PhD Theses
Molecular- and Cell Biology PhD Program

Genetic and taxonomic studies in the genus *Rhizomucor*

Made by
Mária Vastag

Supervisor:
dr. Csaba Vágvölgyi

University of Szeged
Faculty of Sciences
Department of Microbiology

SZEGED
2002

INTRODUCTION

The Zygomycetes class is taxonomicaly a well separeted group of the fungi. Species of Zygomycetes can be isolated from a variety of substrates including soil, dung, fruits and flowers, stored grains and fleshy plant organs, mushrooms, invertebrates, and vertebrates, including humans. Nutritional modes range from saprobic through facultative, weak parasitism of plants to specialized parasitism and predation of animals to obligate parasitism of true fungi. The Mucorales order includes the largest number of species and exhibits the highest morphological diversity among the seven orders of Zygomycetes. Mucorales can be separeted from the other orders of Zygomycetes by their relatively nonspecialized associations with other organisms. Most genera of the order Mucorales are saprobes that occur in dung, soil, humus, and other organic debris. Some Mucorales genera have recently attracted increasing interest due to their abilities to produce valuable microbial products, e.g. industrially important extracellular enzymes or pharmaceutically important polyunsaturated fatty acids. They also find application in the formation of various groups of organic compounds by biotransformation. Mucoraceous fungi are additionaly of value as model organisms in basic research.

Members of the genus *Rhizomucor* are distinct from *Mucor* by virtue of their thermophilic nature and some morphological traits (e.g.

the formation of rhizoids). These fungi are of value in both theoretical and applied microbiology. They are good producers of different extracellular enzymes (e.g. proteases), while in other cases they may be the agents of progressive and frequently fatal opportunistic mycotic diseases. However, as is the case with many other fungi of practical or theoretical importance, this exploited potential is not accompanied by a full knowledge of the genetic background of these organisms. The aim of the present study was to obtain information about the largely unknown genetic organization of these fungi.

THE AIMS OF THE PRESENT STUDY

1. To obtain information about the largely unknown genetic organization of these fungi.
2. To reveal their intraspecific variability.
3. To determine useful markers for a methodically simple, quick and more unambiguous strain identification and for genetic analysis using physiological and molecular methods (carbon source utilization, isoenzyme, ITS-RFLP and RAPD analysis).
4. To construct a representative genomic library from *Rhizomucor miehei.*
5. To clone the glyceraldehyde-3-phosphate dehydrogenase (*gpd*) gene from *R. miehei* and characterize the structural gene and its

adjecent regulatory elements (promoter and terminator regions) at sequence level (molecular organisation, codon usage and homology with *gpd* genes of other fungi).

6. To optimise the protoplast preparation conditions which will be important in the further transformation procedures.
7. To conduct electrophoretic karyotype experiments to obtain some data about the genome size of *Rhizomucor* species.

NEW SCIENTIFIC RESULTS

1. We examined the sexual life cycle of the *Rhizomucor* isolates, and determined their mating type and the sizes of zygospores resulted from the homo- and heterotallic sexual processes. Our results supported the earlier observations that the differences in size and morphology of zygospores are valuable tools in the taxonomy of the genus *Rhizomucor*. Certain approaches, such as determination of the number of nuclei in the sporangiospores or mating studies coupled with determination of the morphological traits of the zygospores, could supply characteristics for the delimitation of these species. However, the differences are not clear-cut in every case, or the procedure is time-consuming. One of our aims was to develop a methodically simple, quick and more unambiguous identification of the *Rhizomucor* species.

2. Numerous *Rhizomucor* isolates were assayed for their ability to utilize 87 various substrates as a single carbon source. Besides a difference in sucrose utilization, distinctive differences were found in the abilities of *R. miehei* and *R. pusillus* in the utilization of glycine, phenylalanine and L-alanine. Under the same experimental conditions, *R. tauricus* utilized (to various extents) few of the same 87 compounds. It was, however, able to grow on three (sucrose, phenylalanine and β-alanine but not glycine) of the four compounds whose utilization was found to be characteristic for *R. pusillus* but not for *R. miehei*. Differences in the abilities of *R. tauricus* and the other *Rhizomucor* species in the utilization of various substrates as a single carbon source could be explained with the occurence of pleiotropic mutation in this single isolate. Assimilation spectra work well for wild-type isolates but could be misleading if a pleiotropic mutation influencing different biochemical functions is present.
3. Five isoenzyme systems were tested (malate dehydrogenase, glutamate dehydrogenase, acid phosphatase, α-estherase, glucose-6-phosphate dehydrogenase) that also proved useful for the determination of markers of distinctive value at a species level. The results of our investigations revealed different levels of intraspecific genetic variability within the *R. pusillus* and *R. miehei* species. *R. miehei* strains were more homogeneous genetically than the heterothallic *R. pusillus* isolates. *R. pusillus* isolates can either

