Ph.D. thesis

MOLECULAR ANALYSIS OF GENES ENCODING IRON TRANSPORT PROTEINS IN OPPORTUNISTIC PATHOGEN ZYGOMYCETES

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Introduction

Several members of the class Zygomycetes have been reported as agents of opportunistic fungal infections designated as zygomycoses. They can cause severe and frequently fatal infections in debilitated or immunocompromised patients. *Rhizopus*, *Mucor*, *Absidia* and *Rhizomucor* species seem to be most frequently involved in human and animal mycotic diseases.

The risk of development of zygomycosis increases first of all in diabetic (particularly associated with ketoacidosis) and immunocompromised patients associated with medical intervention. Neutropenia as a consequence of hematological malignancies is also serious predisposing factor to the development of disseminated zygomycosis. Burn injuries and extreme malnutrition are also risk factors, and patients on dialysis treated with the iron chelator deferoxamine form a specific risk group as well.

Although zygomycoses are relatively rare, the incidence of such infections shows an increasing tendency due to the improved medical techniques (solid organ or bone-marrow transplantations, prolonged steroid treatment or chemotherapy). Therefore the number of patients at risk for zygomycosis is continuously increasing in line with the expanding population of the immunocompromised patients and the continuous rise of diabetes. This fact together with the associated high mortality rate (from 75 to 95 %), the difficulty of their diagnosis and their resistance to the most widely used antifungal drugs underline the importance of the studies on the molecular background of this infections and indicate that the development of new diagnostic and therapeutic methods is urgently needed. Molecular and serological diagnostic methods are still in the experimental phase, and

efforts to identify possible virulence factors of Zygomycetes started only a few years ago.

Recently, it has been recognized that patients with a permanently high level of iron in their serum and those, who are treated with the iron chelator deferoxamine (to remedy the iron overload conditions) have an increased susceptibility to invasive zygomycosis. Based on clinical and experimental observations it has been proved that zygomycetous fungi can uptake and use the deferoxamine-chelated iron efficiently, so it strongly stimulates their growth (BOELAERT et al. 1993). Acquisition of iron in the host is a crucial pathogenetic event in both bacterial and fungal infections. The amount of free iron available for microbial growth in the human serum is extremely low: nearly all iron is present in a form bound to carrier proteins. To overcome this, pathogenic microbes developed different iron-scavenging mechanisms to solubilize the iron and to transport it into the cells. Deferoxamine is a hidroxamate-type siderophore from bacterial origin which is able to chelate serum iron effectively. Studies with radiolabelled deferoxamine indicated that iron was liberated from deferoxamine extracellularly before being taken up into the cell (DE LOCHT et al. 1994). The dissociation of the iron from the deferoxamine-iron complex is an energy-dependent process, which contains a reductive step; so the transport of the iron across the cell membrane is probably due to a high-affinity iron permease. The high-affinity iron permease gene (*ftr1*) of the zygomycete *Rhizopus oryzae* has been cloned and characterized recently by FU et al. (2004). They proved that iron starvation causes strong expression of the *ftr1* gene in the fungus whereas it is suppressed in iron-rich environments. These observations support the central role of the iron metabolism and iron-transport proteins in particular, the high-affinity iron transport system in the

fungal virulence.

Aims

Molecular investigation of the pathogenesis of zygomycetous fungi and examination of factors playing an important role in the fungal infection may lead to the determination of new possible therapeutic targets, the development of new diagnostic methods as well as of effective antifungal treatments. Development of rapid and accurate strain identification and typing methods for Zygomycetes is also beneficial from a biotechnological point of view. It could reveal important data concerning the epidemiology of zygomycoses. It could also help the successful treatment of these infections due to the precise identification of the pathogen species and strains, which have different antifungal susceptibility. The main factor hindering the investigation of the pathogenesis of zygomycetous fungi is the limited availability of genetic manipulation techniques. Development of more efficient transformation systems than those presently applied is a basic requirement for a detailed analysis of the possible virulence factors and for the studies on the molecular and the genetic background of the pathogenicity of Zygomycetes.

