PHD THESIS

CHARACTERIZATION OF SECRETED ASPARTYL PROTEASES IN *CANDIDA PARAPSILOSIS*

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Introduction

C. parapsilosis is a ubiquitous microorganism in natural environments such as soil, seawater, plants, insects, and domestic animals. *C. parapsilosis* can easily be isolated from mucosal surfaces, skin, and nails of human and mammalian hosts where it resides as a commensal host. Being a skin commensal and its unique ability to adhere to physical surfaces makes it a frequent cause of nosocomial infections in the hospitals. Among non-*albicans* species, infections caused by *C. parapsilosis* increasing worldwide and it became the second or third most common yeast isolated from hospitals of Asian and American countries whereas, the increasing incidence in European countries is also reported. Pathogenicity of *C. parapsilosis* is associated with low birth weight (≤1,500 grams) neonates, hospitalized immunocompromised patients, and invasive disease such as fungemia, endocarditis, endophthalmitis, arthritis, peritonitis, all of which usually occur in association with receiving parenteral nutrition, invasive procedures or use of intravascular devices.

Several factors such as adhesion to host surfaces, switching from yeast to pseudohyphal form, biofilm formation, and secretion of hydrolytic enzymes such as lipases, phospholipases, and aspartyl proteases are considered as crucial virulence factors behind *Candida* caused pathogenesis.

Aspartyl proteases are present in a diverse range of microorganisms and play a crucial role in nutrition to pathogenesis in microorganism. *C. parapsilosis* possess three known aspartyl proteases gene designated as *SAPP1*, *SAPP2*, and *SAPP3*. Recently, duplication of *SAPP1* gene was identified in our lab whereas, some homologs with high similarity with *SAPP2* is also been identified. Even though available literature suggests a crucial role of aspartyl proteases of *C. albicans* in its pathogenicity not much is known about their roles in *C. parapsilosis*. Therefore, in the present study we tried to elucidate the roles of *C. parapsilosis SAPP*s mainly in its attachment, potential to damage the epithelial cells, modulating the behavior of macrophages, and their proteases activity against human complement proteins.

Methods

Bacterial, Fungal culture, and transformation:

Cultivation of *C. parapsilosis* and *E. coli*, competent bacterial and yeast cell preparation, transformation techniques.

Molecular methods:

DNA and RNA isolation from yeast cells, plasmid isolation from *E. coli*, molecular cloning using the Gateway system, PCR, qRT-PCR, gel electrophoresis, Southern blot, and Western blot.

Phenotypic characterization:

Growth assays on complex and minimal media, survival tests in the presence of oxidative, cell wall and membrane stressors, biofilm formation (XTT metabolic activity assay, crystal violet staining assay), and morphology comparison (microscopic analyses).

Human monocytes isolation and culturing:

Oral and vaginal epithelial cell culture, PBMC isolation and differentiation, *in vitro* stimulation of epithelial cells, and macrophages with different *C. parapsilosis* strains.

Immunological methods:

Flow cytometry, cytokine analysis by ELISA (enzyme-linked immunosorbent assay).

***In vivo* models:**

In vivo virulence studies using *Galleria mellonella* and *Drosophila melanogaster* as an infection model system.

Results

Reintegration of the *SAPP1*, *SAPP2*, and *SAPP3* genes

To analyze the role of individual *SAPP* gene, we reintegrated *SAPP1*, *SAPP2*, and *SAPP3* genes individually under control of *C. albicans* constitutive promoter (*CaTDH3)* at *C. parapsilosis* neutral locus (*CpNeut5l*) and confirmed the integration in mutant strains by colony PCR and Southern blot. The blotting results confirmed that *SAPP* genes are integrated at correct locus.

Relative transcription of *SAPP* genes in reintegrant *C. parapsilosis* strains

The transcription level of the *SAPP* genes in the reintegrant strains was determined using qRT-PCR. The qRT-PCR analysis revealed that transcription of *RI_SAPP1* and *RI_SAPP2* were nearly similar to transcription of the *SAPP1* and *SAPP2* genes in wild-type *C. parapsilosis*, whereas increased by four fold in case of *RI_SAPP3* strain in comparison with the control when cultured in YCB+0.2% BSA medium.

Detection of protease activities of Sapp in WT and mutant strains of *C. parapsilosis*

To test if reintegrant strains restored protease production capability, WT, *sapp1/2/3*$^{-/-}$, and *RI_SAPP1*, *RI_SAPP2*, *RI_SAPP3 C. parapsilosis* strains were spotted onto YCB + 0.2% BSA containing plates. Plates were stained with amido black and the widths diameter of clearance zones were measured after three days of incubation. Wild-type *C. parapsilosis* showed a clear hallow zone (7.3 mm) in BSA containing plates whereas, *RI_SAPP1* (5.78 mm) and *RI_SAPP2* (5.76 mm) also showed proteolytic activity. In contrast, *RI_SAPP3* and *sapp1/2/3*$^{-/-}$, *C. parapsilosis* strains did not show any proteolytic activity, although the growth of the colonies was nearly similar to WT.