be homothallic and heterothallic, while all *R. miehei* isolates are homothallic. The different levels of genotypic diversity suggest a correlation with the different forms of mating behaviour of these species. These results indicate, in contrast with earlier opinions, the existence of the sexual life cycle of this fungus in nature.

4. PCR primers targeted to highly conserved regions of rDNA could likewise be used to determine molecular markers, especially at the species level. Amplified ITS and intergeneric spacers are highly polymorphic, revealing variations in their length and in the restriction sites they contain. The ITS-1 and ITS-4 primers successfully amplified the ITS region of the zygomycete strains studied. After amplification, *R. tauricus* and the heterothallic *R. pusillus* revealed a PCR product of the same size. In *R. miehei* and homothallic *R. pusillus* strains, a smaller amplification product was detected. The variability of the ITS region was also investigated by restriction enzyme digestion of the amplified DNA. Double digestions with two different restriction enzymes generated sufficient polymorphism. In all cases *R. tauricus* and the heterothallic *R. pusillus* strains shared the same restriction pattern. No common character was found between *R. miehei* strains and either *R. pusillus* strains or *R. tauricus*.
5. RAPD-PCR analysis is an important tool for classification and identification of fungal isolates. There are several advantages of this approach, one of the most important being that RAPD-

generated markers are thought to be more neutral than those obtained, for instance, from the analysis of proteins or different physiological traits. The genetic variability in *R. pusillus* and *R. miehei* isolates was found to differ; the latter revealed less intraspecific polymorphism. The different levels of genotypic diversity suggest a correlation with the different forms of mating behaviour of these species. *R. tauricus* displayed amplification patterns similar to those of the investigated *R. pusillus* strains, reinforcing the assumption that *R. tauricus* does not represent a separate species. Characteristic RAPD markers allowing PCR-based species identification of *Rhizomucor* isolates were determined. Besides these markers, which appeared to be species-specific, one major amplification product was also found which appeared to be genus specific in our experiments.

6. The aims of the further studies were to construct a representative genomic library from *R. miehei* 5282 strain, to clone the glyceraldehyde-3-phosphate dehydrogenase (*gpd*) gene from *R. miehei* and characterize the structural gene and 5’ and 3’non-coding regions (promoter and terminator regions) at sequence level. Lambda Fix II vector and the Gigapack III Gold Packaging Extract kit were used for packaging the 10-20 kb fragments of the partially digested R. miehei genomic DNA. The titer of the genomic library was found to be 4×10^{4}PFU/ml. Polymerase chain reaction was used to synthesize a specific 300 bp fragment of the *gpd* gene of *R.*

miehei and used as a *gpd*-specific probe to screen the genomic library of *R. miehei*. For sequence analysis several subclones were generated, and sequenced with a number of newly synthesized oligonucleotides to obtain the complete nucleotide sequence of the *gpd* gene. The nucleotide sequence of the *R. miehei gpd* gene was compared to the *gpd* gene sequences of several organisms. The *gpd* sequences used in this study were obtained from nucleotide sequence libraries (EMBL, GeneBank). Comparison of the derived amino acid (aa) sequences revealed that there is a large extent of homology among *gpd* proteins in various organisms (about 70% aa sequence identity). This result confirms the fact that the *gpd* gene is a very conservative gene in nature. On the basis of the *gpd* aa sequence zygomycetes show a closer relationship to basidiomycetes than to ascomycetes. Furthermore, our results supported those earlier observations that the *Saccharomyces*-like yeasts differ significantly from the filamentous ascomycetes. The explanation could be that yeasts are unicellular throughout their life cycles whereas the filamentous fungi are multicellular during most of their life cycles. In the course of the sequence analysis of the *R. miehei gpd* gene the positions of five introns were determined and compared with positions of introns *gpd* genes in other organisms. However, these results, in contrast with earlier observations, could not provide information about the systematic relatedness of these organisms.

