GENOME ANALYSIS OF MICROSCOPIC FUNGI BY PULSE FIELD GEL ELECTROPHORESIS

PH.D. THESIS

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1996

1. INTRODUCTION

The genetic analysis of microscopic fungi requires the existence of well-defined genetic markers. Thr application of classical genetic markers (most often auxotrophic and resistance markers) has in several cases promoted knowledge on the genetic material of different fungal species of practical or theoretical importance. However, the creation of such markers (mutations), especially for industrial strains, is unfavourable. A further obstacle for classical genetic studies could be the frequent lack of the sexual cycle in several fungal species. Another possible approach, the observation of condensed metaphase chromosomes by light microscopy (determination of cytological karyotype), is practically impossible because of their small size.

Until the 1980-s, the only method of DNA gel electrophoresis allowed the separation merely of relatively small molecules (20 kb); above this size, the DNA molecules elongated in the electric field and migrated at the same speed, independently of their size.

Klotz and Zimm (1972), founders of the pulsed field gel electrophoresis (PFGE) technique, discovered that under the influence of an electric field DNA molecules change their conformations, but that these are restored after removal of the electric field. They observed that, above a certain molecular size, the time of this relaxation is proportional to the molecular weight of the DNA.

These discoveries concerning DNA conformational changes and their size--dependent relaxation allowed the invention of a new separation technique (Schwartz et al., 1982). PFGE opened up a new way for the separation of large DNA molecules and this method allowed the direct examination of eukaryotic chromosomal sets (Dixon and Kinghorn, 1990).

The essence of this method is that, in two alternatively active, angled electric fields, the DNA molecules undergo continuous reorientation during their migration in the gel (Schwartz and Cantor, 1984; Carle and Olson, 1984). The larger the DNA molecule, the longer the time necessary for its conformational change. The DNA molecules can be separated as a result of the mobility advantage of the small DNA molecules.

Strains of a given fungal species or genus obviously differ in genetic material. The main reason for this is the existence of spontaneous mutation events, characteristic of all genetic material. Such mutations, e.g. the structural mutation of chromosomes, has no direct phenotypic consequences, but can be detected by the analysis of electrophoretic karyotypes.

Structural changes in chromosomes can be either intrachromosomal or interchromosomal. The various chromosomal rearrangements can lead to the formation of CLP (chromosome-length polymorphism): for instance, fungal genomes frequently contain sequences which promote various chromosomal rearrangements (recombination, deletion or translocation) and contribute to the establishment of CLP (Zolan, 1995).

Kistler and Miao (1992) hypothesized that sexual events exert selective effects against chromosomal aberrations: the frequency of meiosis is inversely proportional to the extent of CLP. There are data in support of, but also data that conflict with this hypothesis. However, investigation of the effects of sexual events on intraspecific CLPs is especially difficult because of the lack of data about the molecular events causing CLP.

In most cases, the karyotype of a given fungal strain is stable and does not change in the course of repeated mitotic nuclear divisions. This stability seems to be contradictory with the high variation observed between the strains of a fungal species. An explanation of this phenomenon might be the independent (separate) evolution of these strains during shorter or longer times under different conditions. In such cases different mechanisms (not yet understood perfectly) could create changes in the

chromosomal materials. These changes could be harmful, neutral or, especially in a new environment, beneficial, allowing the formation of new genetic combinations.

The stability of chromosomal profiles and the existence of strain-specific karyotypes has made electrophoretic karyotyping an especially important method in genetic, medical, epidemiological and agricultural studies. In spite of the mentioned limitations, electrophoretic karyotyping is nowadays one of the most effective methods of strain identification.

Carle, G.F. and Olson, M.V. (1984). Separation of chromosomal DNA molecules from yeast by orthogonal-field alternation gel electrophoresis. Nucleic Acids Res. 12, 5647-5664.

Carle, G.F. and Olson, M.V. (1985). An electrophoretic karyotype for yeast. Proc. Natl. Acad. Sci. USA 82, 3756-3760.

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Klotz, L.C. and Zimm, B.H. (1972). Retardation times of deoxyribonucleic acid solutions. II. Improvements in apparatus and theory. Macromolecules 5, 471-481.

Schwartz, D.C. and Cantor, C.R. (1984). Separation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis. Cell 37, 67-75.

Schwartz, D. C., Saffran, W., Welsh, J., Haas, R., Goldenberg, M. and Cantor, C.R. (1982). New techniques for purifying large DNAs and studying their properties and packing. Cold Spring Harbor Symp. Quant. Biol. 47, 189-195.

Zolan, M.E. (1995). Chromosome-length polymorphism in fungi. Microbiological Reviews 59, 686-698.

The aims of the research program were as follow:

1. Characterization of the chromosomal material in fungal genera of theoretical and practical importance (*Aspergillus*, *Claviceps*, *Mucor*, *Penicillium* and *Phaffia*) by means of electrophoretic karyotyping.

