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Genome dynamics and virulence in the human pathogen *Candida glabrata*

Khadija Mohamed Ahmad



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DOCTORAL DISSERTATION

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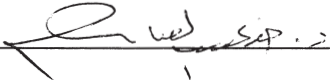
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Abstract Although the yeast <i>Candida glabrata</i> is considered to be a part of the commensal microflora in healthy individuals, during the last years it has been frequently isolated from patients with mucosal and systemic fungal infections. Now it is considered as the second most frequently isolated pathogenic yeast after <i>Candida albicans</i> . Despite its name, <i>C. glabrata</i> is phylogenetically a closer relative to <i>Saccharomyces cerevisiae</i> than to <i>C. albicans</i> . Apparently, <i>C. glabrata</i> has only recently changed its life style and become a successful opportunistic pathogen. It has been found that this yeast can rearrange its genome to cope the surrounding environments, and I show hereby that clinical isolates of <i>C. glabrata</i> show enormous genomic plasticity. How this yeast reshuffles its genome to become a successful human pathogen remains to be elucidated. During the last decades, several studies have been conducted to find out the mechanisms behind the pathogenicity of <i>C. glabrata</i> . Some studies have shown that <i>C. glabrata</i> can adapt to the harsh conditions by changing the number and size of chromosomes but intra- and inter-chromosomal segmental duplications have also been observed. Moreover, <i>C. glabrata</i> has become of great interest for researchers due to its rapid development of antifungal drug resistance. Therefore, the mechanisms involved in genome rearrangement of <i>C. glabrata</i> to survive as a human pathogen and how it tolerates azole antifungal therapy is an interesting aspect to study. In this study I also developed a new tool, RNAi, to study putative virulence genes.		
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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

يَرْفَعُ اللَّهُ الَّذِينَ آمَنُوا مِنْكُمْ وَالَّذِينَ أُوتُوا الْعِلْمَ دَرَجَاتٍ

صَدَقَ اللَّهُ الْعَظِيمِ

In the name of Allah the most gracious the most Merciful

Allah will raise, in degree, those of you who believe and those who are
endowed with knowledge

(From the Holy Quran- Surat Almujaadilah verse11)

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List of publications

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals. The papers are appended at the end of this thesis.

- I. Khadija Mohamed Ahmad, Olena P. Ishchuk, Linda Hellborg, Gloria Jørgensen, Miha Skvarc, Jørgen Stenderup, Dorte Jørck-Ramberg, Silvia Polakova, Jure Piškur (2013). Small chromosomes among Danish *Candida glabrata* isolates originated through different mechanisms. *Antonie Van Leeuwenhoek*. 104: 111-122.
- II. Khadija Mohamed Ahmad, Janez Kokošar, Xiaoxian Guo, Zhenglong Gu, Olena P. Ishchuk and Jure Piškur (2014). Genome Structure and Dynamics of the Yeast Pathogen *Candida glabrata*. *FEMS Yeast Research*. (In press).
- III. Olena P. Ishchuk, Silvia Polakova, Khadija Mohamed Ahmad, Praveen Chakravarthy, Sofia Mebrahtu Wisén, Sofia Dashko, Maryam Bakhshandeh, Leif Søndergaard, Victoria Rydengård, Artur Schmidtchen, John Synnott, Can Wang, Sarah Maguire, Geraldine Butler & Jure Piškur. The haploid nature of *Candida glabrata* is advantageous under harsh conditions. 2014. *Manuscript*.
- IV. Khadija Mohamed Ahmad, Klara Bojanovič, Lydia Kasper, Sascha Brunke, Bernhard Hube, Torbjörn Säll, Jure Piškur and Olena P. Ishchuk. Development of RNAi tools to study putative virulence genes in the yeast *Candida glabrata*. 2014. *Manuscript*.

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My contributions to the presented papers

- I. Khadija Mohamed Ahmad, Olena P. Ishchuk, Linda Hellborg, Gloria Jørgensen, Miha Skvarc, Jørgen Stenderup, Dorte Jørck-Ramberg, Silvia Polakova, Jure Piškur. Small chromosomes among Danish *Candida glabrata* isolates originated through different mechanisms.

I designed the experiment with Jure Piskur. I did the whole experimental part and analyzed the data with other co-authors. I was the main actor behind the writing.
- II. Khadija Mohamed Ahmad, Janez Kokošar, Xiaoxian Guo, Zhenglong Gu, Olena P. Ishchuk and Jure Piškur. Genome Structure and Dynamics of the Yeast Pathogen *Candida glabrata*.

I wrote the review together with Jure Piskur.
- III. Olena P. Ishchuk, Silvia Polakova, Khadija Mohamed Ahmad, Praveen Chakravarthy, Sofia Mebrahtu Wisén, Sofia Dashko, Maryam Bakhshandeh, Leif Søndergaard, Victoria Rydengård, Artur Schmidtchen, John Synnott, Can Wang, Sarah Maguire, Geraldine Butler & Jure Piskur. The haploid nature of *Candida glabrata* is advantageous under harsh conditions.