7. The protoplast preparation conditions have also been optimised for some *Rhizomucor* isolates. These experiments will play an important role in the further transformation procedures.
8. Electrophoretic karyotype experiments have also been conducted to obtain some data about the genome size of the *Rhizomucor* species. Our preliminary experiments reflect that the genome size of *Rhizomucor* species is comparable with that of the *M. circinelloides* genome. To obtain a precise elektrophoretic karyotype of *Rhizomucor* strains the experimental conditions should be further optimised.

PUBLICATIONS

PUBLICATIONS FROM OUR STUDY

1. **Vastag, M.,** Papp, T., Kasza, Zs. and Vágvölgyi, Cs. (1998). Differentiation of *Rhizomucor* species by means of carbon source utilization and isoenzyme analysis. J. Clin. Microbiol. 36, 2153-2156.
2. Vágvölgyi, Cs., **Vastag, M.,** Ács, K. and Papp, T. (1999). *Rhizomucor tauricus*: a questionable species of the genus. Mycol. Res. 103, 612-618.

3. **Vastag, M.,** Papp, T., Kasza, Zs. and Vágvölgyi, Cs. (2000). Intraspecific variation in two species of *Rhizomucor* assessed by random amplified polymorphic DNA analysis. J. Basic Microbiol. 40, 269-277.
4. Vágvölgyi, Cs., **Vastag, M.,** Kasza, Zs., Ács, K. and Schwab, H. (2002). Cloning and sequence analysis of a glyceraldehyde-3-phosphate dehydrogenase gene from a zygomycete fungus, *Rhizomucor miehei*. FEMS Microbiol. Letters (előkészítve).

ABSTRACTS

1. **Vastag, M.,** Papp, T., Nagy, Á., Palágyi, Zs., Ferenczy, L. and Vágvölgyi, Cs. (1997). Delimitation of *Rhizomucor* species on the basis of genetic and physiological markers. Acta Microbiol. Hung. 44, 430-431.
2. Vágvölgyi, Cs., **Vastag, M,** Papp, T., Nagy, Á. and Ferenczy, L. (1997). Physiological and molecular markers to differentiate *Rhizomucor* species. 13th ISHAM, Parma, Abstracts 101.
3. Vágvölgyi, Cs., Nagy, Á. and **Vastag, M.** (1998). DNA amplification polymorphism of *Rhizomucor* species. IMC-6, Jerusalem, Abstract 28.
4. **Vastag, M.,** Nagy, Á., Papp, T., Ács, K. and Vágvölgyi, Cs. (1999). Investigation of the taxonomic position of *Rhizomucor tauricus*. Acta Microbiol. Hung. 46, 134-135.

5. **Vastag, M**., Papp, T**.,** Ács, K. and Vágvölgyi, Cs. (1999). Intraspecific variability of thermophilic *Rhizomucor* species as assessed by randomly amplified polymorphic DNA. Acta Microbiol. Hung. 46, 351.
6. Ács, K., **Vastag, M**., Kasza, Zs. és Vágvölgyi, Cs. (2001). A *Rhizomucor miehei* glicerinaldehid-3-foszfát dehidrogenáz génjének klónozása. MMT 2001. évi Naggyűlése, Balatonfüred, Összefoglalók.
7. Vágvölgyi, Cs., **Vastag, M**., Kasza, Zs. and Ács, K. (2002). Isolation and Characterization of the Glyceraldehyde-3-phosphate dehydrogenase gene from *Rhizomucor miehei*. IMC7, Oslo, Abstracts.

OTHER PUBLICATIONS

1. Palágyi, Zs., Nagy, Á., **Vastag, M.,** Ferenczy, L. and Vágvölgyi, Cs. (1997). Maintenance of fungal strains on cryopreservative-immersed porous ceramic beads. Biotechnol. Tech. 11, 249-250.
2. Vágvölgyi, Cs., Magyar, K., Papp, T**.,** **Vastag, M**., Ferenczy, L. Hornok, L., and Fekete, Cs. (1998). Detection of double-stranded RNA molecules and virus-like particles in different *Mucor* species. Ant. Leeuwenhoek 73, 207-210.

3. Nagy, Á., Palágyi, Zs., **Vastag, M**., Ferenczy, L. and Vágvölgyi, Cs. (2000). Electrophoretic karyotypes of some related *Mucor* species. Ant. Leeuwenhoek 78, 33-37.