2. Analysis of the intraspecific CLP of certain microscopic fungi (*Mucor* and *Phaffia*); determination of its type and extent.

3. Establishment and beginning of detailed genetic analysis in the genera *Phaffia* and *Mucor* through gene assignment experiments.

4. Testing the applicability of PFGE for verification and analysis of hybridization events.

5. Optimization and development of PFGE methods allowing more efficient genetic analysis.

2. MATERIALS AND METHODS

2.1. Fungal species investigated

Candida albicans, Aspergillus nidulans, Candida krusei, Hansenula anomala, Kluyveromyces lactis, Phaffia rhodozyma, Claviceps fusiformis, Claviceps purpurea, Mucor plumbeus, Saccharomyces cerevisiae, Mucor circinelloides, Saccharomyces unisporus, Schizosaccharomyces pombe, Mucor racemosus, Mucor bainieri, Mucor mucedo, Penicillium notatum, Penicillium chrysogenum

2.2. The main methods applied

- DNA gel electrophoresis
- methods of protoplast formation
- methods of DNA labelling (radioactive and non-radioactive) and hybridization
- methods of pulsed field gel electrophoresis (OFAGE and CHEF)

3. RESULTS

PFGE was used for investigation of the chromosomal genome organization in microscopic fungi of theoretical and practical importance.

1. Data were obtained on the genetic material of *Phaffia rhodozyma*, a yeast of biotechnological importance. The size of the chromosomal genome (15.5-23.2 Mb), and the number (8-12) and size (0.83-3.5 Mb) of chromosomal DNA mobility groups were determined. A comparative analysis of several isolates revealed the existence of definite CLP in *P. rhodozyma*; its type and extent were investigated.
2. Numerous *P. rhodozyma* mutants (167 colour mutants and 11 auxotrophs) were created and their electrophoretic karyotypes were investigated. Data were obtained on (a) the frequency of chromosomal rearrangements in the course of mutagenesis; (b) the differences in behaviour of independent chromosomal DNAs; (c) the possible existence of a correlation between a given chromosomal rearrangement and an observed new phenotype. Information was acquired on the ploidy level of these yeasts.

3. The results obtained gave the first insight into the chromosomal constitution of a *Mucor* strain. The electrophoretic karyotypes of several strains of *M. circinelloides* were determined; the existence and extent of intraspecific CLP were studied. Altogether five different *Mucor* species (*M. hainieri*, *M. circinelloides*, *M. mucedo*, *M. racemosus* and *M. plumbeus*) were investigated: data were collected on the organization of the genome and on the CLP inside this genus (genome size: 31.7-40.1 Mb; number of chromosomes: 6-11; size of chromosomal DNA: 2.3-9.3 Mb).

4. Gene assignment experiments were started with *Phaffia rhodozyma* and the investigated *Mucor* species to establish and start a detailed genetic analysis of these genera.
5. The results obtained gave the first information about the genome size and chromosome number of *Claviceps* species.
6. Results were obtained on the electrophoretic karyotyping of industrial *Penicillium* strains.
7. PFGE experiments were also used for the verification and analysis of hybridization events (protoplast fusion) in two fungal species (*P. rhodozyma* and *A. nidulans*). In both cases, the chromosomal set of fusion products and recombinant strains were analysed. The applicability of PFGE for the verification of fusion processes and for the tracking of individual chromosomal segregations was proved.
8. PFGE methods allowing more efficient electrophoretic karyotyping were developed and optimized.

The results reffered to have substantially increased our knowledge of the genome organization of microscopic fungi. They efficiently assist both further genetic studies and practical strain improvement work.

4. LIST OF PUBLICATIONS

4.1. FULL PAPERS

1. **Nagy, Á., Vágvölgyi, Cs., Balla, É. and Ferenczy, L.** (1994) Electrophoretic karyotype of *Mucor circinelloides*. Curr. Genet. 26, 45-48.

2. **Nagy, Á., Garamszegi, N., Vágvölgyi, Cs. and Ferenczy, L.** (1994) Electrophoretic karyotypes of *Phaffia rhodozyma* strains. FEMS Microbiol. Letters 123, 315-318.

3. **Nagy, Á., Vágvölgyi, Cs. and Ferenczy, L.** (1995) Simple method for obtaining chromosomal DNA for orthogonal field alteration gel electrophoresis analysis from *Phaffia rhodozyma*. BioTechniques 18, 63-65.

4. **Varga, J., Vágvölgyi, Cs., Nagy, Á. and Ferenczy, L.** (1995) Isoenzyme, restriction fragment length polymorphism and random amplified polymorphic DNA characterization of *Phaffia rhodozyma* Miller et al. Int. J. Syst. Bacteriol. 45, 173-177.

