PH.D. THESIS

INVESTIGATION AND FUNCTIONAL CHARACTERIZATION OF GENES PLAYING A ROLE IN ZINC HOMEOSTASIS OF *CANDIDA PARAPSILOSIS* DURING HOST-PATHOGEN INTERACTION

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Introduction

Invasive *Candida* infections pose a significant health threat today, particularly for individuals with compromised immune systems, where untreated cases can be fatal. While *C. albicans* is the primary cause of systemic infections, a notable surge in non-*albicans Candida* species, such *as C. parapsilosis*, has been observed in recent decades. *C. parapsilosis*, known for causing numerous nosocomial infections, is particularly prevalent in cases involving premature babies and low-birth-weight newborns often resulting in high mortality rates. Despite of the extensive research on opportunistic fungal pathogenic infections, focus has been put on understanding the determinants of *C. albicans* pathogenicity predominantly, leaving a knowledge gap for *C. parapsilosis*, especially regarding its virulence factors and interaction with the immune system.

Heavy and light metal ions serving as cofactors for essential metalloproteins play a critical role in cell growth and overall cell homeostasis. Maintaining a proper balance of these ions is crucial for cellular functionality and various physiological processes. Zinc in

particular is vital, being a key component of many enzymes and playing a crucial role in both the innate and adaptive immune systems in higher eukaryotes. Cells utilize zinc transporters to regulate zinc absorption and storage, ensuring cellular zinc homeostasis. Host organisms protect themselves against pathogens by sequestering trace element (like zinc ion) binding proteins, rendering them inaccessible to most microbes — a phenomenon referred to as "nutritional immunity". Consequently, successful pathogens must possess efficient zinc transport systems to access and utilize these bound zinc ions. However, zinc can be toxic to pathogens if they fail to transport excess or substantial amounts of zinc ions to their intracellular stores. In the phagolysosome of macrophages, the concentration of zinc ions increases to a level toxic to pathogens, contributing to the elimination of the pathogen.

According to these, our research group aimed to conduct an *in silico* analysis of *Candida parapsilosis* zinc ion transporters, create gene deletion mutants for putative zinc ion transporters, and characterize the mutants phenotypically and the transporters functionally. Our

investigation specifically focused on the roles of zinc transporters *CpZRT21* and *CpZRC1* during interactions with mouse and human macrophages *in vitro*, as well as in the infection of *Galleria mellonnella* larvae and mice *in vivo*.

Methods

Cultivation of Cells Used in Experiments

Maintenance and culture of yeast cells, maintenance and culture of *E. coli* cells, culture of J774.2 mouse and M1 human macrophages

Methods Applied in the Characterization of *C. parapsilosis* Strains

Growth tests in the presence of different stressors, growth tests in liquid nutrient solutions with different pH values and zinc ion content, zincosome staining, zinc transporter GFP labeling

Molecular Techniques

DNA and RNA isolation from yeast cells, cDNA synthesis, PCR, gel electrophoresis, RT-qPCR

Techniques used in fluorescence microscopy

AlexaFluor647 staining, CONA-TRITC cell wall staining, DAPI nucleus staining, Calcofluor white cell wall staining, Fluozin3 zinc ion staining, Zinquin zincosome staining

In vitro J774.2 and M1 human macrophage-fungal assays

Determination of phagocytosis, determination of killing efficiency, determination of intracellular zinc ion quantity, examination of the elimination of *C. parapsilosis* based on zinc ion poisoning

Methods used in *in vivo* studies

Examination of wax moth larval infection and larval survival, implementation of intravenous infection in a mouse model: determination of CFU count after infection

Results

Identification of zinc transporters in *C. parapsilosis*

In collaboration with our collaborator (Duncan Wilson, University of Exeter, Exeter, UK) we performed an *in silico* analysis to identify the orthologs of zinc ion transporters from *C. albicans* SC5314 and *S. cerevisiae* S288C, in *C. parapsilosis* CLIB 214. Six potential zinc transporter-coding genes were identified whose orthologs play roles in zinc uptake (CPAR2_210740, CPAR2_806710, CPAR2_806720, CPAR2_500170), zinc detoxification (CPAR2_212100), and vacuolar zinc ion export (CPAR2_212080). Notably, no homologous gene segment encoding the CaPra1 zincophore protein was found in the *C. parapsilosis* genome, suggesting a unique mechanism for zinc mobilization and utilization compared to other pathogens. We investigated the expression of the identified genes in environments with different zinc ion concentrations and varying pH levels. The gene expression profiles of zinc uptake genes (*CpZRT21, CpZRT22, CpZRT23, CpZRT101*) were found to be similar to the ones of orthologs from *C. albicans*.

