PH.D. THESIS

THE ROLE OF *CANDIDA PARAPSILOSIS* SECRETED ASPARTYL PROTEINASES IN HOST-PATHOGEN INTERACTIONS

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Introduction

During the last three decades *Candidiasis* has become the most commonly diagnosed yeast-related infection worldwide. Although *Candida* species are members of the normal human flora, under specific circumstances they have the ability to turn themselves into pathogenic fungi causing superficial, cutaneous or systemic infections. In most cases *Candida* species are responsible for nosocomial infections. The major risk factors for opportunistic fungal infections are the prolonged hospitalization, the extended use of antibiotics, use of intravenous catheters and the low birth weight in case of premature neonates. According to recent studies, although the most prevalent species causing invasive candidiasis in adults is *C. albicans*, several studies demonstrated, that in special patient groups especially in case of neonates *C. parapsilosis* is even outmarks *C. albicans.* Although, in the last two decades candidial infections caused by non-*albicans* species show an increasing tendency, little is known about the pathomechanism and virulence attributes of these species. It has been shown, that secreted hydrolytic enzymes –

especially lipases – play a crucial role in the pathogenicity and virulence of *C. parapsilosis*. Previous studies demonstrated, that *C. parapsilosis* lipase deficient strain has decreased virulence compared to the wild type in several *in vitro* and *in vivo* infection models. Even the two main secreted aspartyl proteinases of *C. parapsilosis* (Sapp1 and Sapp2) are biochemically well characterized, very little is known about the role of these enzymes during host-pathogen interactions. Although for *C. albicans* several techniques are available for genetic manipulation, the numbers of gene deletion methods in *C. parapsilosis* are limited and, there is no gene over-expression method available for this microbe.

To fill this gap the aims of our study were: 1.: to clarify the role of the *C. parapsilosis* secreted aspartyl proteinase 1 (Sapp1) in host-pathogen interactions, 2.: to generate a "Sapp negative" secreted aspartyl proteinase deficient strain in *C. parapsilosis* in order to study the general role of Sapp proteins in host-pathogen interactions, and finally 3.: to establish an overexpression strategy optimized for *C. parapsilosis.*

Methods

Generation of mutant strains, molecular techniques: Generation of *E. coli* and *C. parapsilosis* competent cells, transformation of competent cells, PCR, generation of vectors, Gateway reaction, DNA extraction, Southern hybridization, RNA extraction from yeast, cDNA synthesis, qRT-PCR, HPLC analysis, flow cytometry, ELISA assay

Cultivation and *in vitro* infection: isolation and differentiation of primary cells, cultivation of yeast cells, *in vitro* infection of primary cells, yeast elimination assay, measurement of host cell damage, serum susceptibility assay of yeast strains

Microscopic techniques: Calcofluor White staining, Concavalin A staining, Wheat Germ Agglutinin staining, Calcofluor White/lysotracker Red staining, scanning electron microscopy, extracellular vesicle analysis

In vivo assay: Infection of *Galeria Mellonella* larvae, *in vivo* survival assay

Results

The role of secreted aspartyl proteinase 1 (Sapp1) in host-pathogen interactions.

The role of *C. albicans* secreted aspartyl proteinases, as virulence factors in host-pathogen interactions has been extensively investigated (Hube and Naglik 2001). It has been demonstrated, that *C. albicans* sap's are able to degrade several structural and immunologically important proteins of the host, thus assist the invasion of the microbe.

In contrast, the role of the two main proteinases (Sapp1 and Sapp2) (Hruskova-Heidingsfeldova, et al. 2009) of *C. parapsilosis* in virulence and pathogenesis was not studied previously.

According to the biochemical studies, the major aspartyl proteinase of *C. parapsilosis* is the Sapp1 protein, thus we first focused on the investigation of Sapp1 in host-pathogen interactions.

The *in silico* analysis of *SAPP1* open reading frame revealed two, identical 2871bp long region in 32kb distance from each other in the *C. parapsilosis* genome, that contained the *SAPP1* ORF. The two copies of the

ORF were identified as *SAPP1a* and *SAPP1b*. In 2009, Geraldine Butler and her colleagues submitted the annotated genome sequence of *C. parapsilosis,* that confirmed the result of our *in silico* analysis (Butler, et al. 2009).

