PH.D. THESIS

CHARACTERIZATION OF GENES AFFECTING THE ADAPTATION OF *MUCOR LUSITANICUS* FOR ENVIRONMENTAL CHANGES

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Introduction

Mucor lusitanicus is a dimorphic fungus that can act as a human opportunistic pathogen. It exhibits hyphal growth in aerobic conditions or multi-budded yeast growth under anaerobic conditions with high hexose concentration. Filamentous form of *M. lusitanicus* is involved in virulence and the yeast-like form is less pathogenic. Dimorphism in *M. lusitanicus* is a complex process regulated by cyclic AMP-dependent protein kinase A pathway, calcineurin pathway, heterotrimeric G proteins and ADP-ribosylation factors.

Despite the aggressive surgery, antifungal treatment and correction of risk factors, the overall mortality rate of mucormycosis remains high, ranging from 50% and up to 90% depending on the infection form and underlying condition of the patient. Adaptation of *M. lusitanicus* for environmental changes complicates the treatment of mucormycosis infections. Till these days information about genes involving in the adaptation of *M. lusitanicus* to unfavorable conditions is limited. Characterization of these genes can broaden the knowledge about the adaptation mechanisms of Mucoralean fungi.

Heat shock factor (HSF)-type transcription factor 1 (Hsf1) plays a crucial role in regulating the expression of genes involved in cellular stress responses, including heat shock, oxidative stress, glucose starvation, heavy metal and proteotoxic stress response.

Quinidine drug resistance protein 2 (Qdr2) is a major facilitator superfamily (MFS) multidrug transporter that is located in the plasma membrane. It exports different compounds such as quinidine and barban across the fungal cell. It is also involved in the resistance to the anticancer agents like cisplatin and bleomycin.

Investigation of the role of *hsf*s and *qdr2*s genes in the adaptation of *M. lusitanicus* for environmental changes could be useful in developing new therapeutic agents.

Aims

The aim of the study was to identify and characterize differentially expressed genes in *M. lusitanicus* under anaerobic conditions, determine their functions, and investigate their role in yeast morphology, antifungal resistance, and pathogenicity.

Objectives of the presented research were the following:

1. Identification of differentially expressed genes under anaerobiosis in *M. lusitanicus* via transcriptomic analysis.

2. Validation and selection of genes for further characterization based on transcription analysis under various cultivation conditions, such as oxygen tension, cultivation time, different temperatures, and antifungal treatments.

3. Functional characterization of the selected genes:

a) Disruption of the selected genes by using a plasmid-free CRISPR-Cas9 method.

b) Morphological and physiological characterization of the disruption mutants (growth under different conditions, pathogenicity test).

Methods

Molecular methods:

DNA extraction; Isolation of DNA from agarose gel; RNA isolation; Agarose gel electrophoresis; Polymerase chain reaction (PCR); Construction of disruptions cassettes; cDNA synthesis (reverse transcription); qRT-PCR; Restriction digestion and ligation; Transformation of *E. coli*; Plasmid DNA extraction; RNA sequencing.

Development of disruption strains: PEG-mediated protoplast transformation using the CRISPR-Cas9 system.

Characterization of disruption strains: Analysis of the growth ability at different temperatures; Analysis of the effect of detergents and cell wall stressors; Sporulation and germination ability; Growth during the copper and potassium deficiency; Antifungal susceptibility test; Susceptibility to quinidine, cisplatin, and bleomycin; Susceptibility to different abiotic stressors; Analysis of lipid composition.

In vivo **pathogenicity models:** Virulence studies conducted using *Galleria mellonella* larval models.

Results

1. Identification of differentially expressed genes under anaerobiosis in *M. lusitanicus* via transcriptomic analysis.

After RNA-sequencing analysis, 539 genes were found to be differentially expressed. *hsf1*, *hsf2*, *nudix*, and *qdr2d* genes were differentially upregulated under anaerobic conditions, while *fen2* and *pho84* genes were differentially downregulated include.

2. Validation and selection of genes for further characterization based on transcription analysis under various cultivation conditions, such as oxygen tension, cultivation time, different temperatures, and antifungal treatments.