5. **Palágyi, Zs., Nagy, Á., Vágvölgyi, Cs. and Ferenczy, L.** (1995) A new mutation protocol for obtaining mutants of the yeast *Phaffia rhodozyma*. Biotechnol. Techn. 9, 401-402.

6. **Palágyi, Zs., Nagy, Á., Papp, T., Ferenczy, L. and Vágvölgyi, Cs.** (1996) Chromosomal DNA preparation from yeasts of biotechnological importance. Biotechnol. Techn. 10, 565-568.

7. **Vágvölgyi, Cs., Magyar, K., Papp, T., Palágyi, Zs., Ferenczy, L. and Nagy, Á.** (1996) Value of substrate utilization data for characterization of *Mucor* isolates. Can. J. Microbiol. 42, 613-616.

4.2. PATENTS

1. **A protocol for production of ergolen-type compounds (especially ergometrin); a selective staining method.**
   Zalai, K., Trinn, M., Manczinger, L., Polestyukne Nagy, Á., Kordik, G., Pécsné Rázsó, Á., Beszedics, Gy., Ferenczy, L., Nagy, L., Robicsek, K. and Szegedi, M.
   Patent No.: 2251-2949/90/5

2. **A protocol for the increased production of elimoclavin; construction of a new strain**
   Trinn, M., Manczinger, L., Polestyukne Nagy, Á., Kordik, G., Pécsné Rázsó, Á., Zalai, K., Beszedics, Gy., Ferenczy, L., Nagy, L., Robicsek, K. and Szegedi, M.
   Patent No.: 2251-2950/90/7

4.3. LECTURES AND POSTER PRESENTATIONS

1. **Vágvölgyi, Cs., Nagy, Á., Varga, J., Palágyi, Zs. and Ferenczy, L.** (1994) Value of some molecular markers for species delimitation in the genus *Mucor*. 7. Int. Congr. Mycol. Div. IUMS, Prague, Abstracts, 324.

2. **Nagy, Á., Vágvölgyi, Cs. and Ferenczy, L.** (1994) Pulsed field electrophoresis of *Mucor* chromosomal DNA. ECFG-2, Luntereń, Abstracts. C3.

3. **Kevei, F., Varga, J., Vágvölgyi, Cs., Kucsera, J., Nagy, Á., Pfeiffer, I. and Ferenczy, L.** (1994) Interpretation of compatibility relations among black *Aspergilli* on the basis of their molecular characters. Acta Microbiol. Hung. 41, 348.

4. **Nagy, Á., Vágvölgyi, Cs. and Ferenczy, L.** (1994) Electrophoretic karyotyping of *Mucor* species. Acta Microbiol. Hung. 41, 353.

5. **Varga, J., Vágvölgyi, Cs., Nagy, Á. and Ferenczy, L.** (1995) Molecular characterization of *Phaffia rhodozyma* strains. Acta Microbiol. Hung. 42, 135.

6. **Nagy, Á., Vágvölgyi, Cs., Palágyi, Zs. and Ferenczy, L.** (1995) Analysis of chromosomal DNA polymorphism of *Phaffia rhodozyma*. Acta Microbiol. Hung. 42, 133.

7. **Nagy, Á., Vágvölgyi, Cs. and Ferenczy, L.** (1995) Preparation of chromosomal DNA from yeasts of biotechnological importance. Acta Microbiol. Hung. 42, 134.

8. **Nagy, Á., Vágvölgyi, Cs., Palágyi, Zs., Papp, T. and Ferenczy, L.** (1996) Investigation of chromosomal length polymorphism of *Mucor* species by electrophoretic karyotyping. Hung. Biochem. Soc. Mol. 1st Workshop Mol. Biol. Div., Seregélyes, Abstracts 135.

9. **Palágyi, Zs., Vágvölgyi, Cs., Nagy, Á. and Ferenczy, L.** (1996) Efficient method of mutant isolation from the yeast *Phaffia rhodozyma*. Acta Microbiol. Hung. 43, 241-242.

10. **Nagy, Á., Palágyi, Zs., Vágvölgyi, Cs., Papp, T. and Ferenczy, L.** (1996) Chromosomal rearrangements observed in auxotrophic and hybrid strains of *Phaffia rhodozyma*. Acta Microbiol. Hung. 43, 242.

11. **Vágvölgyi, Cs., Fekete, Cs., Magyar, K., Papp, T., Palágyi, Zs. and Nagy, Á.** (1996) Detection of double-stranded RNA molecules in *Mucor* species. Congress of Hung. Microbiol. Soc., 1996., Nyíregyháza, Abstracts.

5. ACKNOWLEDGEMENTS

I wish to thank to all my colleagues who have helped in so many ways and supported my research:

Professor Lajos Ferenczy

Gergely L. Nagy
Dr. Zsuzsanna Palágyi
Dr. Mihály Szegedi
Dr. Csaba Vágvölgyi
Dr. Károly Zalai