *SAP9* expression (4-fold higher) compared to the expression level of *SAP10* in *Δsap9*. Recently, Schild *et al.* (2011) showed that although Sap9 and Sap10 exhibit near neutral pH optimum of proteolytic activity they only partially substitute each other in these conditions. In our study a lower expression of *SAP10* may be related to a more limited number of potential substrates (Schild *et al.*, 2011).

In the study, the mutants lacking these proteins showed dissimilar phenotypes of *Δsap9* or *Δsap10*, such as altered morphogenesis under human serum influence. Moreover, our data supported the results by Naglik *et al.* (2004) that Sap9 is probably a key proteinase that does not only promote cell wall integrity, general cell growth and fitness at mucosal surface, but as our data implied is dependent on the morphology under human serum influence. Thus, our study supports the findings of Schild *et al.,* (2011) and in view of our data it is highly probable that Sap9 is involved in polarized growth and morphogenesis in *C. albicans* under human serum influence at pH 7.1–7.4. Previously, quantification of cellular forms of the wild type strain (Staniszewska *et al*., 2011 and 2012) showed that incubation in human serum at 37°C for 18 h resulted in transformation of almost 100% of all the observed cells to pure true hyphal forms. However, our present data demonstrated that deletion of *SAP9* substantially reduces true hyphae formation compared with the wild type strains *i.e.*, strain 83, SC5314 as well as *Δsap10* under these conditions (Fig. 2). Thus, *SAP9* expression may be linked to hyphal morphology even if the pleomorphism is regulated by other factors. Moreover, it was shown that incubation in undiluted serum at human body temperature did not prevent the defective hyphal growth of the mutants *Δsap9* and *Δsap9*/*Δsap10* (Fig. 2), indicating that Sap9 may be involved in the loss of cell polarity in these mutants under hyphae-forming conditions. Moreover, we showed that *SAP7* and *SAP8* expression could not prevent the morphogenesis defect of either *Δsap9* or *Δsap9*/*Δsap10* in undiluted human serum and expression of these genes does not induce hyphae formation. Finally, our data imply that Sap7, Sap8 and Sap10 play a minor role in morphogenesis and these genes do not compensate the *SAP9* disruption in hyphae formation.

Studies investigating the production of *SAP* mRNA (Hube *et al.,* 1994; Taylor *et al.,* 2005) and the virulence of *SAP* knockout strains (Lermann and Morschhäuser, 2008) showed that induction of *SAP* expression is a niche-specific phenomenon. Therefore it was necessary in our study to discover which of the tested protein-