The results of the RNA-sequencing analysis were validated through qRT-PCR. Following the RNA-sequencing analysis and qRT-PCR heat shock transcription factors (*hsf1* and *hsf2*) and quinidine drug resistance 2 transporters (*qdr2a*, *qdr2b*, *qdr2c* and *qdr2d*) were chosen to further investigations. Selected *hsf1*, *hsf2* and four *qdr2* genes play roles in adaptation to temperature and oxygen level changes. *qdr2*s responded differently to antifungal treatments under aerobic and

anaerobic conditions, with all *qdr2* genes upregulated after exposure antifungals under aerobic condition; *qdr2a* and *qdr2b* downregulated, and *qdr2c* and *qdr2d* upregulated in response to antifungal treatment under anaerobic condition.

3. Functional characterization of the selected genes:

a) Disruption of the selected genes by using a plasmid-free CRISPR-Cas9 method.

hsf1, *hsf2*, *qdr2a*, *qdr2b*, *qdr2c* and *qdr2d* genes were disrupted by applying the CRISPR-Cas9 technique.

b) Morphological and physiological characterization of the disruption mutants (growth at different conditions, pathogenicity test).

hsf1 has a role in adaptation to temperatures below optimal, while *hsf2* is involved in low and high-temperature response. Disruption of *hsf1* and *hsf2* did not affect sporulation, germination, and virulence of *M. lusitanicus*. Disruption of one of *qdr2* genes tended to lead to changes in the expression of others, suggesting the existence of genetic compensation phenomenon that regulates the expression of *qdr2* genes in *M. lusitanicus*. Results showed that *qdr2* genes are involved in the

temperature adaptation. Qdr2b and Qdr2d transporters were found to be important for sporulation. Despite the previous studies on other fungal species, disruption of *qdr2* genes in *M. lusitanicus* did not affect cell membrane integrity, susceptibility to abiotic stressors, and potassium and copper sensitivity. *qdr2* genes were differentially expressed in response to antifungals. However, *qdr2* disruption mutants did not exhibit altered susceptibility to antifungals, indicating their limited role in antifungal resistance or the effect of genetic compensatory mechanisms. *qdr2*s disruption attenuated fungal virulence in *G. mellonella*, suggesting their role in the pathogenicity of *M. lusitanicus*. Results showed altered lipid content of *qdr2* mutants, indicating that *qdr2* genes are involved in the regulation of the lipid homeostasis.

Summary

The results of the present study suggested that *hsf1*, *hsf2*, *qdr2a*, *qdr2b*, *qdr2c*, and *qdr2d* genes are differentially expressed during the anaerobic growth of *M. lusitanicus*. In *M. lusitanicus*, Hsf1 and Hsf2 proteins have ability to influence the adaptation to different temperatures, maintenance of cell membrane and cell wall integrity. Qdr2a, Qdr2b, Qdr2c, and Qdr2d proteins have a role in temperature adaptation, cell membrane integrity, sporulation, germination, pathogenesis, and lipid homeostasis in *M. lusitanicus*.

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Articles

Homa, M., **Ibragimova, S.,** Szebenyi, C., Nagy, G., Zsindely, N., Bodai, L., et al. (2022). Differential gene expression of *Mucor lusitanicus* under aerobic and anaerobic conditions. *Journal of Fungi*, *8*(4), 404. **IF$_{2022}$: 5.6**

Ibragimova, S., Szebenyi, C., Sinka, R., Alzyoud, E. I., Homa, M., Vágvölgyi, C., et al. (2020). CRISPR-Cas9-based mutagenesis of the mucormycosis-causing fungus *Lichtheimia corymbifera. International Journal of Molecular Sciences*, *21*(10), 3727. (* Divided first authorship) **IF$_{2020}$: 5.924**

Cumulative impact factor: 11.524

Abstracts

Ibragimova, S., Vágvölgyi, C., Nagy, G., Papp, T. (2023). Characterization of the *qdr2* multidrug transporter genes of *Mucor lusitanicus.* 16th European Conference on Fungal Genetics programme & abstracts book, 854-855.

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K., Márialigeti; O., Dobay, Eds., *Acta Microbiol et Immunol Hung Budapest, Hungary: Akadémiai Kiadó,* 72.

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Vágvölgyi, C., **Ibragimova, S.,** Szebenyi, C., Nagy, G., Papp, T. (2018). Construction of an uracil auxotrophic mutant of the opportunistic pathogen *Lichtheimia corymbifera* using an *in vitro* CRISPR/Cas9 method. *Romanian Journal of Laboratory Medicine*. *26*(3), 57.

Declaration

I declare that the contribution of Sandugash Ibragimova was significant in the listed publications and the doctoral process is based on the publications listed. The results reported in the Ph.D. dissertation and the publications have not been used to acquire any PhD degree previously and will not be used in the future either.

Szeged, 11.01.2024

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