Expression of SAP5 and SAP9 in Candida albicans biofilms: comparison of bloodstream isolates with isolates from other sources

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Secreted aspartic proteases (Sap), encoded by a family of 10 SAP genes, are key virulence determinants in Candida albicans. Although biofilm-associated bloodstream infections (BSIs) are frequently caused by C. albicans, SAP gene expression in C. albicans biofilms formed by BSI isolates has not been evaluated. We compared the expression of two SAP genes, SAP5 and SAP9, in C. albicans biofilms formed by BSI isolates with those formed by isolates from other body sites. Sixty-three C. albicans isolates were analyzed, comprising 35 BSI isolates and 28 from other sites. A denture-strip biofilm model was used, and expression of the two SAP genes was quantified by real-time RT-PCR during planktonic or biofilm growth. Mean SAP5 expression levels of the BSI isolates were 3.59-fold and 3.86-fold higher in 24-h and 48-h biofilms, respectively, than in planktonic cells. These results did not differ from those for isolates from other sites (2.71-fold and 2.8-fold for 24-h and 48-h biofilms, respectively). By contrast, mean SAP9 expression during biofilm formation was higher in BSI isolates (2.89-fold and 3.29-fold at 24 and 48 h, respectively) than in isolates from other sites (1.27-fold and 1.32-fold at 24 and 48 h, respectively; both, P < 0.001). These results show, for the first time, that both SAP5 and SAP9 are upregulated in C. albicans biofilms formed by BSI isolates, and that BSI isolates may have a greater capacity to express SAP9 under biofilm conditions than isolates from other sites.

Keywords Candida albicans, SAP genes, biofilm, bloodstream infections

Introduction

Candida albicans is a commensal organism in healthy individuals, but often causes life-threatening bloodstream infections (BSIs) in hospitalized patients [1]. Recent investigations have shown that C. albicans BSIs are frequently associated with biofilm formation on vascular catheters [2,3]. C. albicans is the third leading cause of intravascular catheter-related BSIs, with the second highest catheter colonization-to-infection rate [3]. Biofilm-associated BSIs are inherently difficult to resolve and may require both long-term antifungal therapy and the removal of catheters to control the infection [2]. Although C. albicans biofilms are an escalating clinical problem associated with significant rates of mortality, the pathogenesis of biofilm-related C. albicans BSIs is complex and incompletely understood.

Secreted aspartic proteases (Saps), encoded by a family of 10 SAP genes (SAP1–SAP10) have long been recognized as a virulence-associated trait of C. albicans [1,4]. The main function of Saps is to degrade proteins, but they also play a role in cell-cell adhesion [1]. Mendes et al. showed that C. albicans biofilms secrete more Saps than do planktonic cells [5], while Ramage et al. showed that an in vitro C. albicans biofilm induced Sap activity, and that SAP8 expression within the biofilm...
correlated with in vivo denture stomatitis severity [6]. Therefore, it is possible that Saps play a role in biofilm-associated BSIIs caused by C. albicans, and that different SAP genes may be differentially expressed in BSI isolates under biofilm conditions, which may contribute to biofilm-associated BSIIs. However, to date, there is a lack of substantial research on quantitative expression of SAP genes in C. albicans biofilms formed by BSI isolates.

Of the 10 SAP genes, SAP4–6 are predominantly expressed in hyphae [1] and hyphae are the predominant forms in biofilm growth in the in vivo model [7]. On the other hand, SAP9- and SAP10-encoded proteins maintain cell surface integrity by processing cell wall proteins, which mediate biofilm formation [8,9]. C. albicans forms biofilms on abiotic and biotic surfaces such as the oral and vaginal mucosas [4, 10]. Naglik has shown that SAP5 and SAP9 are the major SAPs expressed in vivo in mucosal biofilms [4]. In this study, we examined the expression of these two SAP genes, SAP5 and SAP9, in in vitro biofilms formed on abiotic surfaces by C. albicans BSI isolates. Expression of the two SAP genes was determined during the intermediate (24 h) and mature (48 h) phases of biofilm development by real-time polymerase chain reaction (PCR), and the results were compared with those for clinical isolates from other body sites.

