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NEW METHODS FOR BIOTYPING OF *CANDIDA* SPECIES

PHD THESIS

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Introduction

Infections caused by *Candida* species are widespread throughout the world. Among them, C. *albicans* is the most important species encountered as a cause of both superficial and systemic infections. *C. albicans* has been found to be responsible for about 45-60% of human infections, with one of the highest rate (77-80%) observed in Hungary. Clinical manifestations of *C. albicans* include superficial infections such as cutaneous candidiasis, oropharyngeal candidiasis, and vulvovaginitis that are frequent but usually benign in immuno-competent hosts. They are also able to cause severe infections including candidemia and disseminated candidiasis.

Information on the genetic diversity of *C. albicans* population is important in order to adapt prevention policies. Several typing techniques, including random amplified polymorphic DNA (RAPD) analysis, CA3 fingerprinting, electrophoretic karyotyping and multilocus sequence typing (MLST) have been used for characterising *C. albicans* populations. The abovementioned methods employ nuclear markers. As an alternate, the analysis of the extrachromosomal mitochondrial DNA (mtDNA) of *C. albicans* is a successful tool to detect intraspecific genetic variability in this species. The mtDNA of *C. albicans* is circular, with the size of 40420 bp (SC 5314 strain, accession number: AF285261). It contains the standard set of mitochondrial genes. An existing mtDNA based method is the EO3 typing, since the EO3 region (within the duplicated region in its stem-and-loop structure) exhibits a DNA-size polymorphism

Competition for territories and food in the nature had forced organisms to develop different mechanisms for their successful spreading and living. Among these are the antagonistic relationships, where a given organism represses the growth of its competitors. The antifungal property of killer toxins raises the possibility of many agricultural and industrial applications. Analysis of the sensitivity pattern of different yeast species against a killer toxin is a cheap typing method, but difficult to standardize it. Some *Pichia* strains have an outstanding antagonistic effect against several fungal species, including human pathogen yeasts.

Aims of the work:

- study of mtDNA polymorphism of *C. albicans* clinical isolates and strains from culture collections
- physical and partial functional mapping of the mtDNA types
- establisment of new PCR RFLP based typing method
- study of mtDNA polymorphism of *C. parapsilosis* clinical isolates
- whole mitochondrial genome sequencing of *Candida maltosa*
- comparative analysis of mitochondrial introns of the species belonging to *C. albicans* clade
- characterization of an anti-*Candida* toxin produced by *Pichia anomala* VKM Y-159 strain
- identification of its biochemical properties, the cell wall receptor and reveal of its mode of action

Methods

- Classical microbiological methods
    - Maintenance of the strains
    - Production of the toxins
    - UV mutagenesis
- Molecular methods
    - Isolation of mitochondrial DNA
    - mtDNA RFLP
    - RFLP PCR
    - Physical mapping
    - Cloning
    - Transformation
    - Random sequencing
    - PFGE (pulsed field gelelectrophoresis)
- Protein based techniques
    - SDS PAGE method
    - Native PAGE method
    - Ion-exchange chromatography
    - Affinity chromatography
- Methods for characterization of the mechanism of action of the toxin
    - Competition analysis
    - Flow cytometric assays:
        - Viability test: PI (propidium iodide)
        - Cell cycle analysis: DAPI

Results

The study of mtDNA polymorphism among clinical *Candida* isolates

The opportunistic human pathogen yeast *C. parapsilosis* has linear mitochondrial DNA with tandem repeats at the termini. Earlier study demonstrated the very stable RFLP pattern of the mtDNA within group I isolates of this species. Our survey on 23 Hungarian clinical isolates confirmed these results.

The occurrence and genetic variability of *Candida albicans* isolates in a Hungarian hospital were examined. Among the 103 *Candida* isolates, 44 (42.7%) proved to be *C. albicans*. The mitochondrial DNA profiles of the 44 clinical isolates of *C. albicans* were analysed. Total DNA purified from the strains were cut with restriction enzymes, which have plenty of recognition sites on nuclear DNA, and less on mitochondrial DNA (*Hae*III digestion gave identical pattern, *Hin*6I gave two, while the digestion with *Hinf*I resulted in four different patterns). The majority of the strains (23) were found to belong to the group labeled as type I. MtDNA types II and III include 11 and 9 strains, respectively, while type IV was exhibited only by a single isolate. The variability was also studied using isolated mtDNAs of selected isolates. The restriction enzymes were selected on the basis of the frequency of their recognition sites. *Bgl*II and *Eco*RV have a numerous recognition sites on the mtDNA of *C. albicans*. Both of these enzymes gave distinct pattern on each type, supporting the results originated from total DNA digestion. Determining of the fragments' size we observed that every type have an appr. 40 kb-genome size.

