PH.D. THESIS

INVESTIGATION OF THE MOLECULAR MECHANISMS UNDERLYING *CANDIDA PARAPSILOSIS* VIRULENCE

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Introduction

Globally, *Candida* species are still one of the most frequent causes of opportunistic mycoses. Several members of this genus cause a severe threat to immunosuppressed patients especially at intensive care units. The frequency of nosocomial infections caused by *Candida* species is continuously rising since the last decades. Although, the majority of cases are caused by *Candida albicans*, the so-called non-*albicans Candida* species –e.g. *Candida parapsilosis*- are also of rising concern. Besides being frequently isolated from immunocompromised patients, *C. parapsilosis* also threatens children and low birth weight neonates.

One of the major characteristics of this species is that in contrast with *C. albicans*, it is able to spread horizontally and to cause disease without primary colonization. Furthermore, *C. parapsilosis* is capable of forming biofilm on a variety of medical equipment such as on catheters, prostheses or other implanted devices and cells of this species can proliferate rapidly in parenteral nutrition. All of these clinically relevant features enhance the need to explore *C. parapsilosis* pathogenesis, the virulence factors involved in this process and their regulatory mechanisms.

The purpose of our study was to identify regulatory genes that might play a central role in *C. parapsilosis* virulence. In order to examine and characterize the targeted genes, we generated a deletion

mutant library. This approach enabled us to identify genes that may be key factors of *C. parapsilosis* virulence.

Methods

Cultivation and transformation:

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

Molecular techniques:

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

Methods for mutant library characterization:

Growth assays on complex and minimal media, survival tests in the presence of oxidative, cell wall and membrane stressors, biofilm formation (MTT metabolic activity assay) and morphology comparison (microscopic analyses), antifungal susceptibility testing (microdilution method)

Other methods:

Fluorescent staining and fluorescent confocal microscopy

In silico protein structure analysis

In vivo virulence studies with *Galleria mellonella* larvae

Results

Identification of *C. parapsilosis* virulence regulatory genes and preparation of a mutant library

Previously, in our laboratory we used a novel targeted approach in order to identify *C. parapsilosis* virulence regulatory ORFs. After analyzing the gene expression profile of fungal cells shortly following host-pathogen interaction, we found that several fungal genes showed altered expression. Using these data we created a list including mainly transcriptional factor and protein kinase encoding ORFs as potential regulators of *C. parapsilosis* virulence. We selected further targets to the pre-existing list as other supposedly virulence controlling genetic factors.

We aimed to examine these genes via the generation and characterization of homozygous deletion mutant strains. Each of the ORFs were removed using deletion constructions generated by the fusion PCR method. Gene elimination was achieved by auxotrophy complementation of a *C. parapsilosis* double auxotrophic strain (CPL2H1). Using this technique we successfully generated a deletion mutant library of 30 genes including 2-2 independent null mutant strains per target ORF.

Large-scale screen of the deletion mutant library

During the screening process we first examined the viability of each strain on complex and differently supplemented minimal solid

media. The collection was further tested in the presence of oxidative (H_2O_2) stressor, membrane perturbing agent (SDS) and different cell wall stressors (calcofluor white, congo red, caffeine). Cell morphology and biofilm formation of each strain was also examined. Furthermore, we also tested the antifungal susceptibility of the generated mutant strains.

According to our results, out of the 30 null mutants 9 strain showed defective growth in YPD complex and/or in minimal media, 14 strains showed altered sensitivity to the presence of oxidative, cell wall and / or cell membrane stressors, and 7 mutants showed altered susceptibility to azole and echinocandin type antifungals when compared to the wild type strain. Furthermore, 3 deletion mutant strains showed altered morphology in the presence of serum. In one case we detected only yeast forms, while in case of the other two abnormally elongated, long pseudohyphae were present in the medium in comparison with the reference strain. Another 3 mutant strain generated abnormal biofilm when compared to the wild type strain. Two out of the three strains showed decreased biofilm forming ability, while the remaining one generated biofilm more efficiently than the reference strain. According to the experimental results we suppose that several of the identified genes have pleiotropic effects as their removal led to 2 or more phenotypic aberrations. In summary, a total of 17 out of the 30 mutants showed a somehow altered phenotype relative to the wild type strain. Since most of the applied conditions chosen, somehow interfere with the fungi's virulence, we can conclude that at least half of the identified genes might play a role in the fungal pathogenicity.