Materials and methods
C. albicans isolates

A total of 63 C. albicans isolates were analyzed, which included 35 BSI isolates and 28 clinical isolates from other body sites. The latter were obtained from cultures of the oral cavity (n = 9), sputum (n = 10), and vaginal discharge (n = 9) of patients who were not wearing dentures or other artificial prostheses. All 35 BSI isolates were collected from patients with candidemia in whom central venous catheters were in place. All isolates were obtained from patient as part of the routine diagnostic procedures that were conducted at Chonnam National University Hospital from 2006–2010. Duplicate isolates from the same patient were excluded. C. albicans was identified by colony morphology on CHROMagar Candida (BBL, Becton Dickinson, Sparks, MD, USA), along with a commercially available biochemical identification system, i.e., the API 20C system (bioMérieux, Marcy L’Etoile, France) or the Vitek 2 system (Vitek 2 ID-YST, bioMérieux).

Biofilm formation

For biofilm formation, we used a denture strip model with 12-well tissue culture plates that has been used with Candida species [11,12]. Briefly, a standard inoculum of 10^7 cells/ml from an overnight culture of the C. albicans strain was applied to the surface of a 1.5-cm² denture strip (Nunc 174969 Thermofax Coverslips; diameter, 15 mm; Naperville, IL, USA), which was then placed in a well of a 12-well tissue culture plate with 4 ml of phosphate-buffered saline (PBS). The cells were then allowed to adhere for 90 min at 37°C, with non-adherent cells subsequently removed from the strips by gentle washing with 5 ml of PBS. The strips were then submerged in 4 ml of yeast nitrogen base medium supplemented with 50 mM dextrose (YNBD) and incubated for 24 and 48 h at 37°C on a rocker. Planktonic cultures were grown in the same way as the biofilms, except that glass tubes were used, and denture strips were not added [5,13]. For each biofilm, biofilm biomass was measured using a colorimetric XTT [2,3-bis-(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide] assay, as reported previously [12].

Real-time PCR analysis of C. albicans SAP gene expression

Total RNA was isolated from biofilms (24 and 48 h) and planktonic cells [11] and was analyzed by real-time reverse transcription (RT)-PCR [4]. SAP5 and SAP9 expression was quantified by real-time RT-PCR using a ROTOR Gene 3000 system (Corbett Research, Sydney, Australia). Primers and fluorescent probes were used as reported previously [4], and the calibration and efficiency of the primer/probe sets was assessed in titration experiments using serial dilutions of C. albicans SC5314 genomic DNA (500 ng to 5 pg). The expression of each gene was calculated with reference to the respective standard curve using Corbett Research software and normalized as the ratio of the target (SAP5 and SAP9) and housekeeping (ACT1) gene expression levels from separate reactions. Each reaction was performed in triplicate. Real-time PCR data were normalized with the geometric mean of ACT1 [10], and data are presented as mRNA transcript (arbitrary units [AU]) relative to ACT1 (10,000 AU) [4]. For all biofilms, relative gene expression levels were reported as the fold change determined as the mean normalized expression relative to that of corresponding planktonic cells (set as 1.0). The levels of SAP or ACT1 expression between biofilms and planktonic cells were compared by Wilcoxon matched-pairs signed rank test using the SPSS Win 18.0 program. The levels of SAP expression or biofilm biomass formation between BSI isolates and isolates from other sites were compared by using Mann-Whitney U test. Correlations between the SAP gene expression and biofilm biomass formation were examined by using Spearman’s test. Differences
between groups were considered significant when \( P \) was < 0.05.