C. parapsilosis WT, *sapp1/2/3*$^{-/-}$, and *RI_SAPP* strains grow similar rate

Effect of the deletion and reintegration of *SAPP* genes in growth rate of *C. parapsilosis* was examined in YPD and YCB+0.2% BSA growth medium for 24 h. Growth curve results indicated that *SAPP* genes in *C. parapsilosis* do not influence its growth.

C. parapsilosis SAPP3 reintegrant strain and *sapp* null mutant strain is more sensitive to normal human serum

To investigate the effect of the fungicidal effect of human serum, yeast cells were cultivated in 20% normal human serum and heat-inactivated serum; then, CFU determinations were performed at different time intervals. Whereas all *C. parapsilosis* strains grown similarly in 20% heat-inactivated serum, the viability of the *RI_SAPP3* and *sapp1/2/3*$^{-/-}$ *C. parapsilosis* strains in intact serum was reduced significantly after 18 and 24 h incubation, respectively. There were no significant differences between the growth of WT, *RI_SAPP*, and *sapp1/2/3*$^{-/-}$ mutant strains in heat-inactivated human serum at any time point of treatment.

Deletion and reintegration of *SAPP* genes did not alter the biofilm formation in *C. parapsilosis*

To check if deletion and reintegration of *SAPP* genes affect the biofilm forming ability of *C. parapsilosis* we choose MTT reduction assay and crystal violet (CV) staining method. Both strains are inoculated in YNB and RPMI media and allowed to grow biofilm for 48 h at 37 °C and formed biofilm level was determined by MTT and CV stain. No significant differences were observed in biofilm forming abilities WT strain and mutant strains.

Secreted aspartyl proteases of *C. parapsilosis* affect its adhesion

We examined whether the reintegration or deletion of *SAPP* genes in *C. parapsilosis* affect adhesion to artificial surface and epithelial cells. Results have clearly shown that all three reintegrant strains exhibited significant reduced number of cells (CFU counts) adhered to the solid surface when compared to the WT. Highest reduction in adhesion was observed in *sapp1/2/3*$^{-/-}$ mutant strains (nearly 40%), whereas as lowest reduction was reported in *RI_SAPP1* mutant strain (20%) followed by *RI_SAPP*2 (25%) and *RI_SAPP3* (25%) reintegrant mutant strains. A significant reduction in adhesion of *RI_SAPP3*, moderately lower adhesion of *sapp1/2/3*$^{-/-}$ and *RI_SAPP2* to oral epithelial cells (TR-146) and vaginal epithelial cells (A-431) was also observed. However, *RI_SAPP1* strain restored its adhesion capacity to epithelial cells nearly to WT. These findings clearly indicated the role of *SAPP1* and *SAPP2* in adhesion of *C. parapsilosis*.

SAPP null mutant is less capable of causing host-cell damage whereas reintegration of *SAPP1* and *SAPP2* gene restored its damage causing capabilities

The ability of the WT, *RI_SAPP*, and *sapp1/2/3*$^{-/-}$ mutant strains to damage human peripheral blood mononuclear cells - derived macrophages (PBMC-DMs) were monitored with lactate

dehydrogenase (LDH) release assay at 24 and 48 h. WT and *RI_SAPP1* and *RI_SAPP2* strains caused similar damage whereas, *RI_SAPP3* and *sapp1/2/3*$^{-/-}$ caused significantly less damage after 24 h incubation with PBMC-DMs. WT reported to cause significantly higher damage to PBMC-DM at both 24 h and 48 h. LDH released by A-431 cells infected with WT *C. parapsilosis* is significantly higher compared to *RI_SAPP2*, *RI_SAPP3,* and *sapp1/2/3*$^{-/-}$ strains indicating that *SAPP1* and *SAPP2* are crucial in *C. parapsilosis* to cause host cell damage.

Macrophages phagocytos *RI_SAPP3* and *sapp1/2/3*$^{-/-}$ mutants more efficiently than WT , *RI_SAPP1* and *RI_SAPP2* cells

To examine if *C. parapsilosis* Sapps can modulate the phagocytic efficiencies of PBMC-DMs, *C. parapsilosis* yeast cells were labeled with the fluorescent dye Alexa Fluor 488 (a succimidyl ester), washed, co-incubated with PBMC-DMs for 2 h and then analyzed with flow-cytometer. Our results indicated that PBMC-DMs ingested *RI_SAPP3* and *sapp1/2/3*$^{-/-}$ mutant strains more efficiently compared to the WT strain highlighting the importance of *SAPPs* in modulating phagocytosis of macrophages.