I performed the mouse experiments and a part of the fly experiment and analyzed the final data.
- IV. Khadija Mohamed Ahmad, Klara Bojanovič, Lydia Kasper, Sascha Brunke, Bernhard Hube, Torbjörn Säll, Jure Piškur and Olena P. Ishchuk. Development of RNAi tools to study putative virulence genes in the yeast *Candida glabrata*.

I performed a part of RNAi cloning into putative virulence genes and I performed a part of the data analysis. I wrote the manuscript together with Olena and Jure.

Abbreviations

<i>ACE2</i>	Transcription activator encoding (factor required for septum destruction after cytokinesis)
<i>CDR</i>	<i>Candida</i> drug resistance
<i>EPA</i>	Epithelial adhesion genes
<i>FLO</i>	Flocculation genes
<i>HML</i>	Mating type cassette – Left
<i>HMR</i>	Mating type cassette – Right
HO	The HO gene encodes an endonuclease responsible for initiating mating-type switching
<i>IME1</i>	Inducer of meiosis
<i>MDR</i>	Multidrug resistance
<i>MFALPHA2</i>	Mating Factor ALPHA
<i>MTL</i>	Mating type like
NADH	Nicotinamide adenine dinucleotide
NA	Nicotinic acid
PDRE	Pleiotropic drug response elements
<i>SIR</i>	Sirtuin family, involved in subtelomeric silencing
<i>SMK1</i>	Sporulation-specific MAP kinase
<i>SNF1</i>	Sucrose non-fermenting-1, Putative serine/threonine protein kinase required for trehalose utilization
<i>STE2</i>	Cell type-specific sterile gene encodes alpha-factor receptor
<i>STE3</i>	Cell type-specific sterile gene encodes a factor receptor

Abstract

Although the yeast *Candida glabrata* is considered to be a part of the commensal microflora in healthy individuals, during the last years it has been frequently isolated from patients with mucosal and systemic fungal infections. Now it is considered as the second most frequently isolated pathogenic yeast after *Candida albicans*. Despite its name, *C. glabrata* is phylogenetically a closer relative to *Saccharomyces cerevisiae* than to *C. albicans*. Apparently, *C. glabrata* has only recently changed its life style and become a successful opportunistic pathogen. It has been found that this yeast can rearrange its genome to cope the surrounding environments, and I show hereby that clinical isolates of *C. glabrata* show enormous genomic plasticity. How this yeast reshuffles its genome to become a successful human pathogen remains to be elucidated.

During the last decades, several studies have been conducted to find out the mechanisms behind the pathogenicity of *C. glabrata*. Some studies have shown that *C. glabrata* can adapt to the harsh conditions by changing the number and size of chromosomes but intra- and inter-chromosomal segmental duplications have also been observed. Moreover, *C. glabrata* has become of great interest for researchers due to its rapid development of antifungal drug resistance. Therefore, the mechanisms involved in genome rearrangement of *C. glabrata* to survive as a human pathogen and how it tolerates azole antifungal therapy is an interesting aspect to study. In this study I also developed a new tool, RNAi, to study putative virulence genes.

Introduction

Candida glabrata is currently the second most usual cause of yeast infections. This yeast is phylogenetically more related to *Saccharomyces cerevisiae* than to *Candida albicans*. Many systemic infections have recently been found associated with *C. glabrata* yeast. Apparently, this yeast can easily reshuffle its genome and this is one of the topics of my thesis.

During the last decades a few studies have been conducted to find out the mechanisms behind the pathogenicity of *C. glabrata*. Some of these studies have found that *C. glabrata* can adapt to the harsh conditions by changing the number and the size of chromosomes also intra- and inter-chromosomal segmental duplications have been observed. One part of my study was to focus on the mechanisms involved in the genome rearrangements that are likely a way how to survive in the human and to become resistant to azole antifungal therapy.

C. glabrata is an asexual yeast and only haploid isolates have been found so far. Organisms can adapt to a new environment by rearranging their chromosomes. The ploidy and genomic instability have been reported to be associated with increased virulence.

In my thesis we generated hybrids of *C. glabrata* isolates and we let them grow under different stressful conditions including high temperature and the presence of azole. The aim of this experiment was to find out which strain, haploid or diploid, was more resistant to harsh environments. The competition was conducted *in vitro* and *in vivo* using the fly and mouse models. The genes that were highly expressed to overcome the stress were elucidated by microarrays.

Like other budding yeasts, *C. glabrata* has lost RNA interference pathway which is involved in the regulation of gene expression. In human, this pathway has been reported to play a crucial role in silencing genes related to some diseases. The scientists were able to silence some genes in *Saccharomyces cerevisiae* by reconstitution of RNAi system from *Saccharomyces castellii*. The beauty of this tool is that one can study the function of essential genes which can otherwise not be deleted. RNAi tool was successfully designed in our laboratory and we could silence *URA3* and *ADE2* and putative virulence genes and could study the resulting strains in the macrophage cell cultures.