4. Papp, T., **Vastag, M.,** Nagy, Á., Michailides, T.,J. and Vágvölgyi, Cs. (2001). Genetic variability of the postharvest pathogen *Gilbertella persicaria*: identification of randomly amplified polymorphic DNA (RAPD) markers correlating with (+) and (-) mating types. Ant. Leeuwenhoek 80, 301-309.

ABSTRACTS

1. Palágyi, Zs., Nagy, Á, Vágvölgyi, Cs., **Vastag, M.** and Ferenczy, L. (1997). Application of "Protect" bacterial storage system for fungal strain maintenance. EBC-8, Budapest, Abstracts 108.

2. Papp, T., **Vastag, M.** and Vágvölgyi, Cs. (1999). Genetic variability of the postharvest pathogen *Gilbertella persicaria.* Acta Microbiol. Hung. 46, 343-344.

3. Vágvölgyi, Cs., **Vastag, M**., Papp, T**.** and Kasza, Zs. (1999). Isoenzyme and random amplified polymorphic DNA (RAPD) analysis of the homothallic *Mucor genevensis.* Acta Microbiol. Hung. 46, 352.

4. Garas, K., **Vastag, M.,** Somogyvári, F. and Vágvölgyi, Cs. (1999). Applicability of the *ATB Fungus* system for rapid antifungal

susceptibility testing of *Rhizomucor* isolates. Acta Microbiol. Hung. 46, 355.

5. Papp, T., Fekete, Cs., **Vastag, M.,** Nagy, Á. and Vágvölgyi, Cs. (1999). Presence of double-stranded RNA molecules and virus-like particles in *Rhizopus* strains. Acta Microbiol. Hung. 47,333.
6. **Vastag, M**., Garas, K., Papp, T., Ács, K. and Vágvölgyi, Cs. (1999). The antifungal activity of lovastatin against *Rhizomucor* strains. Acta Microbiol. Hung. 47, 383-384.
7. Papp, T., Nagy, Á., Palágyi, Zs., **Vastag, M**. and Vágvölgyi, Cs. (1999). Genetic studies on sexual processes of *Gilbertella persicaria*. Acta Microbiol. Hung. 47, 327-328.
8. Papp T., Nagy, Á., Palágyi, Zs., **Vastag, M**. and Vágvölgyi, Cs. (1999). Genetic studies on sexual processes of *Gilbertella persicaria*. 13th. International Congress of the Hungarian Society for Microbiology, Budapest, Abstracts 75.
9. **Vastag, M**. Garas, K., Papp, T., Ács, K. and Vágvölgyi, Cs. (1999). The antifungal activity of lovastatin against *Rhizomucor* strains. 13th. International Congress of the Hungarian Society for Microbiology, Budapest, Abstracts 109.
10. Papp, T., **Vastag, M.**, Ferenczy, L. and Vágvölgyi, Cs. (2000). Investigation of the genetic variability of *Gilbertella persicaria* via molecular markers. ECFG-5, Arcachon, Abstracts 314.
11. Papp, T., **Vastag, M**., Ferenczy, L. and Vágvölgyi, Cs. (2000). Phylogenetic position of *Gilbertella persicaria* inferred from

glyceraldehyde-3-phosphate dehydrogenase gene sequences. ECFG-5, Arcachon, Abstracts 315.

12. **Vastag, M.,** Palágyi, Zs., Nagy, Á., Ferenczy, L. and Vágvölgyi, Cs. (2000). Investigation of the genetic variability of *Phaffia rhodozyma* via RAPD markers. ECFG-5, Arcachon, Abstracts 338.
13. Ács, K., Kasza, Zs., **Vastag, M.** és Vágvölgyi, Cs. (2000). A gliceraldehide-3-foszfát dehidrogenáz gén klónozása és részleges szekvenciaelemzése *Mucor circinelloides*ben. MMT 2000. évi Nagygyűlése, Keszthely, Összefoglalók.
14. Papp, T., Nyilasi, I., Ács, K., **Vastag, M**. és Vágvölgyi, Cs. (2000). A *Gilbertella persicaria* taxonómiai helyének vizsgálata a glicerinaldehid-3-foszfát dehidrogenáz gén és magi riboszómális-DNS szekvenciák alapján. MMT 2000. évi Nagygyűlése, Keszthely, Összefoglalók.