SAPP2 influences the phagosome-lysosome fusion

We analyzed the phagosome-lysosome co-localization after co-incubating pHrodo stained *Candida* cells with PBMC-DMs for 2 h. WT and *RI_SAPP2* infected PBMC-DMs showed less phago-

lysosomal fusion compared to the *RI_SAPP1*, *RI_SAPP3*, and *sapp1/2/3*$^{-/-}$ mutant strains suggesting the corresponding genes influence on phagosome maturation.

SAPP null and *RI SAPP3* mutants are killed more efficiently by PBMC-DMs and human whole blood compared to *RI SAPP1/2* strains

Further, we examined if alteration of phago-lysosome maturation by *C. parapsilosis* is related to yeast cell killing efficiency of PBMC-DMs. Our data showed that PBMC-DMs were able to kill significantly higher numbers of *C. parapsilosis RI_SAPP3* mutant cells compared WT cells. Notably, PBMC-DM killed significantly more [32% WT vs 53.9% *sapp1/2/3*$^{-/-}$ and 50.39% *RI_SAPP3*]. However, killing efficiency of macrophages was nearly similar against WT and *RI_SAPP1*, *RI_SAPP2* indicating significant contribution of *SAPP1* and *SAPP2* in modulation of killing capacity of macrophages. In addition to PBMC-DMs, we also examine the killing efficiency of whole human blood. Our result showed higher killing of *RI_SAPP3* and *sapp1/2/3*$^{-/-}$ by human whole blood indicating protective roles of *SAPP1* and *SAPP2*.

SAPPs regulate the cytokine response of host microphages

In order to examine the immunological responses triggered by the *SAPP* reintegrant mutant and *sapp1/2/3*$^{-/-}$ strains, we stimulated human PBMC-DMs for 24 h with each strain and measured cytokine

IL-1β, TNFα, IL-6, and chemokine IL-8 responses. The obtained results indicated that PBMC-DMs stimulated with either WT, *RI_SAPP1*, and *RI_SAPP2* strains produced similar IL-1β, IL-8, and TNFα levels. In contrast, macrophages stimulated with *sapp1/2/3*$^{-/-}$ produced significantly less IL-1β and IL-6, and moderately but not significantly less IL-8 compared to the WT. PBMC-DMs stimulated with *RI_SAPP3* produced significantly lower IL-8 and moderately low IL-6, however, no significant differences were observed in the production of IL-1β and TNFα compared to WT.

Sapp1p and Sapp2p have differential cleavage capacity against human complement proteins

To test if *C. parapsilosis* Sapp proteins can also cleave human complement proteins we incubated human complement proteins C3b and C4b with the purified Sapp proteins (Sapp1p and Sapp2p)**.** Result indicated that Sapp1p and Sapp2p can efficiently cleave the human C4b whereas cleavage efficiency of Sapp1p is higher against C3b compared to Sapp2p.

Sapp2p but not Sapp1p can cleave FHR-5

We also measured the capacity of Sapp1p and Sapp2p to degrade factor H (FH) and FH family proteins FHL1, FHR1, and FHR5. Sapp1p and Sapp2p both cleaved FH. Interestingly, Sapp2p but not Sapp1p cleaved FHR5 indicating differential proteases activities of *C. parapsilosis* Sapps against human complement proteins. Co-

incubation of Sapp1p or Sapp2p with FHL-1 or FHR-1 revealed that the proteases cannot cleave these human complement proteins as visualized by Western blot.

Sapp1p and Sapp2p can not alter the expression of CR3 and CR4 on macrophage surface

To understand the effect of *C. parapsilosis* Sapp1p and Sapp2p proteins on the cell surface expression of CR3 and CR4, PBMC-DMs were treated with these proteases. Unexpectedly, we did not found any substantial differences in the CR3 and CR4 expression on macrophages either after Sapp1p or Sapp2p treatment indicating in contrast to *C. albicans*, *C. parapsilosis* can not utilize their Sapp proteins to alter CR3 and CR4 expression on macrophages.

