PH.D. THESIS

CHARACTERIZATION OF GENES ENCODING SPORE SURFACE PROTEINS IN *MUCOR CIRCINELLOIDES*

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Introduction

Mucormycosis is an invasive fungal infection caused by certain members of the filamentous fungal order Mucorales. It most frequently occurs in patients who have an underlying immunocompromised status due to immunosuppressive treatment or haematological malignancy. The species most frequently identified as the etiological agents of mucormycosis belong to the genera *Rhizopus*, *Lichtheimia* and *Mucor*. The frequency of systemic mucormycosis has been increasing, mainly because of the elevating ratio of susceptible population.

Furthermore, Mucorales fungi display intrinsic resistance to the majority of routinely used antifungal agents (e.g., echinocandins and azoles), which also limits the number of possible therapeutic options. All of the above mentioned issues urge the improvement of molecular identification methods and the discovery of new antifungal targets.To achieve these goals, clarification of the pathomechanism of mucormycosis, understanding the interaction of these fungi with their hosts, and the identification of potential virulence factors and new biomarkers are essential. All these studies need the adaptation and routine application of molecular and genetic manipulation methods. Appropriate tools for genetic

manipulation, including efficient and reliable methods for genetic transformation, are basic requirements of cell biological and molecular studies, as well as of strain improvement by genetic and metabolic engineering.

As recent results have pointed out the importance of the CotH protein family in connection with virulence, our research was focused mainly on the extensive analysis of these genes and the clarification of their role in the virulence. However, that only a subset of the putative spore surface proteins identified in the *Mucor* genome showed homology to *Rhizopus* proteins associated with fungal pathogenicity. Thus, we also had to consider the possibility that the CotH family is a diverse group of proteins involved in many biological processes, and so forth we designed several experiments to elucidate the role of spore surface proteins in *Mucor*. Based on this, we attempted to perform the functional analysis of the CotH proteins, which involved monitoring the phenotypic alterations of genetically stable mutants created using CRISPR-Cas9 system. To reveal whether CotH proteins play a role in the pathogenesis and other biological processes of the *Mucor circinelloides* fungus.

Aims

Although the clinical significance of infections caused by Mucorales fungi is increasing, we have limited information about the factors that determine their virulence. The identification and comprehensive investigation of these mechanisms may form the basis for new, more effective, targeted therapeutic treatments in the future.

Taking into account all these aspects, we set the following objectives:

1. Application of the CRISPR-Cas9 system for genetic modification of a Mucoral fungus;
2. Identification and *in silico* characterization of the *cotH* gene family in *Mucor circinelloides*;
3. Disruption of *cotH* genes potentially involved in host-pathogen interaction from the genome and development of a disruption mutant library by CRISPR-Cas9;
4. Morphological, physiological and genetic characterization of the members of the established disruption library;
5. Investigation of the role of CotH proteins in pathogenicity in *in vitro* and *in vivo* models.

Methods

Molecular methods: Extraction of genomic DNA from fungal cells; Agarose gel electrophoresis; DNA recovery from agarose gel; RNA purification from fungal cells; cDNA synthesis (reverse transcription); Polymerase chain reaction (PCR) technique; Construction of fusion constructs; qRT-PCR

Development of disruption strains: Protoplast transformation of fungal cells; Gene editing *via* the CRISPR-Cas9 system; Isolation of monosporangial colonies and investigation of mitotic stability after gene disruption.

Characterization of disruption strains: Analysis of the growth ability of strains; Examination of spore structure by transmission electron microscopy (TEM) and the surface of fungal spores by scanning electron microscopy (SEM); Examination of the spore wall with a fluorescent dye; *In vivo* interaction with macrophage-like mouse cell line J774.2 with spores of *Mucor circinelloides* strains; Investigation of acidification of phagosomes containing *M. circinelloides* spores; Analysis of the elimination of *M. circinelloides* spores by macrophages.

***In vivo* pathogenicity models:** *Galleria mellonella*; *Drosophila melanogaster*; DKA mouse.

Results

1. Application of the CRISPR-Cas9 system for genetic modification of a Mucoral fungus

In our gene engineering experiments using the NHEJ error repair mechanism, we transformed the *M. circinelloides* double auxotrophic MS12 (*leuA*- and *pyrG*-) and a wild-type strain CBS277.49 to disrupt the *carB* gene. Using 100 μM gRNA and Cas9, transformation (i.e. disruption) frequencies were found to be 1.25×10^4 and 2×10^4 colonies per 10^5 protoplasts for MS12 and CBS277.49, respectively. Deletion was confirmed by sequencing the region containing the *carB* gene. However, more than 2.3 kb long deletions were detected upstream from the protospacer adjacent motif (PAM) sequence in the resulting mutants, also affected the adjacent *carRP* gene. To achieve HDR-based disruption of the *carB* gene, we created a disruption cassette, which served as the template DNA containing the *pyrG* gene as a selection marker and two fragments homologous to the target site to direct the HDR. When *carB* was targeted, transformation frequency was two colonies per 10^5 protoplasts. Gene disruption occurred *via* the integration of the selection marker at the appropriate sites, indicating the usefulness of this methodology to obtain targeted gene disruption and/or integration in *Mucor*. Stability

of the mutants was proven. No signs of degradation or reorganization of the integrated DNA were found. Thus, in Mucoral fungi, we successfully applied the CRISPR-Cas9 system for the first time.

