**PH.D. THESIS**

# FUNGAL EICOSANOID BIOSYNTHESIS INFLUENCES THE VIRULENCE OF *CANDIDA PARAPSILOSIS*

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# Introduction

The increased incidence and high mortality rate of fungal infections in immunocompromised patients have become a serious concern in hospitals since the early 1990s. Among different pathogenic fungi, *Candida* spp. remain the most prevalent cause of invasive fungal infections, exceeding invasive aspergillosis and mucormycosis. Although, *C. albicans* is still the most common cause of invasive candidiasis, bloodstream infections caused by non-*albicans Candida* species such as *C. glabrata*, *C. krusei*, *C. auris*, *C. parapsilosis* and *C. tropicalis*, altogether have risen to account for approximately one-half of all candidemia cases. *C. parapsilosis* is ubiquitous in nature and commonly found on the surface of the human skin as a commensal. It is also frequently isolated from the gastrointestinal tract. It is the leading cause of invasive fungal infections in premature infants. Among non-*albicans Candida* spp., the incidence of *C. parapsilosis* is increasing in this particular patient group and in some hospitals, it even outnumbers *C. albicans* infections. Risk factors that are associated with *C. parapsilosis* driven neonatal candidiasis includes low birth weight (<1500 g), prematurity, prior colonization, the use of parenteral nutrition, intravascular catheters and prolonged treatment with antibiotics or steroids. This species is especially capable of forming biofilm on abiotic surfaces such as catheters, prostheses or other implanted devices, further increasing the chances for invasive infections.

It has been shown before that *C. parapsilosis* can produce immunomodulatory prostaglandin molecules from exogenous arachidonic acid. But the pathways regarding the prostaglandin production is still not known. So, the purpose of this study was to identify genes in *C. parapsilosis* associated with the production of immunomodulatory eicosanoid molecules and decipher their role in host pathogen interactions. Furthermore, we also investigated the role of a homologue of a multicopper oxidase in pseudohyphae and biofilm formation in this medically relevant fungus.

## Methods

### Cultivation and transformation:

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

### Molecular methods:

DNA and RNA isolation from yeast cells, cDNA synthesis, plasmid isolation from *E. coli*, molecular cloning using the Gateway system, targeted gene deletion from yeast strains, PCR, Fusion PCR, qRT-PCR, gel electrophoresis, RNA sequencing and generation of fluorescent tagged *C. parapsilosis* strains.

### Phenotypical 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).

### Cell isolation and culturing:

PBMC isolation, macrophage differentiation, *in vitro* stimulation of PBMCs/macrophages with different *C. parapsilosis* strains.

**Immunological methods**

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

**Other methods**

LC/MS and related data analysis, RNA sequencing data analysis, fluorescent confocal microscopy, *in vivo* virulence studies with a mouse model of systemic infection.

# Results

## Identification of genes involved in extracellular eicosanoid production in *C. parapsilosis*

Previously, it has been shown in our group that *C. parapsilosis* can produce fungal prostaglandin from externally supplied arachidonic acid (AA). Although, prostaglandin production is not regulated by the faty acid desaturase gene *OLE2* in *C. parapsilosis* unlike in its closely related specis *C. albicans*. After transcriptomic analysis in presence of arachidonic acid we have identified three genes- a multi copper oxidase (CPAR2_603600), an Acyl-CoA thiolase (CPAR2_80020) and an Acyl-CoA oxidase (CPAR2_807710) –that are potentially involved in prostaglandin production. Following their deletion, we throughoutly examined the corresponding deletion mutant strains' characteristics. The LC/MS data for the secretory eicosanoid analysis revealed that the deletion mutant strains of

CPAR2_603600, CPAR2_800020 and CPAR2_807710 produced less prostaglandin $D_2$ ($PGD_2$), prostaglandin $E_2$ ($PGE_2$), 15-keto-prostaglandin $E_2$ (15-keto-$PGE_2$) and $5D_2$-IsoProstane compared to the CLIB 214 wild type strain. These reductions were significant for $PGD_2$ and $PGE_2$ in *603600*Δ/Δ; $PGD_2$ and $PGE_2$ in *800020*Δ/Δ; $PGE_2$ and 15-keto-$PGE_2$ in *807710*Δ/Δ and $5D_2$-isoP in *800020Δ/Δ*.

## The deletion mutants are more efficiently phagocytosed and killed by human macrophages

Human peripheral blood monocyte derived macrophages (PBMC-DM) were used to characterize the virulence properties of mutant strains with altered eicosanoid producing profiles. We first examined the phagocytic activity of PBMC-DM by fluorescence-activated cell sorting (FACS). Our results indicated that PBMC-DMs ingested each of the mutant strains more efficiently than the wild type strain. We also examined the yeast cell killing efficiency of PBMC-DMs by comparing the recovered fungal CFUs. Our data showed that each of the mutant strains were killed more effectively by PBMC-DMs in comparison with the wild type strain.