In order to investigate the role of the SAPP1 genes we applied the deletion constructs pSFS2Sapp1a and pSFS2Sapp1b. Using the deletion vectors, *Δ/Δsapp1a, Δ/Δsapp1b* (by the deletion of the duplicated region of *SAPP1b* locus) and *Δ/Δsapp1a-Δ/Δsapp1b* homozygous mutants were generated.

The loss of *SAPP1* allels was validated in RNA and in protein level as well. Quantitative real-time PCR analysis was performed to measure the transcript levels of *SAPP1, SAPP2* and *SAPP3* (a putative secreted proteinase like ORF) genes. The wild type strain in proteinase inducing medium showed 80-fold overexpression of *SAPP1* while in the *Δ/Δsapp1a-Δ/Δsapp1b* double deletion mutant no *SAPP1* transcription level was detectable. Interestingly, the *SAPP2* expression level was significantly increased in the

Δ/Δsapp1a-Δ/Δsapp1b mutant in compare to the wild type under inductive conditions. The putative, yet not characterized gene the *SAPP3,* showed ten-fold overexpression in wild type yeast in induction media. However, under the same conditions the homozygous *Δ/Δsapp1b* strain displayed almost no *SAPP3* expression. To clarify this finding further sequence analyses were performed. The results indicate a possible deletion of the regulatory region of the *SAPP3* gene that was caused during the generation of the *SAPP1b* deficient mutant.

To investigate the enzyme activities of Sapp1 and Sapp2 proteins, quantitative HPLC measurement was performed using the fluorescent substrate DABCYL-Glu-His-Val-Lys-Leu-Val-Glu-EDANS. In the wild type strain both specific peaks of Sapp1 and Sapp2 were detected. Interestingly, the Sapp2 activity in the *Δ/Δsapp1a-Δ/Δsapp1b* mutant strain was significantly increased. Based on the elevated transcript and enzyme activity level of Sapp2 we assume a possible feedback mechanism that monitors the levels of the produced aspartyl proteinase levels.

It has been shown, that *C. albicans* Sap proteins are able to degrade the complement proteins of human serum (Gropp, et al. 2009) and enhance the dissemination of the microbe. To investigate whether the *C. parapsilosis* Sapp1 has similar role, serum susceptibility assay was performed. The presence of the heat inactivated human serum did not alter the growth capacity of the wild type nor the mutant cells. In contrast, the intact human serum inhibited the growth rate of the *Δ/Δsapp1a-Δ/Δsapp1b* mutant strain in compare to the wild type significantly. It suggests that *C. parapsilosis* Sapp1 is also able to inactivate some of the defense protein components (complement components, immunoglobulin, defensins etc.) of the human serum.

Macrophages and other phagocytes play a crucial role in the host innate immune response. The pattern recognition receptors (PRRs) of macrophages are able to recognize the evolutionally conserved pathogen associated molecular patterns (PAMPs) of the pathogens. After the recognition, macrophages are able to phagocytose the invading pathogens. Following the phagocytosis, as a final step the phago-lysosome fusion

takes place, that leads to the elimination of the ingested pathogen (Netea, et al. 2008).

To study the process of phagocytosis flow cytometry (FACS) and fluorescent microscopic analysis were performed. The phagocytic capacity of human macrophages (PBMC-DM) were investigated followed by the co-incubation of wild type and homozygous deletion mutant cells. The results showed increased phagocytic capacity of PBMC-DM infected with the *Δ/Δsapp1a-Δ/Δsapp1b* mutant strain. To further dissect the process of engulfment, fluorescent microscopy analysis was performed. Increased phago-lysosome fusion was observed in macrophages infected with *Δ/Δsapp1a-Δ/Δsapp1b* mutant strain in compare to the wild type. These results strongly suggest that Sapp1 has an important role in host-pathogen interaction, during the intracellular survival most likely due to the inhibition of phago-lysosome fusion.

To investigate the final stage of host-pathogen interaction killing assays were performed. Using PBMC and PBMC-DM cells the killing efficiency was

monitored after the co-incubation of host cells with wild-type and *SAPP1* homozygous mutant strains. Both the PBMC, and PBMC-DM showed significantly increased elimination efficiency against the *Δ/Δsapp1a-Δ/Δsapp1b* mutant cells. Additionally, the damage of the host cells caused by *Δ/Δsapp1a-Δ/Δsapp1b* mutant strain was significantly decreased compared to the damage caused by the wild-type strain. These results suggest that *C. parapsilosis* Sapp1 has an important role in the maintenance of virulence by inhibiting the intracellular killing efficiency of host cells and by propagating the intracellular survival.

