PH.D. THESIS

CHARACTERIZATION OF NOVEL HYDROPHOBIC SURFACE BINDING PROTEINS IN *MUCOR CIRCINELLOIDES*

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Introduction

Mucormycosis is a life-threatening opportunistic fungal infection, caused by a group of moulds belonging to the order Mucorales. It is the third most commonly occurring invasive fungal infection, followed by aspergillosis and candidiasis. The importance of Mucoralean species has grown in recent years since there is an increase in the number of patients with predisposing factors for mucormycosis. Very poor outcome has been observed despite the current treatment options, like correction of the underlying risk factors, antifungal therapy, and aggressive surgery. This is mainly due to less knowledge or poor understanding on the pathogenesis of the infection, as well as the role of specific virulence factors and the interaction with host immune system. Hence rises the urgency to identify various virulence factors of these pathogenic fungus that could pose as a potential diagnostic and therapeutic target.

Hydrophobic surface binding protein A (HsbA) are small galactomannoproteins of fungi that could either be bound to the cell wall or secreted to outside the cell. It is suggested that these proteins may have role in the adhesion to hydrophobic surfaces, in recruiting cutinases and hydrolytic enzymes on hydrophobic surfaces and colonization and penetration into the plant tissues; *hsbA* genes were found to be

upregulated in human pathogen fungi during the infection. The aim of the present study was to characterize the hsbA genes of the Mucoralean model organism *M. circinelloides*. Since, *M. circinelloides* is also one of the most frequently isolated species from clinical samples of patients suffering from mucormycosis.

Methods

Culture maintenance and transformation of bacteria and Fungi:

Cultivation of *M. circinelloides* f. *lusitanicus*, *Escherichia coli* and *Pichia pastoris*, competent cell preparation for bacteria and fungi, transformation techniques.

Molecular methods:

Plasmid isolation from *E. coli*, DNA and RNA isolation from fungal cells, gene cloning using PCR, qRT-PCR, gel electrophoresis, construction of plasmids for transformation.

Morphological and Physiological characterization:

Growth assay on minimal and cell wall stressor containing media, biofilm formation (safranine staining assay), morphology comparison (Light microscopy, Scanning electron microscopy), sporulation and germination ability, biofilm, hydrophobicity measurement (alcohol percentage test).

Interaction studies:

Phagocytosis assay using murine alveolar macrophage J774.2 cell lines.

In vivo models:

Virulence studies conducted using *Galleria mellonella* larval models

Purification of Heterologously expressed proteins:

Nuvia cPrime cation exchange chromatography, size exclusion chromatography with a HI prep 16/60 Sephacryl S200HR column.

Results

Identification of *hsbA* genes in the *M. circinelloides* genome

Six potential *hsbA* genes were found on carrying out BLAST searches using the *L. corymbifera hsbA1* gene in the *Mucor* genome database, named as *hsbA1a*, *hsbA2*, *hsbA1b*, *hsbA3*, *hsbA4* and *hsbA5*, respectively. Interestingly the coding sequences of *hsbA1a* and *hsbA1b* are totally the same, despite the differences between their promoter and terminator regions, hence indicating a recent duplication of the *hsbA1* gene in *M. circinelloides*. *hsbA5* was the least similar to *L. corymbifera hsbA* gene. Because they showed the highest similarity to the *L. corymbifera hsbA* gene, HsbA1a (which is referred as *hsbA1* in our further analysis), HsbA2, HsbA3 and HsbA4 were selected for further detailed analysis.

Transcription of the *hsbA* genes of *M. circinelloides*

qRT-PCR analysis indicated that the tested *hsbA* genes (i.e. *hsbA1a*, *hsbA2*, *hsbA3* and *hsbA4*) are expressed throughout the whole life cycle and especially from the second day of cultivation, i.e. in the late hyphal stage. At the higher temperature 35 °C, all four genes proved to be upregulated compared to their transcription activity at 25 °C, indicating a clear temperature regulation in their expression. All four

genes were downregulated by anaerobiosis (especially *hsbA1a* and *hsbA2*, which were inactive under anaerobic growth) indicating that they are linked to the aerobic and/or hyphal growth. Presence of human serum upregulated *hsbA1a*, *hsbA2* and *hsbA4* but did not affect *hsbA3* suggesting different roles in the adaptation for environmental changes. Presence of lignocellulosic material in the cultures induced only the transcription of *hsbA3* suggesting that this gene may participate in the degradation of plant material.