**Results**

Figure 1 presents the expression of the *SAP5* and *SAP9* genes, relative to that of *ACT1* (10,000 AU), in 63 clinical isolates of *C. albicans* under planktonic and biofilm conditions. The expression of *ACT1* did not differ significantly throughout the experiment between biofilms and planktonic cells. For all 63 isolates, median (range) *ACT1* copy number per 100 ng RNA was 758 (177–2677) in 24-h biofilms, and 1178 (304–2918) in 48-h biofilms, both of which did not show significant differences, compared with corresponding planktonic cells [789 (323–2021) and 1154 (213–2328) at 24 h and 48 h, respectively] (both, \( P > 0.05 \)). For all 63 isolates, mean (standard deviation) *SAP5* expression levels were 3452 ± 2861 AU in 24-h biofilms, and 3862 ± 3567 AU in 48-h biofilms, both of which were higher than those in corresponding planktonic cells [1563 ± 1923 and 2052 ± 2816 AU at 24 and 48 h, respectively] (both, \( P < 0.0001 \)). In addition, mean *SAP9* expression in 24- and 48-h biofilms were 11,357 ± 5237 and 9761 ± 7006 AU, respectively, which were also higher than in those in corresponding planktonic cells [6539 ± 3127 and 5022 ± 3016 AU at 24 h and 48 h, respectively] (both, \( P < 0.0001 \)). No correlation was found between *SAP5* and *SAP9* expression levels in 63 clinical isolates of *C. albicans* under biofilm conditions.

Figure 2 shows the relative gene expression of biofilms, presented as the fold change relative to the corresponding planktonic cells, for 35 BSI isolates and 28 isolates from other sites. Mean *SAP5* gene expression levels in the 35 BSI isolates were 3.59-fold and 3.86-fold higher in the 24-h and 48-h biofilms, respectively, similar values to those for the 28 isolates from other body sites (2.71-fold and 2.80-fold higher in the 24-h and 48-h biofilms, respectively). Mean *SAP9* expression levels in all BSI isolates were 2.89-fold and 3.29-fold higher in the 24-h and 48-h biofilms, respectively, and significantly higher than those in all isolates from other sites (1.27-fold and 1.32-fold at 24 and 48 h, respectively; \( P < 0.001 \) for both 24-h and 48-h biofilms). The majority of clinical isolates of *C. albicans* showed higher levels of *SAP5* expression (> 1.0-fold) in 24-h biofilms (89% of BSI isolates and 100% isolates from other sites) and in 48-h biofilms (97% of BSI isolates and 82% isolates from other sites). Higher *SAP9* gene expression (> 1.0-fold) under 24-h and 48-h biofilm conditions was observed in 97% (34/35) and 100% (35/35) of BSI isolates, respectively, compared with 75% (21/28) and 68% (19/28) of isolates from other sites, respectively (\( P < 0.01 \) for both 24-h and 48-h biofilms).

The levels of biofilm biomass formation determined by XTT assay (values expressed in OD units at 492 nm) were highly variable among BSI isolates (0.087–0.364), or those from other sites (0.066–0.352). The mean OD (standard deviation) did not differ significantly between BSI isolates and those from other sites at 24 h (0.253 ± 0.062 versus 0.229 ± 0.079, \( P > 0.05 \)) or 48 h (0.224 ± 0.057 vs. 0.202 ± 0.056, \( P > 0.05 \)). In addition, no significant correlations were found between levels of *SAP5* or *SAP9* expression levels in 63 clinical isolates of *C. albicans* under biofilm conditions.

**Fig. 1** Expression of *SAP5* and *SAP9* genes in 63 clinical isolates of *Candida albicans* during planktonic or biofilm (24 h and 48 h) growth. Quantification was performed using real-time RT-PCR. Expression of SAP genes is shown in arbitrary units (AU) relative to 10,000 AU of Actin. Each symbol represents individual isolate, and the AU values are averages of three independent experiments for each isolate. Horizontal lines indicate the medians. \( ***p < 0.001 \), planktonic cells vs. biofilms.

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expression and biofilm biomass formation among 63 clinical isolates.