All these data demonstrate that Sapp1 is an important virulence factor of *C. parapsilosis*. Sapp1 is able to degrade host defense proteins, inhibits the phagocytosis, phago-lysosome fusion and promotes the intracellular survival of the pathogen.

The general role of *C. parapsilosis* secreted proteinases in virulence and host-pathogen interactions.

The *C. albicans* secreted aspartyl proteinase gene family consists of ten *SAP* genes (*SAP1-SAP10*), that are well characterized (Hube and Naglik 2001, Naglik, et al. 2003), however the generation of a *SAP* deficient strain in *C. albicans* due to the high number of encoding genes is almost impossible. *C. parapsilosis* has only three *SAPP* genes (*SAPP1-SAPP3*), where *SAPP1* and *SAPP2* are actively transcribed, while *SAPP3* is considered as a pseudogene. For this reason, the generation of a *C. parapsilosis SAPP* deficient strain is much feasible. Our second aim was to investigate the general role of *C. parapsilosis* Sapps during host-pathogen interactions by generating a *SAPP* minus strain.

We validated the loss of *SAPP2* in transcriptional level by Quantitative Real-Time PCR in inductive (1x YCB+ 2% BSA) and non inductive (YPD) conditions. In the deletion mutant strain neither *SAPP1,* nor *SAPP2* mRNA was detectable. The transcriptional level of *SAPP3* was also significantly decreased.

To measure the activities of Sapp1 and Sapp2 enzymes the concentrated supernatants of the wild-type, *Δ/Δsapp1a-Δ/Δsapp1b* and the *Δ/Δsapp1a-Δ/Δsapp1b-Δ/Δsapp2* strains were applied. The *Δ/Δsapp1a-Δ/Δsapp1b-Δ/Δsapp2* strain showed no proteinase activity, which also validated the loss of *SAPP1* and *SAPP2* genes.

Our previous results suggested, that *C. parapsilosis* Sapp1 plays an important role by digesting the serum proteins. To investigate, whether the loss of Sapp2 has similar effect, serum susceptibility assay was performed. In intact human serum the growth of the *Δ/Δsapp1a-Δ/Δsapp1b-Δ/Δsapp2* strain was significantly decreased after 24 and 48 hours of cultivation. Compared to our previous result, there was no difference between the growth rate of *Δ/Δsapp1a-Δ/Δsapp1b* and *Δ/Δsapp1a-Δ/Δsapp1b-Δ/Δsapp2* strains in human serum, suggesting that only Sapp1 has the role in the degradation of the host immune proteins of the human serum.

Similarly, there was no difference in the ratio of phagocytosis by macrophages between *Δ/Δsapp1a-Δ/Δsapp1b* and *Δ/Δsapp1a-Δ/Δsapp1b-Δ/Δsapp2* strains. This result suggests, that Sapp1 has the major role in the inhibition of phagocytosis.

It has been shown, that *C. albicans* Sap1, Sap2 and Sap6 proteins are able to induce the secretion of IL-1β, IL-6, TNF-α and additionally, Sap3 is able to induce the production of IL-1β and TNF-α (Pietrella, et al. 2010). To investigate whether *C. parapsilosis* Sapp proteins can also modulate the host response, the level of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) produced by PBMC-DM were measured. Although, the infection of the macrophages with *Δ/Δsapp1a-Δ/Δsapp1b-Δ/Δsapp2* cells resulted in a decreased level of IL-1β and IL-6 production, these differences were not significant in compare to the wild type infection. Imoprtantly, we still can not exclude the potential immune modulatory role of the *C. parapsilosis* Sapps , however this need further investigations, using different

cell lines and by measuring the anti-inflammatory cytokines as well.

The killing efficiency of the human macrophages was also measured after the coincubation with the Sapp minus deletion strain. We detected a slight increase in the killing efficiency against *Δ/Δsapp1a-Δ/Δsapp1b-Δ/Δsapp2* cells compared to the *Δ/Δsapp1a-Δ/Δsapp1b* mutant, suggesting that Sapp2 could have an additive effect in the intracellular survival of the microbe.

To analyse the *in vivo* effect of the secreted proteinases, a non-conventional animal model (*Galleria mellonella* larvae) was applied. The survival of *G. mellonella* larvae infected with wild-type and *Δ/Δsapp1a-Δ/Δsapp1b-Δ/Δsapp2* strains were monitored for 14 days after infection, however no significant differences could be observed between the survivals of the infected larvae.