The *Pvu*II cleavage gave almost identical RFLP pattern in each type; only an appr. 50 bp difference could be detectable in the molecular weight of the smallest double *Pvu*II fragments (position itself inside the repetitive region of the mtDNA). Primers were designed on this region, the fragment was amplified and sequenced. Results revealed a 55 bp deletion in Type I., II. and IV. compared to Type III, in the upstream (intergenic) sequence of *cox3* gene. This *Pvu*II fragment contains the aforementioned EO3 region.

Mapping of the *Candida albicans* mtDNA types

The physical maps of the mtDNAs of the four types (I: 10930, II: 9132, III: 5796, IV: 17471) were generated by *Eco*RI-*Eco*RV. The *Eco*RI pattern of the mtDNAs seemed identical (6 fragments in each type), whereas the *Eco*RV pattern is distinctive. *In silico*, we generated the *Eco*RI-*Eco*RV map of *C. albicans* strain SC5314 found in the database. This strain is identical to Type III, hence we use it as a basic map. The differences in the organization are due to point mutations (e.g. GAT/ATC–GGT/ATC), which led to the distinctive *Eco*RV patterns. Partial genetic map was also constructed with the following hybridization probes: *cox1, cox2, cox3, cob, nad1, nad2, nad4, nad5* and *atp6*. The hybridizations explored the same gene order in the four types, as in SC 5314 strain.

Typing methods of *Candida albicans* isolates

We could manage to group our clinical isolates into the types Miyakawa and co-workers established: no. 5796 isolate (Type III.) belongs to L, no. 9132 (Type II.) and no. 10930 (Type I.) belongs to MII, while no. 17471 (Type IV.) is MI. Neither of the examined isolates represent type S.

In order to establish a new typing method, we chose a region, which differ in the four types corresponding to the *Eco*RV sites. The chosen FZRV region is involved in the largest *Eco*RI fragment. We designed primers to this region (FZRV) to amplify it. The PCR product (1551 bp) was digested with *Eco*RV. Type I contains no *Eco*RV site within this region (1551 bp), Type II. carries two *Eco*RV sites (920bp, 403 bp, 228 bp), while Type III and Type IV possess the same one *Eco*RV site (1323 bp, 228 bp), but Type IV could be differentiated from Type III by *Bgl*II digestion. We examined the PCR-RFLP pattern of the FZRV region of several geographically distinct *C. albicans* isolates. The survey of PCR RFLP pattern of the region we chose to differentiate among clinical isolates is a good alternative epidemiological tool, beside the abovementioned EO3 region analysis.

Karyotyping of clinical *Candida albicans* isolates

The electrophoretic karyotypes of strains belonging to the different mtDNA types were analyzed. One additional chromosomal DNA band was detected in type II strains compared to the *C. albicans* 1006 strain. This band located between chromosomes number 3 and 4 (1.8 Mb and 1.7 Mb). Another well characterized *C. albicans* strain, WO-1 also carries an extra chromosome compared to strain 1006, however, the size of that chromosome (1.65 Mb) is different from that observed in mtDNA type II isolates (The *Candida albicans* physical map site; http://albicansmap.ahc.umn.edu/).

Analyzing of *Candida maltosa* mt genome on the level of nucleotide sequence

The *Eco*RV-*Pst*I restriction map of *C. maltosa* was generated, thereafter we carried out the sequencing of the whole mt genome. The size of the CBS 5611 mtDNA is 62949 base pairs and it is circular. It has the standard set of genes: *atp*6-8-9, *cox*1, *cox*2, *cox*3, *cob, nad1-2-3-4-4L-5-6*, *rns, rnl* and 26 tRNA. Three of the genes (*cox*1, *cob* and *rnl)* contain 2-2 introns. The genome contains two repeat regions: one is on the direct strand (12859-27237 nt); the other is on the complement strand (40937-55315 nt).

Comparative analysis of mitochondrial introns of the species belonging to *C. albicans* clade

The study of introns can be a tool to establish additional typing methods. One can take advantage of their presence or absence, furthermore of their differing size to design primers on the flanking exon regions, to generate species specific PCR products. Among the fourteen examined strains of the *C. albicans* clade (outgroup: *D. hanseniaspora*), only *L. elongisporus* proved to be intron-free. The number and the size of the introns are not in correlation with the whole mt genome size. Four genes, namely *cox*1, *cob*, *rnl* and *nad5*

contain introns, the majority of them are Group I. introns, Group II. intron appear only in four cases.