## Host cell damage is decreased by the mutant strains

We next examined the mutant strains' abilities to cause host cell damage by measuring the amount of LDH released by human PBMC-DMs following infection. We found that the PBMC-DMs showed significantly lower LDH release when infected with the mutants compared to the wild type. Our results indicated that *603600*Δ/Δ, *800020*Δ/Δ and *807710*Δ/Δ mutant strains show a lower

host cell damaging capacity than wild type cells.

## Macrophages favor the uptake of *603600*Δ/Δ, *800020*Δ/Δ and *807710*Δ/Δ strains over the wild type

By using differently labeled *Candida* strains: a GFP tagged wild type strain and mCherry labeled mutant strains, we tested the uptake efficiency of the mutants by human PBMC-DMs using a competition assay and compared them to the wild type strain. By using confocal fluorescence microscopy, the percentage of internalized cells was calculated for each strain, and the values for the mutants were compared to those of the wild type's. Overall our results revealed that, PBMC-DMs significantly preferred the uptake of *603600*Δ/Δ, *800020*Δ/Δ and *807710*Δ/Δ cells over the wild type.

## Reduction in prostaglandin production alters the cytokine response

In order to examine the immunological responses triggered by the eicosanoid mutants, we stimulated human PBMC-DMs for 24 hours with each strain and determined the amount of cytokine and chemokine production. During the experiments we measured pro-IL1β, TNFα, Interleukin-1 receptor antagonist (IL-1ra), interleukin-6 (IL-6), interleukin-8 (IL-8) and interleukin-10 (IL-10) levels.

PBMC-DMs infected with *603600*Δ/Δ or *800020*Δ/Δ produced significantly higher Pro-IL-1β, IL-1ra, Il-6 and TNFα levels compared to the wild type strain. Whereas PBMC-DMs stimulated with *807710*Δ/Δ showed a higher amount of Pro-IL1β, TNFα and

IL-8 production, but no significant difference was observed in the IL-1ra and IL-10 levels compared with the reference strain. Notably, all mutant strains induced significantly higher amounts of IL-6 secretion.

We also analyzed the cytokine production of human primary PBMCs following fungal stimuli. PBMCs infected with 807710Δ/Δ produced higher amounts of IL-1β and TNFα, although there was no difference with the other mutant strains. These data suggest that fungal eicosanoids may also influence the host cytokine response.

## Deletion mutant strains show attenuated virulence in a mouse model of systemic infection

Following *in vitro* studies, we also aimed to examine the virulence of the *603600*Δ/Δ, *800020*Δ/Δ and *807710*Δ/Δ *in vivo* using a mouse model of disseminated candidiasis. Following the intravenous infection of BALB/c mice, the fungal burdens of different organs were determined three days after the infection. After CFU recovery, we found that mice infected with *603600*Δ/Δ showed significantly reduced fungal burdens in the liver and kidneys, while *800020*Δ/Δ inoculated mice also revealed lower fungal burdens in the liver and kidneys. Finally, CFUs recovered following *807710*Δ/Δ injection were significantly less in the spleen and kidneys compared to those recovered from wild type-infected mice (Fig 23). These results indicate that these eicosanoid biosynthesis gene play a role in *C. parapsilosis* virulence.

## Iron dependent growth of the *603600*Δ/Δ mutant strain

To investigate the role of CPAR2_603600- a homologue of the *C. albicans FET3* multicopper oxidase gene- in iron homeostasis regulation in *C. parapsilosis*, we examined the growth of the two independent homozygous deletion mutants in the presence of iron. Both mutants grew similarly to the wild type strain on YPD agar plates, indicating that CPAR2_603600 is not essential for viability in complex media. Next, the growth of the mutants was examined in the presence of an iron chelator, BPS (Bathophenanthrolinedisulfonic acid) chelating ferrous iron(II). Under iron-limited conditions, both mutants trains showed a growth deficiency compared to the wild type strain. These results suggest that CPAR2_603600 is required for growth under low iron conditions in *C. parapsilosis.*

## Phenotypic characterization of the *603600*Δ/Δ mutant

Phenotypic characterization was performed under 18 various growth conditions, including different temperatures, pHs and the presence of stresors such as cell wall, cell membrane, osmotic, oxidative and heavy metal stressors. YPD plate was used as an untreated control. The mutants grew slowly at lower temperatures and under alkaline conditions. They were highly sensitive to the cell wall stressor Congo red, to the cell membrane stressor SDS as well as to the presence of the metal ion chelator EDTA, and the oxidative stress inducer menadione and cadmium ($CdSO_4$).

## Significant reduction in yeast to pseudohypha production in case of the 603600Δ/Δ mutant

One of the most important traits of *C. parapsilosis* is its ability to change morphology. Previously, it has been shown that *TUP1* in *C. albicans* is, required for filamentous growth and is also involved in iron transport regulation. Therefore, we examined whether the deletion of CPAR2_603600 has any effect on pseudohypha formation in *C. parapsilosis*. Interestingly, the homozygous deletion mutant showed a significant reduction in pseudohypha formation in both solid and liquid psuedohypha inducing media. Specifically, the mutant strains showed a smooth colony morphology rather than a wrinkled phenotype observed in case of the wild type. Percentage of pseudohypha was calculated from the bright field microscopic images which revealed a significantly lesser amount of pseudohyphae present in the mutant strains in serum supplemented YPD, spider, YPS and in Lee's media. We also quantified the amount of pseudohypha production in the examined strains in hypoxic condition (5 % $CO_2$) by flow cytometry after cell wall staining. The mutant strain showed lesser percentage of pseudohypha compared to the wild type strain in dfferent media condition.