However, *CpZRC1*, implicated in zinc detoxification and storage, exhibited a distinct expression pattern. This uniqueness hinted at a potential divergence in zinc-related processes compared to other species.

Next, we have created homozygous deletion mutants of the six identified potential zinc transporter genes. Their viability was assessed under diverse stress conditions, including different temperatures and exposure to various stressors (e.g., cell wall and membrane stressors, acidic and alkaline chemicals, and light and heavy metals). Deletion of the *CpZRT21* gene resulted in a growth defect in acidic low zinc concentration environments, while *CpZRC1* was identified as essential in environments with high zinc ion content.

Combining *in silico* analyses and experimental results, our study suggests that the zinc uptake system in *C. parapsilosis* is unique and more intricate compared to known pathogens. This novel insight enhances our understanding of zinc homeostasis mechanisms in this species.

Analysis and functional characterization of the *CpZRT21* gene and the zinc uptake in *in vitro* macrophage and *in vivo* models

The *CpZRT21*, *CpZRT22*, and *CpZRT23* genes of *C. parapsilosis* exhibit substantial similarity to the *Sc/CaZRT2* genes, renowned for functioning in an acidic environment and zinc uptake. *CpZRT22* and *CpZRT23*, sharing a high degree of similarity which can be regarded as paralogs. This gene expansion is presumed to compensate for the absence of the zincophore protein Pra1. While *CpZRT21* is essential in an acidic, zinc-ion-limited setting, phenotypic studies reveal that *CpZRT22* and *CpZRT23* are not essential. Howevery, the loss of *CpZRT22* or *CpZRT23* results in increased *CpZRT21* expression, indicating potential shared regulatory mechanisms between the paralogs.

In vitro experiments involving phagocytosis and fungal elimination with mouse J774.2 and human M1-type macrophages demonstrate that *CpZRT21* deletion enhances the pathogen's survival during macrophage interaction. The Δ/Δ*CpZRT21* mutant exhibits partial

protection against high phagolysosomal zinc ion concentrations, as it lacks the principal plasma membrane zinc importer. However, *in vivo* experiments using *Galleria mellonella* insects and BALB/c wild-type mice reveal that, unlike the well-established role of CaZrt2 in *C. albicans*, *CpZRT21* does not have any impact on the virulence of *C. parapsilosis*.

Detailed characterization of the zinc detoxification system in *C. parapsilosis* and functional investigation of the vacuolar zinc transporter CpZrc1 in *in vitro* macrophage and *in vivo* models

We have identyfied the *S. cerevisiae* and *C. albicans* ortholog *ZRC1* (CPAR2_212100, *CpZRC1*) in *C. parapsilosis* as a zinc detoxification transporter. We observed a reduction in fungal viability upon *CpZRC1* deletion in environments with 5 mM zinc ions or more which underlines the zinc detoxifying role of CpZrc1. *C. parapsilosis* demonstrates the capability to form intracellular zinc ion stores, termed zincosomes in high zinc ion environments in a Zrc1 independent way (in

contrast to *C. albicans*). Our functional studies confirm the vacuolar membrane localization of the CpZrc1 transporter, resembling the zinc detoxification system in baker's yeast and *Cryptococcus neoformans*.

Our *in vitro* experiments with J774.2 mouse and human M1-type macrophages show that the absence of the *CpZRC1* gene does not have any impact on macrophage phagocytosis but leads to increased death of Δ/Δ*CpZRC1* cells lacking a localized zinc importer in the vacuolar membrane. *In vivo* experiments using *G. mellonella* larvae did not reveal any changes in the virulence in the lack of *CpZRC1*. However, in a wild-type mouse model, enhanced *CpZRC1* function significantly boosts resistance to *C. parapsilosis* cells in the spleen.