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

1. Identification, cloning and comparison of genetic elements that are homologous with the high-affinity iron permease gene (*ftr1*) in zygomycetous fungi.
2. Elaboration of a rapid and routinely applicable species and strain identification method based on the *ftr1* gene.
3. Working out selection and transformation methods, which are

essential for the investigation of the genetic background of fungal pathogenesis. Investigation of the applicability of the *Agrobacterium tumefaciens*-mediated transformation (ATMT) method in Zygomycetes.

4. Construction of transforming vectors based on the isolated *ftr1* gene and generation of *ftr1* deleted mutant strains with gene disruption.

Future plans: Investigation of the role of the high-affinity iron permease in the virulence of *Rhizomucor pusillus*.

Methods

DNA based techniques:

- DNA extraction
- Polymerase chain reaction (Inverse PCR, SON-PCR, TAIL-PCR)
- Cloning of DNA fragments
- DNA sequencing
- Plasmid construction
- Transformation of bacteria
- Plasmid DNA extraction
- Southern hybridization

Analysis of the nucleotide and the amino acid sequence data:

- Checking of the nucleotide sequences
- Analysis and comparison of the nucleotide sequences, deduction of the amino acid sequences from nucleotide sequences
- Alignment of the nucleotide and the amino acid sequences
- Construction of phylogenies

Genetic transformation of fungi:

- Optimization of hygromycin B selection
- Generation of protoplasts
- PEG-mediated integrative transformation
- *Agrobacterium tumefaciens*-mediated transformation
- Generation of monosporangial clones
- Mutant enrichment
- Fluorescent microscopy

Results

Identification and comparison of high-affinity iron permease genes (*ftr1*) of different zygomycetous fungi (Nyilasi et al. 2005b).

In the course of our work, *ftr1* fragments were amplified from twenty-six zygomycetous strains representing five different genera (e.g. *Rhizopus*, *Rhizomucor*, *Syncephalastrum*, *Mucor* and *Backusella*) Degenerated primers were designed on the basis of *Rhizopus oryzae* and *Candida albicans ftr1* gene sequences. The amplification products' size varied between 585 and 740 bp. The nucleotide and the deduced amino acid sequences were determined and all sequence data obtained in the study were deposited in the EMBL nucleotide sequence database.

The nucleotide and the deduced protein sequences of the amplified *ftr1* fragments were aligned and compared, and a phylogenetic analysis was carried out. The phylogenetic trees calculated from these sequences were in agreement with the phylogenies based on 18S and 28S ribosomal DNA sequences. Our results supported the need of the revision of the presently used taxonomic system of the Zygomycetes, which was based mainly on morphological characters. The close relationship between *R. schipperae*, a newly described, thermophilic *Rhizopus* species and *R. microsporus* var. *rhizopodiformis* was verified. At the same time the need to handle *R. schipperae* as a separate species was confirmed. Our results also supported that *R. niveus* is a representative of a separate species.

Development of methods based on *ftr1* sequences for the identification of clinically relevant Zygomycetes (Nyilasi et al. 2005c, Nyilasi et al. 2008b).

An identification method based on PCR amplification of the high-

affinity iron permease 1 was described. A partial fragment of the *ftr1* gene was used to generate a sequence dataset in order to find characteristic motifs applicable for oligonucleotide design. Species-specific PCR primer pairs were established and amplification methods were tested for the rapid and accurate detection of clinically important Zygomycetes. *R. oryzae*, *R. microsporus* var. *rhizopodiformis*, *R. microsporus* var. *oligosporus*, *R. schipperae*, *R. niveus* and *R. stolonifer* isolates could be identified at the species, while *Rhizomucor*, *Syncephalastrum* and *Mucor* isolates could be identified at the genus level with this method.