Highlighted regulatory genes identified following host-pathogen interaction

Later we aimed to analyze the exact function of *C. parapsilosis* genes that were identified after the host-pathogen interaction.

The iron uptake and alternative carbon source utilization regulator: Cpar2_100540 transcriptional factor

During the general characterization of the collection, the *CPAR2_100540Δ/Δ* mutant strain showed increased sensitivity to alcalic pH and to the oxidative stressor H_2O_2. The closest orthologue of the identified ORF in *C. albicans* is called *HAP5*. The protein encoded by *HAP5* is involved in alternative carbon source utilization and also influences iron uptake. Iron uptake in both alcalic pH and iron limited environmental conditions is achieved with the contribution of ferric reductases (e.g. Frp1). In *C. albicans*, one of the major regulators of Frp1 is Hap5, since the removal of this ORF resulted in decreased *FRP1* expression and severe growth defects under both inducing conditions. In our experiments, the *CPAR2_100540Δ/Δ* deletion strain showed abnormal growth in both alcalic pH (pH 8) and under low-iron conditions. Furthermore, expression analyses revealed low *FRP1* expression levels in the mutant cells. Thus, the *CPAR2_100540* - similarly to the *C. albicans HAP5* - is required for iron uptake in *C. parapsilosis*.

Strains that are unable to utilaze altenative carbon sources, are usually suspected to have a respiratory chain defect. In both *S. cerevisiae* and *C. albicans*, the Hap5 influences the alternative carbon

sourse utilization via the regulation of certain respiratory chain members (cytochrome – c, *CYC* and cytochrome-c oxidase, *COX* elements). Although, the regulatory mechanism is opposite in the two species as deletion of *HAP5* results in decreased *CYC* and *COX* expression in *S. cerevisiae*, while effector gene expression elevates in *C. albicans*. According to our experimental results, removal of the *CPAR2_100540* resulted in weak growth in the presence of lactate and aminoacids (as sole carbon sources). In addition, the *CYC1* and *COX4* expression levels elevated in the null mutant strain. Thus, the Cpar2_100540 transcriptional factor is also involved in alternative carbon source usage, similarly to the *C. albicans* Hap5 protein.

Decreased virulence of *CPAR2_100540Δ/Δ* cells in *G. mellonella* infection model led us to the conclusion, that this regulatory factor is required for the pathogenesis is *C. parapsilosis*.

The yeast-to-filamentous growth switch regulator: Cpar2_200390 transcriptional factor

A unique characteristic of the *CPAR2_200390Δ/Δ* homozygous deletion strain is, that mutant cells form long aggregating pseudohyphae rapidly. The closest orthologous of this gene is *SPT3* that is involved in yeast-to-filamentous growth switch in both *C. albicans* and *S. cerevisiae*. Although, this regulatory mechanism is also opposite in the two species, as deletion of *SPT3* resulted in yeast-locked cells in *S. cerevisiae*, while in *C. albicans* –besides numerous other defects- a hyper-filamentous phenotype appeared. In *C. parapsilosis*, loss of the *CPAR2_200390* ORF also caused hyper-filamentous growth, thus the

identified gene -similarly to the *C. albicans SPT3*- is also a negative regulator of filamentous growth.

During the characterization of the *CPAR2_200390Δ/Δ* deletion strain we also found resistance to the cell wall stressor calcofluor white. Following the cell wall examination we detected elevated chitin and chitin oligomer content in the mutant cells. Gene expression analyses of four chitinase and another four chitin synthase encoding genes suggested altered chitin biosynthesis in the *CPAR2_200390Δ/Δ* strain.

Decreased virulence of the deletion mutant strain indicated that the Cpar2_200390 transcriptional factor is also required for the virulence of *C. parapsilosis*.