Discussion

*C. albicans* infections are often associated with biofilm formation [2,3]. Various model systems have been developed to study *C. albicans* biofilm biology on abiotic and mucosal surfaces [4,10]. In this study, we examined the expression of two SAP genes in *in vitro* *C. albicans* biofilms grown on an abiotic surface using the denture-strip biofilm model, which may reflect *in vivo* biofilm formation on indwelling medical devices, such as intravascular catheters. All BSI isolates used in this study were from patients in whom central venous catheters were in place. Our results show that expression levels of both SAP5 and SAP9 were upregulated in *C. albicans* biofilms formed by BSI isolates, and that SAP9 was more likely to be upregulated in *C. albicans* biofilms formed by BSI isolates than those formed by isolates from other sites. We believe this to be the first study to compare the quantitative expression of SAP5 and SAP9 genes on *in vitro* *C. albicans* biofilms according to their clinical sources.

We used real-time quantitative PCR methods established by Naglik et al. [4] to measure SAP gene expression in *C. albicans* planktonic cells and biofilms. Using these methods, Naglik et al. demonstrated that SAP5 and SAP9 were the most highly expressed SAP genes in mucosa samples from patients with oral or vaginal candidiasis [4]. Our results show that the expression levels of both SAP5 and SAP9 genes in *in vitro* biofilms formed by most clinical isolates of *C. albicans* were significantly higher than those in planktonic cells, supporting the previous findings that *C. albicans* biofilms on abiotic surfaces secrete more Saps than planktonic cells [5,6]. A recent study to investigate whether SAP genes are highly expressed in biofilms associated with abiotic surfaces, showed that all SAP genes (except for SAP3) were upregulated in the various biofilm model systems formed by a reference strain, *C. albicans* SC5314 [10]. Our results using 63 clinical isolates of *C. albicans* disclosed the strain-dependent differences in the expression level of SAP5 and SAP9 genes under biofilm conditions.

Of the 10 Saps, expression of SAP4–6 has been demonstrated in all mucosal and systemic infections by *C. albicans* examined [1,4,14]. In addition, *C. albicans* expresses the SAP4–6 ubiquitously in all Candida carriers and patients with oral and vaginal candidiasis, regardless of the infection model, suggesting that this proteinase subfamily plays an important role in *C. albicans* colonization and infection [1]. Our data show that most clinical isolates of *C. albicans* from blood, the oral cavity, sputum, and vaginal discharge expressed SAP5 in *in vitro* biofilms, and no differences were found in mean biofilm SAP5 expression levels between the BSI isolates and those from other sites. These data suggest that SAP5 may also play an important role in biofilm-associated colonization and infection caused by *C. albicans*, regardless of type of infection (mucosal or bloodstream).

In contrast to other Saps, Sap9 and Sap10 are not secreted by the fungus, but are glycosylphosphatidylinositol (GPI)-anchored proteins that play a role in cell surface integrity [1,15,16]. Our study using 63
clinical isolates of C. albicans showed no correlation between SAP5 and SAP9 expression levels under biofilm conditions. Schild et al. recently proposed that Sap9 and Sap10 influence distinct cell wall functions by proteolytic cleavage of covalently linked cell wall proteins [9], which mediate biofilm formation and promote adherence to host cells and invasion into epithelial cell layers [16]. Our results show that C. albicans BSI isolates have a higher ability to express SAP9 under biofilm conditions than isolates from other clinical sites, suggesting that the SAP genes expressed or required by C. albicans to cause biofilm-associated infections may vary depending on the type of infection (i.e., mucosal or systemic), and that SAP9 is more likely to play an important role in biofilm-associated BSIs than other infections by C. albicans.

Although the SAP genes expressed by C. albicans under in vitro biofilm conditions may not equate to the SAP genes expressed in vivo, these findings suggest that a BSI may result from selecting C. albicans strains with higher expression of both SAP5 and SAP9 in association with biofilm formation. No significant correlations between levels of SAP5 or SAP9 expression and biofilm biomass formation among clinical isolates suggest that expression of these two SAP genes may not be directly related to the level of biofilm formation. The higher SAP9 expression of BSI isolates of C. albicans under biofilm conditions, together with strain-specific differences of SAP gene expression, may reflect the fact that strain-specific factors secreted from C. albicans within the biofilm may be more important in causing biofilm-associated BSIs than the presence of a biofilm alone or the level of biofilm formation [6], although this hypothesis requires further investigation.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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