All the species under study were differentiated by PCR–restriction fragment length polymorphism (PCR-RFLP) analysis. Amplification products obtained with the degenerated primers were digested with *Alu*I restriction enzyme yielding fragment patterns characteristic of the species studied. *Mucor* species undistinguishable by *ftr1* specific PCR could be divided into three subgroups using the PCR-RFLP. In the same time *R. miehei* and *R. pusillus* isolates could be distinguished at the species level. Moreover, *R. oryzae* and *R. stolonifer* isolates could be identified at the subspecies level with this method.

Isolation and characterization of high-affinity iron permease genes in *Rhizomucor miehei* and *Rhizomucor pusillus* (NYILASI et al. 2005d).

The whole *ftr1* gene was identified in *R. miehei* and *R. pusillus* with the inverse PCR and SON-PCR methods using specific primers designed on the basis of the partial *ftr1* sequences. The promoter and terminal regions of these genes were also determined. The *ftr1* genes were cloned and analyzed: a 2671 bp long sequence was determined in *R. miehei* and a 2253 bp long sequence in *R. pusillus*. The 1270 bp long *ftr1* gene in

R. miehei and the 1280 bp long *ftr1* gene in *R. pusillus* contain the 1149 bp long open reading frames (ORF), respectively. Both ORFs encode a 382 amino acid long protein. Besides the coding regions a 646 bp (*R. miehei*) and a 470 bp (*R. pusillus*) long promoter region and a 755 bp (*R. miehei*) and a 503 bp (*R. pusillus*) long terminal region were determined as well. The whole nucleotide and the deduced amino acid sequences were deposited in the EMBL nucleotide sequence database.

Consensus sequences were also identified in the regulator regions of the *R. miehei* and the *R. pusillus ftr1* genes and the codon usage of the genes was also investigated. We concluded that the two *ftr1* genes are not constitutive genes, GATA elements identified in the promoter also indicate this finding.

Comparing the *R. miehei* and the *R. pusillus ftr1* genes, 86 % of the nucleotides were found to be identical, whereas 90 % identity was found at the amino acid level. We did not find any homology between the introns, promoter and terminal regions of the two *ftr1* genes; however the position of the introns was the same in both species. The N-terminal and the central regions of *R. miehei* and *R. pusillus* putative FTR1 proteins revealed significant homology with other fungal FTR1 proteins, while the C-terminal region was more variable.

Development of selection and transformation methods required for the investigation of the background of the pathogenesis (NYILASI et al. 2005a, NYILASI et al. 2008a).

Different selection and transformation methods were tested for the investigation of the background of the pathogenesis and the role of the high-affinity permease in the fungal virulence. Conditions for a new direct

selection system based on hygromycin B resistance were elaborated for zygomycetous fungi. The selective medium supplemented with rose bengal and dichloran was suitable for the isolation of hygromycin B resistant *Mucor* transformants. Our experiments revealed that a combination of these compounds not only reduced the colony size and suppressed the production of aerial hyphae in *Mucor* but also increased the sensitivity of the fungus to this antibiotic.

The genetic transformation of the zygomycetous fungus *Mucor circinelloides* with the *A. tumefaciens*-mediated transformation method was worked out using the hygromycin B resistance-based selection. Several conditions of transformation were optimized and the method was adapted to other zygomycetous fungi as well (such as *Backusella lamprospora* and *R. pusillus*).

Hygromycin B resistant fungal clones were isolated on selective medium. The presence of the hygromycin B resistance gene in the genome of the *Mucor* transformants was verified by polymerase chain reaction and southern hybridization: the latter analyses revealed the single copy integration of the transforming sequence in the host genome. In each transformant, the *hph* gene was integrated in different chromosomal sites.

Binary vectors based on the Ti plasmid of the *A. tumefaciens* were constructed. Transforming plasmids carried the hygromycin B resistance gene (*hph*) and the green fluorescent protein (*gfp*) gene. Both genes were placed under the control of the promoter and terminator regions of the *M. circinelloides gpd1* gene. The expression of the *gfp* gene served as a direct evidence for the successful transformation.