A master regulator of *C. parapsilosis* biofilm formation: Cpar2_602840 transcriptional factor

Deletion of the *CPAR2_602840Δ/Δ* ORF resulted in increased biofilm formation. Neither orthologues gene of *CPAR2_602840* has been previously associated with biofilm regulation, therefore the hereby-identified ORF has a unique role in *C. parapsilosis* biofilm formation. Thus, we examined the expression of all previously identified biofilm associated *C. parapsilosis* genes in the mutant strain. Our results suggest that the Cpar2_602840 is a master regulator of biofilm formation in this species, as all of the examined gene's expression levels significantly elevated in the *CPAR2_602840Δ/Δ* deletion strain. Furthermore, the cell wall of the mutant cells showed a markedly elevated mannose level, that might be a result of the hyper-biofilm forming phenotype.

Interestingly, we detected a moderate decrease in the virulence of the *CPAR2_602840Δ/Δ* strain when using the *G. mellonella* infection model. To further study the physiological relevance of the obtained phenotype we are planning to use the rat catheter model.

Regulation of adaptation to temperature change and oxidative stress: Cpar2_303700 protein kinase

Elimination of *CPAR2_303700* resulted in temperature sensitivity. Other features of the mutant strain included general growth defect and sensitivity to oxidative stressors. The closest orthologue of the identified gene is *CGI121* in *S. cerevisiae* that is a subunit of the highly conserved KEOPS/EKC complex. Due to the low level sequence identity, we aimed to compare the tertiary structure of Cpar2_303700 and Cgi121 *in silico* in order to search for similarities. Our results showed that the two proteins are highly similar. For further evidence, we examined if the Cpar2_303700 can establish a stable conformation with the closest interacting partner of Cgi121, the Bud32 subunit of the KEOPS/EKC complex. As a result we found a stable interaction between Cpar2_303700 and Bud32 *in silico* and the obtained conformation was similar to the original configuration. Thus the Cpar2_303700 and the Cgi121 might be similar proteins.

When studying telomer maintance in *S. cerevisiae*, previous authors reported that deletion of *CGI121* rescued defective phenotypes originated from telomer defects. Such phenotypes included thermosensitivity, oxidative stress sensitivity and general growth defects. According to our results, deletion of Cpar2_303700 led to the

above mentioned defects, therefore opposite regulatory mechanisms may be present in the two species. Although, in the future further experiments are required to support this hypothesis.

Infection studies of *CPAR2_303700Δ/Δ* resulted in attenuated virulence which suggested that Cpar2_303700 might also play a role in *C. parapsilosis* pathogenicity. According to our recent knowledge, this is the first time when the KEOPS/EKC complex is associated with the virulence of a pathogenic species.

Summary

During this project we have:

1. generated a *C. parapsilosis* deletion library of 30 members (with two independent homozygous strains/targeted gene) and
2. identified several null mutant strains (57% of the collection) that showed altered phenotype compared to the wild type. Out of the mutant collection
   - 30% of the tested strains showed decreased growth in complex and/or in minimal media,
   - 47% showed altered response to the presence of oxidative, cell wall and/or cell membrane perturbing agents,
   - 10% changed morphology in the presence of serum,
   - another 10% had defective phenotype in terms of biofilm formation,
   - and 23% of the null mutant strains showed altered susceptibility to the presence of antifungal drugs when compared to the wild type strain.

3. We have further investigated the function of four *C. parapsilosis* regulatory genes directly involved in host-pathogen interactions:
   - a transcriptional factor (TF) encoded by *CPAR2_100540* is involved in nutrient acquisition by controlling iron uptake (via regulation of a ferric reductase - *FRP1*) and alternative carbon

source utilization (via regulation of respiratory chain members -*CYC* and *COX*),

- a TF coded by *CPAR2_200390* is a novel negative regulator of filamentous growth and also controls the cell wall chitin content (through the regulation of chitinases and chitin synthases) thus may interfere with host recognition,
- a protein kinase translated from *CPAR2_303700* ORF is possibly involved in adaptation to oxidative stress and to temperature change in the host environment, therefore may promote survival,
- and a TF encoded by *CPAR2_602840*, that is a yet unidentified master biofilm regulator and is also responsible for the cell wall mannose homeostasis that may influence host recognition.

Virulence studies with *G. mellonella* confirmed each of the four ORF's involvement in *C. parapsilosis* pathogenicity.

Publications

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