## The *603600*Δ/Δ mutant strain is defective in biofilm formation

One of the major factors associated with *C. parapsilosis* pathogenicity is its ability to form biofilm on abiotic surfaces. Biofilms produced by this fungus are composed of both yeast cells

and pseudohyphae. Thus, the mutant's defect in pseudohypha formation inspired us to examine whether this alteration affected the strain's biofilm forming ability. Biofilm forming abilities were quantified by using a metabolic assay (XTT reduction assay) and also by crystal violet staining (specific staining for biomass measurement). The deletion mutants showed a significant reduction in their biofilm forming ability compared to the wild type strain.

## Iron supplemented media restored pseudohyphae formation and biofilm formation

To check whether the defects in pseudohyphae and biofilm formation in the mutant depends on iron availability, all mutant strains were grown on YPD with additional 2 mM $FeCl_3$ in the preculture media before analyzing the pseudophyphae and biofilm forming abilities. We found that both the wild type and the two-homozygous deletion mutant strains showed similar colony morphology on different pseudohyphae induction media. Percentage of pseudohyphae was also calculated which showed that the pseudohyphae formation was partially rescued in the mutant strains.

We also analyzed whether the addition of additional iron in the preculture can recover the biofilm forming defect of the mutant by XTT assay and crystal violet staining. Our results clearly indicated that addition of iron also partially rescued the biofilm forming defects of these mutant strains.

## Overexpression of genes related to iron metabolism in the *603600Δ/Δ* strain

We also checked, if the lack of this multicopper oxidase gene alters the expression of other *C. albicans* ortholog genes related to iron uptake and metabolism. By qRT-PCR analysis we found that the genes *CFL5*, *HEM15*, *FTH1* and *FTR1* were highly overexpressed (fold change >5), while *CCC2*, *SEF1*, *HMX1*, *RBT5* and *HAP43* were slightly overexpressed (fold change >2). This indicates that in absence of the CPAR2_603600 gene, orthologous genes of ferric reductases, ferrous iron transporters and ferrous iron permeases which play a role in iron transport and metabolism were upregulated in *C. parapsilosis*.

## Summary

During the study we have shown that:

- 14.5% genes related to lipid biosynthesis process were upregulated when *C. parapsilosis* grown in presence of arachidonic acid.

- Three genes (CPAR2_603600, CPAR2_800020 and CPAR2_807710) involved in the production of eicosanoids such as $PGD_2$, $PGE_2$, 15-keto-$PGE_2$ and $5D_2$-isoP in *C. parapsilosis*.

- The eicosanoid mutants were more phagocytosed and killed by the human PBMC derived macrophages.

- The eicosanoid mutants induced altered cytokine response in the human PBMC derived macrophages.

- The mutants were less virulent in mouse model of systemic infection.

- Homologous of a multicopper oxidase gene CPAR2_603600 (*FET3*) needed for growth in iron limited condition in *C. parapsilosis*.

- Homozygous deletion mutant of CPAR2_603600 showed reduction in pseudohyphae formation in both liquid and solid pseudohyphae inducing media.

- The mutant also showed reduction in biofilm formation on abiotic surface.

- Addition of extra iron ($FeCl_3$) in the preculture media rescued the defects of pseudohyphae and biofilm formation in the homozygous deletion mutant.

- Homologue of iron homoeostasis genes were upregulated in absence of the CPAR2_603600 gene in *C. parapsilosis*.

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# Publications

**1. Tanmoy Chakraborty**, Ernst Thuer, Marieke Heijink, Renáta Tóth, László Bodai, Csaba Vágvölgyi, Martin Giera, Toni Gabaldón, Attila Gácser. Eicosanoid biosynthesis influences the virulence of *Candida parapsilosis.* Virulence (In press 2018). IF-4.665.

**2.** Varshney N, Schaekel A, Singha R, **Chakraborty T**, van Wijlick L, Ernst JF, Sanyal K. A surprising role for the Sch9 protein kinase in chromosome segregation in *Candida albicans*. Genetics. 2015;199(3):671-4. doi: 10.1534/genetics.114.173542. IF-5.963.

Cumulative impact factor: 10.628

## Declaration

I declare that, the contribution of Tanmoy Chakraborty was important in the following publication and the thesis is based on this publication:

**Tanmoy Chakraborty**, Ernst Thuer, Marieke Heijink, Renáta Tóth, László Bodai, Csaba Vágvölgyi, Martin Giera, Toni Gabaldón, Attila Gácser. Eicosanoid biosynthesis influences the virulence of *Candida parapsilosis.* Virulence (In press). IF-4.665.

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, 2018

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Dr. Gácser Attila