The ATMT method was adapted to *B. lamprospora* and the introduction and expression of the *hph* and *gfp* genes into the *Backusella*

genome were demonstrated. The presence of the introduced *hph* and *gfp* genes in the transformants was verified by PCR. The introduced genes could also be amplified directly from the spores. The transformants proved to be resistant to hygromycin B and their hyphae exhibited intensive fluorescence, indicating that the regulator sequences of the *M. circinelloides gpd1* gene were able to ensure the transcription of the introduced heterologous genes.

Currently, transformation experiments of a *M. circinelloides* uracil auxotrophic mutant using ATMT are proceeding. We are planning to investigate the possibility of the construction of stable integrative transformants by this method. Our aim is to investigate the stability of the introduced endogenous and exogenous genes and to study the processes following the transformation and the integration events. Transforming vectors using the Ti plasmid of the *A. tumefaciens* were constructed, in which the orotidine 5'-phosphate decarboxylase (*pyrG*) gene of *M. circinelloides* and the *gfp* gene were inserted.

A selection method based on uracil auxotrophy was elaborated and uracil auxotrophic *R. pusillus* strains harbouring mutations in the orotidine 5'-phosphate decarboxylase (*pyr4*) coding gene were created. One of these strains was used in the gene disruption experiments.

Construction of transforming vectors based on the isolated *ftr1* gene and creation of *ftr1*$^-$ deletion mutants.

Vectors harbouring the isolated *ftr1* genes were constructed to investigate the role of the high-affinity iron permease in the virulence of *R. pusillus*. For disruption of the genomic *ftr1* gene, transforming vectors containing the *pyr4* gene previously inserted into the *ftr1* gene were constructed. Transformation of *R. pusillus* was carried out with the ATMT

method, the proof of the disruption of the *ftr1* gene is still proceeding. In the future, we are planning to investigate the role of the high-affinity iron permease in the virulence of *R. pusillus* using *ftr1*⁻ mutant strains in animal models of systemic zygomycosis.

Summary

Our results can be summarized in the following points:

1. Partial *ftr1* fragments were amplified from twenty-six zygomycetous strains representing five different genera. The *ftr1* fragments were cloned and the nucleotide and the deduced amino acid sequences were determined.
2. Phylogenetic analysis was carried out based on the nucleotide and the deduced amino acid sequences of the amplified *ftr1* fragments.
3. PCR diagnostic methods based on the *ftr1* gene sequences were elaborated and tested for the identification of clinically important zygomycetous fungi.
4. The entire high-affinity iron permease gene of *R. miehei* and *R. pusillus* was determined using inverse PCR and SON-PCR methods. The regulator regions of these genes were also determined.
5. Conditions for a new direct selection system for the isolation of transformants based on hygromycin B-resistance have been elaborated and tested for *Mucor*.
6. We described the genetic transformation of the zygomycetous fungus *M. circinelloides* with the *A. tumefaciens*-mediated transformation method using the selection based on hygromycin B resistance. The method was optimized and adapted to other zygomycetous fungi as well (*B. lamprospora*, *R. pusillus*).
7. The presence of the introduced genes in the genome of the transformants was verified by polymerase chain reaction and southern hybridization analysis.

8. Binary vectors were constructed using the Ti plasmid of the *A. tumefaciens*.
9. A uracil auxotrophic *R. pusillus* strain mutant in the orotidine 5'-phosphate decarboxylase (*pyr4*) coding gene was created.
10. Transforming vectors harbouring the isolated *ftr1* genes were constructed, and transformation of *R. pusillus* was carried out with the ATMT method.

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

Nyilasi I, Papp T, Takó M, Nagy E and Vágvölgyi Cs (2005) Iron gathering of opportunistic pathogenic fungi. Acta Microbiol Hung. 52: 185-197.

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