Disruption and overexpression of *hsbA1*, *hsbA2* and *hsbA3* genes in *M. circinelloides*

Single disruptions of *hsbA1*, *hsbA2* and *hsbA3* were carried out by integrating the *pyrG* selection marker gene into the corresponding hsbA gene via the CRISPR-Cas9 technique using the CRISPR-Cas9 method. From which we obtained deletion mutants from each and confirmed through PCR, qRT-PCR analysis. *hsbA1* and *hsbA2* whole genome were sequenced and confirmed presence of no off targets.

Three overexpressed mutants were obtained for the *hsbA1*, *hsbA2* and *hsbA3* genes, which were confirmed through PCR, sequencing, and qRT-PCR analysis.

Characterization of *hsbA* mutants

Disruption and overexpression of the three *hsbA* genes had only a slight effect on the growing ability of the fungus. Overexpression of *hsbA2* led an increased sporulation indicating that function of this gene has a role in the sporangiospore production. Interestingly, overexpression of all three genes decreased the germination ability of the sporangiospores while gene disruption did not affect this feature. Overexpression mutants also displayed increased sensitivity to cell wall and membrane stressors suggesting structural alterations in the outer layers of the fungal cells.

Effect of the *hsbA* genes on the biofilm formation of *M. circinelloides*

Biofilm forming capacity of the mutants, in which the *hsbA* genes were, disrupted somewhat decreased indicating that HsbA may contribute to the biofilm formation of *M. circinelloides.*

Influence *hsbA* genes on the hydrophobicity of the *M. circinelloides* mycelium

Hydrophobicity tests on the mycelial surface of the overexpression mutants proved to be more hydrophobic due to higher concentrations of ethanol were able to penetrate

through the surface of the mycelia when compared to those of the disruption mutants and the control strain where lower concentrations of ethanol could easily penetrate the mycelial surface.

Scanning electron microscopic analysis of mutants

SEM image analysis of overexpressed and deletion mutants further revealed that surfaces of MS12+pAV1 small spores were more granulated, when compared to the parental MS12 strain. Whereas large spores of all deletion mutants were slightly granulated or smooth surface when compared to parental strains, hence suggesting the involvement of HsbA in the cell surface integrity of microspores and macrospores. Although the exact mechanism behind the cause of this variations are yet to be determined.

Phagocytosis assay

Disruption and overexpression of the three *hsbA* genes had no effect on the phagocytosis of *M. circinelloides* by J774.2 cells. Hence suggesting that HsbAs play no role in the recognition and phagocytosis of *M. circinelloides* by J774.2 macrophages

Virulence of the *hsbA* mutants in *Galleria mellonella* non-vertebrate model

In *Galleria* non-vertebrate model, overexpression of *hsbA2* resulted in significantly decreased virulence while that of all deletion mutants significantly increased. This result may suggest that the HsbA level and/or the hydrophobicity of the mycelium may affect the pathogenicity of *M. circinelloides*.

Heterologous expression of *M. circinelloides* HsbA1 in *P. pastoris* KM71H

For future analysis of the proteins and their functions recombinant HsbA1 was expressed in a *Pichia pastoris* heterologous expression system and the produced protein obtained in the 7^{th} day culture supernatant. The purified protein was obtained after purification process with size exclusion chromatography with a HI prep 16/60 Sephacryl S200HR column. The protein was confirmed to be HsbA1 protein through mass spectrometric analysis.

Summary

The results of the present study suggest that HsbA proteins are hydrophobic surface-active proteins that are differentially expressed during the aerobic growth of *M. circinelloides*. In *M. circinelloides*, HsbAs have ability to influence the cell wall integrity of spores and hyphae, sporulation and germination capacity of the spores, hydrophobicity of the hyphal surfaces, biofilm formation and virulence.

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Declaration

I declare that the contribution of Amanda Grace Vaz 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, 29 .09.2020

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