Characterization of the *C. parapsilosis* elimination system of murine and human macrophages based on zinc poisoning

Based on our *in vitro* investigations of phagocytosis and fungal elimination involving human macrophages of

the J774.2 and M1 type macrophages, we observed distinct outcomes when *CpZRT21* or *CpZRC1* gene was deleted.In the absence of the CpZrt21 being responsible for zinc uptake, fungal cells exhibited enhanced survival during interaction with macrophages. Conversely, the lack of the vacuolar zinc transporter CpZrc1 resulted in increased cell death compared to the control strain in the presence of both J774.2 and M1 type macrophages.

Our findings suggest a commonality in the effects of *C. parapsilosis* mutants deficient in zinc uptake (Δ/Δ*CpZRT21*) and zinc detoxification (Δ/Δ*CpZRC1*). When macrophages phagocytose the fungi, it appears that the fungal cells are eliminated with the assistance of toxic levels of zinc ions. This observation was recognized for both mouse J774.2 and human M1 type macrophages. Additionally, we noted that intracellular free zinc ions within macrophages co-localize with *C. parapsilosis* cells as early as half an hour after infection, with this phenomenon becoming more pronounced after 4 hours of incubation. These results are in line with the recent literature supporting the idea that macrophages have the

ability to augment phagolysosomal zinc levels, thereby promoting the effective elimination of pathogens.

Summary

1. In the course of our work, we identified six potential zinc transporter coding genes in *C. parapsilosis in silico*, and then generated and characterized deletion mutants of these genes.

2. CpZrt21 exclusively mediates *C. parapsilosis* growth in acidic environments, two additional Zrt2 orthologs in this species may share regulatory mechanisms with *CpZRT21* under zinc ion-limited conditions.

3. *C. parapsilosis* was found to accumulate zinc ion stores, termed as zincosomes, in environments with high zinc content independent of Zrc1 which is distinct from the one known from *C. albicans.* We identified a vacuolar membrane localized zinc transporter, CpZrc1, crucial for unique zinc detoxification in *C. parapsilosis* that also protecting the fungus from elimination by murine macrophages.

4. Our results support the notion that upon phagocytosis by murine and M1 type human macrophages, zinc ions

accumulate in the phagolysosome and contributing to fungal cell death.

List of publications

1. Németh, M. T., **Takács, T.**, Wilson, D., Gácser, A., (2018). Identification and functional characterization of zinc transporters in the human fungal pathogen *Candida parapsilosis*. Medical Mycology, **IF (2018): 2.9**

2. Takács, T., Németh, M. T., Vágvölgyi, C., Wilson, D., & Gácser, A. (2021) Investigation of the zinc uptake system of the human fungal pathogen *Candida parapsilosis*. Acta Microbiologica et Immunologica Hungarica, **IF (2021): 2.3**

3. Takács, T., Németh, M. T., Vágvölgyi, C., Wilson, D., & Gácser, A. (2021): Investigation of the parts of zinc homeostasis in the human fungal pathogen *Candida parapsilosis*. Acta Microbiologica et Immunologica Hungarica, **IF (2021): 2.3**

4. Mendoza, S., Zamith-Miranda, D., **Takacs, T.**, Gacser, A. Nosanchuk, J. D., Guimarães, A., (2021).

Complex and Controversial Roles of Eicosanoids in Fungal Pathogenesis. Journal of fungi, **IF (2021): 5,7**

5. Takács, T., Németh, M. T., Szilovics, Z., Vágvölgyi, C., Wilson, D., & Gácser, A. (2022). Investigation of the zinc uptake system of the human fungal pathogen *Candida parapsilosis.* Acces Microbiology, **IF (2021): 2.3**

6. Takács, T., Németh, M. T., Bohner, F., Vágvölgyi, C., Jankovics, F., Wilson, D., & Gácser, A. (2022). Characterization and functional analysis of zinc trafficking in the human fungal pathogen *Candida parapsilosis.* Open biology, **IF (2022): 5,8**

7. Bohner, F., Papp, C., **Takacs, T.**, Varga, M., Szekeres, A., Nosanchuk, J. D., Vágvölgyi, C., Tóth, R., & Gacser, A. (2023). Acquired Triazole Resistance Alters Pathogenicity-Associated Features in *Candida auris* in an Isolate-Dependent Manner. Journal of fungi, **IF (2023): 4,7**

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