In vivo virulence of *C. parapsilosis* WT and mutant strains

To check the virulence of *C. parapsilosis* mutant strains *G. mellonella* was infected with WT and *RI_SAPP* strains. The course of infection was followed for 24 h for determination of fungal load and seven days for survival. *G. mellonella* infected with WT shows higher fungal burden compared *RI_SAPP2*, *RI_SAPP3,* and *sapp1/2/3*$^{-/-}$ to all the reintegrant strain. We recovered nearly similar CFU from *Galleria* infected either with WT or with *RI_SAPP1* indicating the role of *SAPP1* in *C. parapsilosis* virulence. However, the larvae were infected with the WT strain, the survival rate decreased moderately but not significantly compared to larvae

infected *sapp1/2/3*$^{-/-}$ mutant strain. In addition, larvae infected with three *SAPP* mutant strains showed no any significant difference in survival during the whole window of time after infection.

Drosophila melanogaster survival

We also used *Drosophila melanogaster* as a model to characterize the virulence of *C. parapsilosis RI_SAPP* strains. After infection of flies with *C. parapsilosis*, we did not find any significant difference in survival either in wild-type Oregon R-S (OreR) flies or in MyD88 knock out flies (MyD88$^{-/-}$) mutant flies.

Summary

Main fundings of the study are:

- Aspartyl proteases of *C. parapsilosis* do not affect its pseudohyphae formation, biofilm formation, cell wall composition, and stress tolerance abilities.

- *RI_SAPP1* and *RI_SAPP2* but not *RI_SAPP3*, affect its adhesion and damage-causing efficiencies to oral as well as to vaginal epithelial cells.

- The *RI_SAPP3* and *sapp1/2/3*$^{-/-}$ mutants were more phagocytosed and killed by the human PBMC-DMs.

- The *SAPP* mutants induced altered cytokine response from

the human PBMC-DMs.

- Sapp1p and Sapp2p can not alter the expression of complement receptor-3 (CR3) and CR4 on macrophage surface.

- Sapp1p and Sapp2p both can cleave human complement proteins C3b, C4b, and FH. However, interestingly, only Sapp2p but not Sapp1p can cleave FHR5.

- *C. parapsilosis SAPP1* and *SAPP2* play role in its virulence as indicated by the higher fungal burden in *Galleria mellonella* infected with *RI_SAPP1* and *RI_SAPP2*.

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Publications

Singh DK, Németh T, Papp A, Tóth R, Lukácsi S, Heidingsfeld O, Dostal J, Vágvölgyi C, Bajtay Z, Józsi M, Gácser A. Functional characterization of secreted aspartyl proteases in *Candida parapsilosis*. mSphere. 2019; 4(4). pii: e00484-19. doi: 10.1128/mSphere.00484-19. IF- 4.28

Verma DK[1], **Singh DK**[1], Gupta S, Gupta P, Singh A, Biswas J, Singh S. Minocycline diminishes the rotenone induced neurotoxicity and glial activation via suppression of apoptosis, nitrite levels and oxidative stress. Neurotoxicology. 2018; 65:9-21. doi: 10.1016/j.neuro.2018.01.006. IF- 3.553 ([1]Sahred first author)

Navarro-Arias MJ, Defosse TA, Dementhon K, Csonka K, Mellado-Mojica E, Dias Valério A, González-Hernández RJ, Courdavault V, Clastre M, Hernández NV, Pérez-García LA, **Singh DK**, Vizler C, Gácser A, Almeida RS, Noël T, López MG, Papon N, Mora-Montes HM. Disruption of protein mannosylation affects *Candida guilliermondii* cell wall, immune sensing, and virulence. Front Microbiol. 2016; 7:1951. PMID: 27994582. IF- 4.259

Singh DK, Bose S, Kumar S. Role of microRNA in regulating cell signalling pathways, cell cycle, and apoptosis in non-small cell lung cancer. Curr Mol Med. 2016; PMID: 26743518. IF- 2.254

Singh DK, Bose S, Kumar S. Regulation of expression of microRNAs by DNA methylation in lung cancer. Biomarkers. 2016; 21(7):589-99. doi: 10.3109/1354750X.2016.1171906. IF- 2.016

Singh DK, Antony SP. Molecular characterization and phylogenetic analysis of family *Adenoviridae* from marine environment as inferred from the hexon protein gene. IJBR . 2015; 3(3): 035-044. IF- 0.7658

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Declaration

I declare that the contribution of Dhirendra Kumar Singh was significant in the following publication and the thesis is based on bellow mentioned publication:

Singh DK, Németh T, Papp A, Tóth R, Lukácsi S, Heidingsfeld O, Dostal J, Vágvölgyi C, Bajtay Z, Józsi M, Gácser A. Functional characterization of secreted aspartyl proteases in *Candida parapsilosis*. mSphere. 2019; 4(4). pii: e00484-19. doi: 10.1128/mSphere.00484-19. IF- 4.28

The results reported in the PhD dissertation and the publication were not used to acquire any PhD degree previously and will not be used in future either.

Szeged, 2019

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Prof. Dr. Attila Gácser