Previous studies indicated, that *C. albicans* Sap9 and Sap10 proteins are GPI anchored and localized in the fungal cell wall. Additionally, they have an important role in the maintenance of cell wall integrity of *C.*

albicans (Schild, et al. 2011). Therefore, we analysed the cell wall composition of wild-type, *Δ/Δsapp1a-Δ/Δsapp1b* and *Δ/Δsapp1a-Δ/Δsapp1b-Δ/Δsapp2* strains. Notably, the chitin content of the cell wall was decreased in both deletion mutant, while the glucan content of the *Δ/Δsapp1a-Δ/Δsapp1b-Δ/Δsapp2* cells showed elevated level. Scanning electron microscopy demonstrated that the cell wall surface of the *Δ/Δsapp1a-Δ/Δsapp1b-Δ/Δsapp2* strain is smoother, compared to wild-type strain. These data demonstrate, that *C. parapsilosis* secreted aspartyl proteinases are not only important virulence factors, but also play a crucial role in the cell wall maintenance.

In summary, *C. parapsilosis* secreted aspartyl proteinases are important factors for the virulence and pathogenesis. They have a major role in the neutralization of immune proteins of the host, inhibition of phagocytosis and they can promote the intracellular survival of the pathogen. Additionally, *C. parapsilosis* secreted aspartyl proteinases may contribute to the maintenance of the cell wall integrity.

Decelopment of an overexpression strategy for *C. parapsilosis*

Members of the CUG clade are constitutively diploid and lack the sexual cycle. For this reason, in these species, gene deletion process is time intensive and technically challenging. Furthermore, the gene deletion methods are not suitable to investigate the role of essential genes. To bypass this problem and to adequately study a function of a gene of interest, a possible solution is to overexpress the targeted gene. Until date, no gene overexpression strategy is avaliable for *C. parapsilosis*. Thus, our aim was to develop a new overexpression system for *C. parapsilosis*.

First, we generated a *C. parapsilosis* acceptor strain, that is able to overexpress our gene of interest. Using the *caSAT1* flipper system, the *RPS10* locus of *C. albicans* was integrated into the locus of the *RPS10* locus of the *C. parapsilosis* CLIB 214 leucine auxotrophic strain.

To investigate, that the generated strain is able to overexpress the gene of interest, a TDH3-GFP containing plasmid was transformed into the acceptor strain. The

TDH3 promoter is a strong, constitutive promoter, which allows the active transcription of the GFP reporter gene. Fluorescent microscopic studies revealed, that the transformants are GFP positive, demonstrating that the acceptor strain is suitable for overexpression.

To generate the vectors for overexpression, the Gateway cloning strategy was applied. As a proof of principle, we selected the *WOR1* gene for overexpression. The orthologous of this gene in *C. albicans* is responsible for white-opaque transition and regulates sexual cycles. The *WOR1* containing destination vector was transformed to the *C. parapsilosis* acceptor strain. After transformation the gene expression level of *WOR1* were monitored by qRT-PCR and resulted a more than 10 fold overexpression in the transformants.

In summary, we successfully generated an overexpression system for *C. parapsilosis,* using the highly efficient Gateway cloning system. This strategy will facilitate the development of overexpression libraries and will definitely help to understand the molecular basis of the pathogenesis and virulence of the important human fungal pathogen, *C. parapsilosis*.

Summary

- Generation of *C. parapsilosis SAPP* gene deletion mutant strains
- Investigation of *SAPP* gene expression and Sapp1 and Sapp2 protein activities
- Increased serum susceptibility in case of *Δ/Δsapp1a-Δ/Δsapp1b* and *Δ/Δsapp1a-Δ/Δsapp1b-Δ/Δsapp2* strains
- Increased phagocytosis and killing efficiency of *Δ/Δsapp1a-Δ/Δsapp1b* and *Δ/Δsapp1a-Δ/Δsapp1b-Δ/Δsapp2* strains by macrophages
- Measurement of proinflammatory cytokines produced by host cells during *in vitro* infection
- *In vivo* survival assay using *G. mellonella* larvae
- Generation of *C. parapsilosis* acceptor strain for gene overexpression
- GFP transformation of the acceptor strain
- Generation of overexpression vectors
- Overexpression of *WOR1* gene in *C. parapsilosis*

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