2. ***Identification of cotH-like genes in the Mucor circinelloides genome and in silico analysis of the encoded proteins***

17 *cotH*-like genes were identified in *M. circinelloides* f. *lusitanicus* genome database. Based on predictions by NCBI Blast, the highest similarity between *Mucor* CotH-like proteins and *Rhizopus* CotH3 was found in CotH4 (49.3%), CotH6 (52.1%) and CotH13 (72.9%). It is important to note that these three proteins carry at least a part of the AA sequence described as "CotH motif". We predicted the putative intracellular localization of CotH-like proteins identified in *M. circinelloides*, based on which they were found to be predominantly extracellular in nature, and also examined the possible presence of signal peptide and GPI anchor in the proteins. Based on the *in silico* analysis of the CotH protein family, it has possibly a wide variety of functions, and some of its members exhibit great similarity with proteins recognised in *R. delemar* and *P. blakesleeanus*, whose function is unknown.

3. *Disruption of cotH genes potentially involved in host-pathogen interaction from the genome and development of a disruption mutant library by CRISPR-Cas9*

To disrupt the *cotH1-6* genes, disruption cassettes were generated containing the promoter and 5 'UTR regions of the target genes, as well as the 3' UTR and terminal regions and the *pyrG* gene, which encodes orotidine-5′-phosphate decarboxylase and complements the uracil auxotrophy of MS12 and were used as a template DNA to disrupt five *cotH* genes. To prove the specific gene disruption in *cotH* genes we performed conventional PCR, and to detect the template DNA-carrying properties of homozygous disruption strains, qRT-PCR and Sanger sequencing. In the case of the *cotH3* and *cotH4* mutants, the presence of disruption was validated using WGS. All of the mutants proved to be mitotically stable. Transformation frequency observed during the disruption of *cotH* genes was 2-6 colonies/10^5 protoplasts. Based on the phenomena observed during attempts to disrupt the *cotH6* gene we concluded that its disruption may be lethal for the fungus.

4. *Morphological, physiological and genetic characterization of the members of the established disruption library*

During cultivation at the optimum growth of the fungus (28 °C) the MS12-*ΔcotH4+pyrG* disruption mutant showed significantly decreased and MS12-*ΔcotH3+pyrG* had significantly increased growth. The MS12-*ΔcotH4+pyrG* strain retained its characteristic growth defect at lower and higher temperatures as well, and its growth was less affected by the cultivation on higher temperatures than the control strain. Strains *cotH1*, *cotH2*, *cotH3*, and *cotH5* were found to be more sensitive to a higher temperature compared to the control strain. The *cotH3* mutant proved to be heat sensitive at 20 °C and 35 °C.

The MS12-*ΔcotH3+pyrG* and MS12-*ΔcotH5+pyrG* disruption mutant strains had increased sensitivity to congo red (CR) dye, and the MS12-*ΔcotH4+pyrG* mutant proved to be more resistant to the CR cell wall stressor. Clacofluor white (CFW) showed a significant effect on the growth of all *cotH* mutants, which was shown to be more sensitive to the stressor in case of *cotH1*, *cotH2*, *cotH3*, and *cotH5* mutants and to be more resistant in case of the *cotH4* mutant strain. A possible explanation for the change in susceptibility to CV and CFW dye may be the structural change in the cell wall of *cotH* mutants, which may also be related to the increased sensitivity of MS12-*ΔcotH4+pyrG* spores to hydrogen peroxide. The MS12-*cotH4+pyrG* disruption strain had increased resistance

to SDS membrane detergent. The effect of SDS was observed from the third day of the cultivation time in case of the *cotH1*, *cotH2*, *cotH3* and *cotH5* strains, at which time intensive spore-forming processes of the fungus take place.