The analysis of the spectra of *Pichia anomala* VKM Y-159 strain

We tested the inhibitory effect on several yeast strains: some human pathogen yeast are inhibited (*C. norvegica*, *C. tropicalis,C. guilliermondii, C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. inconspicua, C. lypolitica* and *C. viswanathii*), while others seemed to be resistant to the toxin. Therefore, the killer sensitivity pattern against this toxin can be a typing tool for discriminating several *Candida* species.

Optimizing of the toxin production of *Pichia anomala* VKM Y-159 strain

One of the most important conditions for toxin production is the pH of the culture medium. As a rule, mycocins are most active at pH 4 - 5. The temperature range 15–20°C is optimal for incubation when assaying for mycocinogenic activity. We propose, that this strain has two different toxins, one (N toxin) has inhibitory effect only against *C. norvegica*, the other (GN toxin) has broader spectrum. Killer activity is only expressed under acidic conditions: the anti-*C. norvegica* N toxin has activity at pH values between 2 and 6, while the GN toxin is active only on pH values 3 and 4.

Characterization of the toxins produced by *P. anomala* VKM Y-159 strain

The toxins showed high stability up to 50°C. The toxins are stable against proteinase K and pronase E, suggesting that the toxins are either a small oligopeptide or a lipid. The lipidic nature can not been ruled out in the case of N toxin, which is smaller than 10 kDa.

The cytocidic or cytostatic effect was investigated on *C. guilliermondii* and *C. norvegica* by means of plate assays and flow cytometric measures. The toxins have cidic activity, and neither of them arrests the cell cycle.

Several principal cell wall components can be involved in the binding of a killer toxin. In the case of GN toxin β-1,6 D-glucan (pustulane) is the receptor of the toxin, contrary to the most *Pichia sp.* toxin, while N toxin binds through general ionic interactions, especially via bivalent (e.g. Mg^{2+}) cations.

Protein analysis of the GN toxin

To determine the size of the toxins, SDS PAGE method was used. The exact size of GN toxin was not yet defined, however it is either one of the detected proteins between 6-14 kDa or it is a protein between 38-49 kDa. Chromatography experiments can not rule out the possible size of 62-98 kDa. The extracellular protein pattern of non-killer mutants does not differ from the pattern of the wild type.

Analysing of the genetic background of toxins

The genetic base of the killer toxin was examined. Neither plasmid nor viral elements was found using PFGE technique. Mycocin production is presumed to be determined by nuclear genes, as in most *Pichia* species.

Conclusions

- The mtDNA polymorphism among *C. albicans* isolates was studied
- Fourty-four clinical isolates were tested, four mtDNA types were established
- Physical (*Eco*RI-*Eco*RV) and partial functional map of the types was generated
- FZRV region was chosen, with distinctive *Eco*RV pattern - a good typing tool for differentiation of *C. albicans* strains, besides the existing EO3 typing.
- Twenty-three clinical *C. parapsilosis* isolates were screened for mtDNA polymorphism all the isolates have the same mtDNA RFLP pattern
- Sequencing of the whole mt genome of *Candida maltosa* CBS 5611 strain revealed a size of 62949 bp, and circular conformation.
- The comparative analysis of mitochondrial intron content of the species belong to *C. albicans* clade was carried out.
- We tested the spectra of the toxin produced by *Pichia anomala* VKM Y-159 strain.
- We propose, that this strain produces at least two kinds of toxin, one is active only against *C. norvegica* (N toxin), the other has a broader spectrum (GN toxin).
- The toxins can be applied for biotyping of several *Candida* species
- We optimized the killer toxin production of *Pichia anomala* VKM Y-159 strain
- Both of the toxins are resistant to high temperature and protein degrading enzymes
- The N toxin is lipid, while the GN toxin has proteinaceous nature
- Both of the toxins have cidic effect, and they do not arrest the cell cycle. Presumably they make pores on the plasma membrane.
- The specific cell wall receptor of GN toxin is ß-1,6 D-glucane, while the binding of N toxin based upon general ionic interactions.
- The exact size of GN toxin is not yet determined.
- No extrachromosomal element beside the mtDNA could be detected, so we can conclude that the killer phenotype is coded on the chromosome.

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