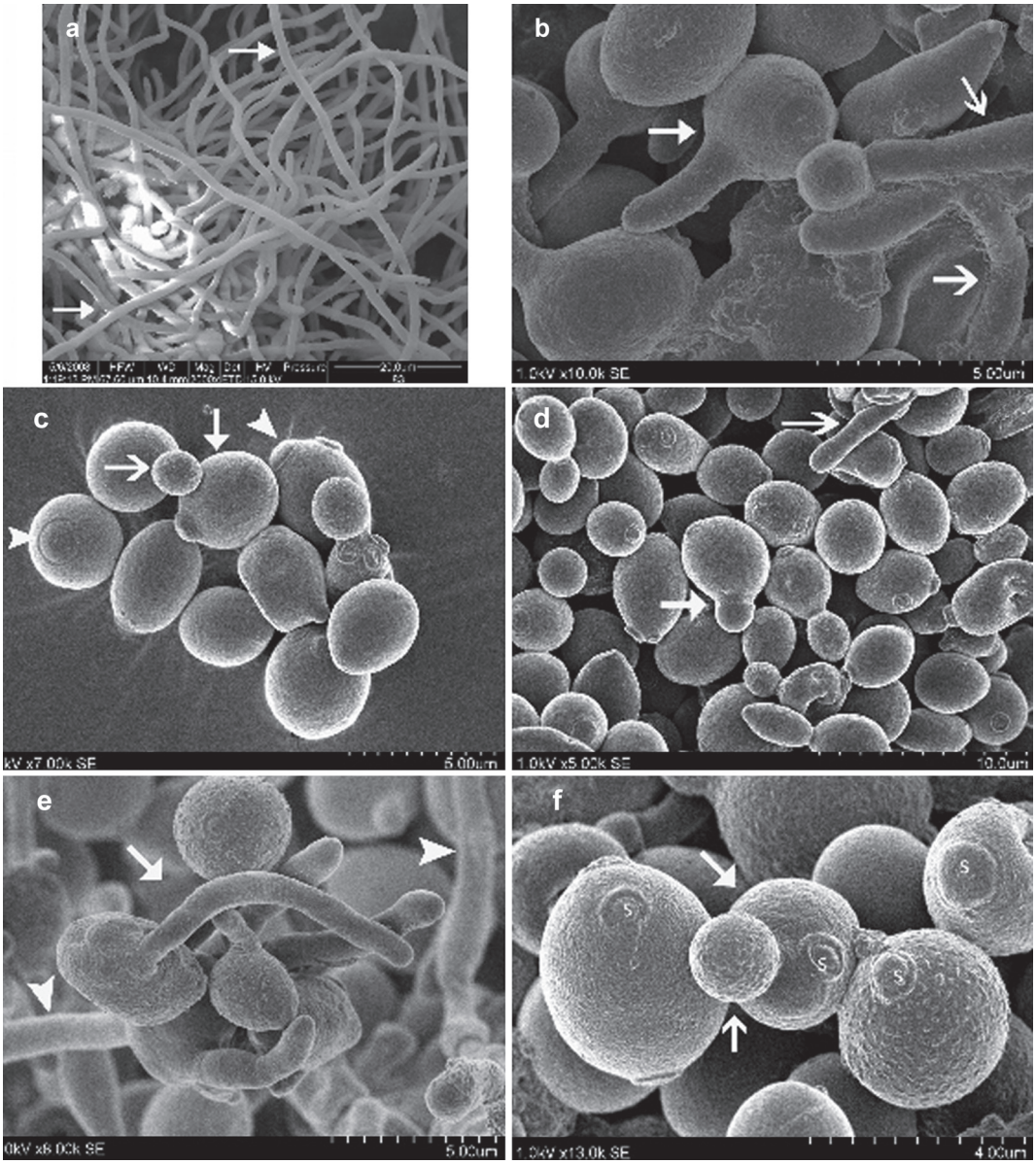

Fig. 2. Characteristics of *Candida albicans* strains' phenotypes grown in undiluted human serum at 37°C for 18 h under static conditions in plastic Eppendorf vials.

(A) Micrograph showing true hyphal phenotype of the *C. albicans* clinical strain 83. Note the dominant filament morphotypes (arrows). (B) Clumps of morphological forms of strain SC5314 can be seen. Note the germinating blastoconidial cell, indicated by an arrow and true hyphal morphologies (open arrows). (C, D) The *Δsap9* mutant exhibits budding phenotype and failure of hyphal development. Fine details of blastoconidia are discernible. (C) Oval blastoconidial mother cell (arrow) with a bud (open arrow) can be seen. Scars (arrowheads) localized polarly are observed. (D) Blastoconidial cells morphology and cellular budding (arrow) typical of strain the *Δsap9* are visible. Mutant causes filamentous growth only in a small percentage of yeast cells. Note singular hyphal morphology (open arrow). (E) The *Δsap10* mutant. Clumps of germinating blastoconidia can be seen (arrow). Hyphal induction has occurred, note long true hyphal cells indicated by arrow heads. (F) The *Δsap9*/*Δsap10* mutant phenotype. Yeast cells defective in cellular filamentation process are observed. Fine details of blastoconidia are discernible. Oval blastoconidial cell (arrow) and a new bud (open arrow) can be seen. Scars (s) localized polarly are observed.

ases are expressed at higher level during artificial blood infection. We observed that human serum favours *SAP7* expression. One reason for this unexpected result may be found in the fact that *SAP7* plays a role in blood dissemination followed by interaction with human endothelial cells. These results demonstrated that *SAP7* is required for the growth of *C. albicans* clinical isolates in human serum as the sole nitrogen source and Sap7 is normally expressed in the wild type strains and over-expressed when the remaining genes are expressed at a very low level. It was likely in our study that when *C. albicans* cells have to adapt and survive within the blood environment they upregulate *SAP7* expression, while other genes important for growth and viability in serum are downregulated (Fradin *et al.*, 2003; Hube *et al.*, 1994). We therefore concluded that there is little

correlation between the expression of the remaining *SAP* genes tested and the role of Sap proteins during blood infection. Furthermore, our findings were in agreement with the view that protein production is reduced in the later stage of blood infections (Fradin *et al.*, 2003). Thus, in our opinion future experiments using patients' blood samples should bring us closer to identifying the *SAP* genes and proteins that are directly involved in candidaemia in humans. Furthermore, a gradual observation of *SAP7* expression during blood infection ought to be conducted which may show whether *SAP7* is induced as a consequence of infection. Taylor *et al.,* (2005) utilizing the *Δsap7* mutant showed a role of proteinase Sap7 in the intravenous model of systemic candidiasis in mice which was in agreement with our *in vitro* blood infection model in humans. Furthermore, our study was a successful attempt in finding conditions which induce *SAP7 in vitro* while it was discussed (Taylor *et al.*, 2005) that an *in vitro* induction of *SAP7* mRNA level has not yet been detected. In conclusion, these data provided the first *in vitro* evidence of *SAP7-10* expression in *C. albicans* strains under human serum influence. In this paper, due to the strong induction of *SAP7*, we hypothesize that it is essential for *C. albicans* survival and help the cells to escape from bloodstream. Thus, *SAP7* may help the fungus to cause systemic infections. We also report *SAP9* expression in serum, which was associated with hyphal invasive growth at the site of infection.

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