Based on TEM measurements, the spores of the MS12+*pyrG* strain used as control have a profile area of 7.77-75.35 μm, a cross-section of the spores were 2.71-7.87 μm and a longitudinal cross-section of the spores were 3.65-12.19 μm. Disruption of the *cotH1*, *cotH2* and genes did not affect either the shape or size of the spores. CotH4 and CotH5 proteins play a role in spore size formation. The examination of the spore wall layers by TEM revealed that *cotH* genes play a significant role in the formation of all the three layers, however, the role of these genes in the formation of the wall of circular and ellipsoidal spores may be different. In the wall of the circular spores of the MS12-*ΔcotH3+pyrG* strain, a thinner middle layer was observed, at the same time the thickening of the outer wall also took place. A decrease in the thickness of the middle layer could also be detected in the case of ellipsoidal spores. Based on all this, we can say that the CotH3 protein plays role mostly in the formation of the middle layer of the spore wall. The *cotH4* gene is essential for the formation of the inner layer of the cell wall of circular spores. In its absence, the middle layer abnormally thickens, however, in the

case of ellipsoidal spores this affects the inner layer. The spore wall of *cotH4* mutant strains, regardless of the shape of the spore abnormally thickens, resulting in the appearance of a characteristic phenotype. CotH5 protein is likely to play role in the formation of all three layers of circular spores, whereas in the case of ellipsoidal spores the formation of the middle layer of the spore wall.

Following mutation in the *cotH4* gene, the total chitin content of the spore wall was significantly increased, which is probably related to a change in some layers of the cell wall. There is also the possibility that the cell wall and cell membrane are separated and material accumulation between the two structures, e.g. accumulation of chitin occurs. Fluorescent staining of young hyphae has demonstrated that changes in chitin content are limited to fungal spores for strain MS12-*ΔcotH4+pyrG.*

To explore the efficiency of J774.2 macrophages in the recognition and elimination of spores produced by the mutant strains, the interaction of spores with macrophages was monitored using a flow cytometer. Although a significant change in the spore size and spore wall structure of MS12-*ΔcotH3+pyrG*, MS12-*ΔcotH4+pyrG* and MS12-*ΔcotH5+pyrG* strains was detected, no significant difference was found in the proportion of phagocytic macrophages,

however, J774.2 macrophages was able to ingest more than four spores in case of the MS12-*ΔcotH3+pyrG* and MS12-*ΔcotH4+pyrG* strains. Subsequently, J774.2 cells were coincubated with the labelled spores and then the ratio of pHrodo™ Red + macrophages was examined by imaging flow cytometry. Acidification of phagosomes is not affected by the examinated CotH proteins, and the absence of CotH proteins did not affected the survival of spores after *in vitro* interaction with macrophages.

5. Investigation of the role of CotH proteins in pathogenicity in in vitro and in vivo models

The *cotH3*, *cotH4*, and *cotH5* mutant strains showed reduced virulence in an *in vivo Drosophila* infection model. The *in vivo* viability studies in *G. mellonella* also confirmed the role of CotH4 protein in virulence. We first examined the infectivity of wild-type fungi (CBS277.49) in mice. Viability studies in DKA mice demonstrated that CotH3 and CotH4 proteins affect the pathogenicity of *M. circinelloides*.

Summary

1. For the first time in Mucoral fungi, the CRISPR-Cas9 system was successfully applied through targeted disruption of the *carB* gene encoding phytoene dehydrogenase.

2. The genetic engineering tool we optimized for *M. circinelloides* filamentous fungus proved to be a reliable genome editing method without the use of plasmids, nor any *off-target* effects, which also allowed us to perform gene disruption by the NHEJ and HDR error repair mechanisms.
3. 17 *cotH*-like genes were identified in *M. circinelloides* f. *lusitanicus* genome, the *in silico* analysis of which has expanded our knowledge of CotH proteins.
4. Successful disruption of five *cotH* genes was performed using the CRISPR system.
5. We validated our results by WGS analysis of two mutant strains.
6. The CotH1, CotH2, CotH3, CotH4, and CotH5 proteins play a role in adaptation to different temperatures as well as in developing the cell wall structure.
7. The CotH3, CotH4, and CotH5 proteins are involved in spore wall formation.
8. The CotH5 protein has a role in the sporangial wall formation.
9. The spore size formation is a process dependent on the *cotH4* and *cotH5* genes, in the absence of which smaller fungal spores are formed.

10. The CotH4 protein affects the total chitin content of the cell wall of spores, thereby affecting the composition of the spore wall formation.
11. The role of CotH3 protein in virulence was confirmed in *D. melanogaster* and DKA mouse models
12. The role of CotH4 protein in virulence was confirmed in *D. melanogaster*, *G. mellonella* and DKA mouse models.

The results summarized in the Ph. D. thesis were published in the following articles:

Ibragimova S *****, **Szebenyi C ***, Sinka R, Alzyoud EI, Homa M, Vágvölgyi C, Nagy G, & Papp T (2020). CRISPR-Cas9-based mutagenesis of the mucormycosis-causing fungus *Lichtheimia corymbifera*. *International journal of molecular sciences*, *21*(10), 3727. (* Divided first authorship.) **IF: 4.556**

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Cumulative impact factor: 31,719

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Further publications: MTMT ID: 10055593

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