1. Kingdom of Fungi

The fungi form a separate kingdom including up to 1.5 million different species. Among these species some adopted a unicellular life form and are called yeast. Fungi are abundant worldwide, occupy diverse ecological niches, differ enormously by their morphology and metabolism and can be living as symbionts or commensals and they have been associated with humans for a long time. Some fungi, like baker's yeast *Saccharomyces cerevisiae*, are used as model eukaryotic organisms in molecular biology studies. Fungi play a crucial role in decomposition of organic matters and nutrient recycling. They are sources of drugs (e.g. penicillin and other antibiotics), food products (baking, brewing products and edible mushrooms) and are used in heterologous protein production. Some fungi can also be pathogenic and cause several diseases in humans and other living organisms.

2. Human pathogenic yeasts

In spite of a great number of different fungal species, fewer than 100 species are known to cause human diseases (Butler, 2010). Yeast pathogens include organisms from both *Ascomycota* (like *Candida* spp.) and *Basidiomycota* (like *Cryptococcus* spp.) phyla.

The genus *Candida* with 150 different species includes both non-pathogenic and opportunistic human pathogens. *Candida* species comprise one of the largest groups of pathogenic fungi and are the most frequent fungal pathogens worldwide. The most common human pathogens are *Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis* and *Candida krusei* (Shoham & Levitz 2005) (Fig. 1).

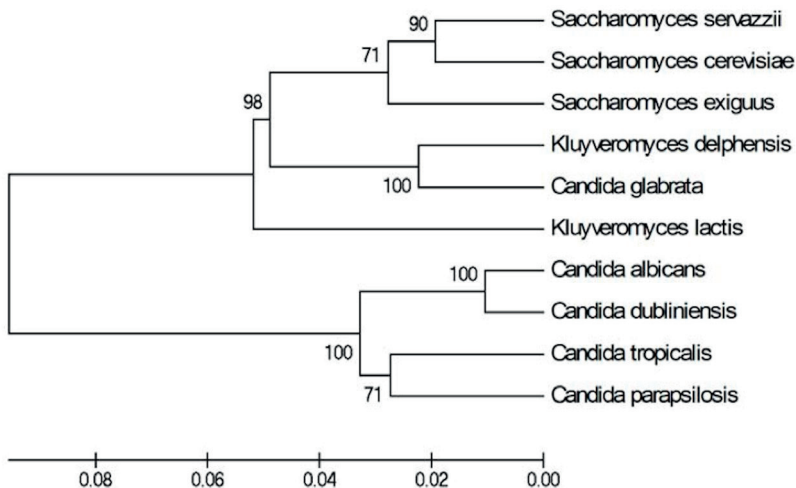


Figure 1: Phylogenetic relationship between *Candida* species and other hemiascomycetes based on analysis of their 26S rDNA. 587 bp of the sequences were aligned using Clustal X, and MEGA 4.0 method was used to construct the tree. The scale bar represents the number of base substitutions per site. Note that *C. glabrata* and *S. cerevisiae* are phylogenetically closely related and are quite distant from the other pathogenic *Candida* species. (adapted from Polakova, 2008).

According to the old classification, the *Candida* species belong to the class Fungi Imperfecti, the order Moniliales and the family Cryptococaceae (Sinnott *et al.*, 1987;

Kwon-Chung *et al.*, 1992). *C. albicans* is a dimorphic pathogen and usually found as a commensal of the human digestive system and vaginal tract, and under certain conditions (e.g. changed pH or temperature) it can switch between a mycelium and a yeast cell form (Pla *et al.*, 1996). Also, *C. albicans* has the ability to form chlamydospores, thick-wall entities, at the terminal ends of hyphae. Among all *Candida* species this property is exhibited by *C. albicans* as well as *C. dubliniensis*. Compared to *C. albicans*, *C. glabrata* does not form hyphae (nondimorphic yeast) and exists as small blastoconidia (1-4µm in size) under all environmental conditions. On CHROMagar (identification selective medium of yeast) (Yücesoy & Marol, 2003) *C. glabrata* has pink to purple colonies. In contrast, *C. albicans* colonies appear from green to blue-green (Fidel *et al.*, 1999). Morphologically, *C. dubliniensis* is similar to *C. albicans* as it forms both germ tube and chlamydospores and on CHROMagar medium (Fig. 2), it shows green colonies like *C. albicans*. In spite of these similarities, the species can be distinguished from each other by the growth at 45°C, at this temperature *C. dubliniensis* does not grow.

C. krusei is one of the most common causes of Candidemia (presence of *Candida* in the blood) because of its innate resistance to the antifungal agent fluconazole. The blastoconidia of this yeast are elongated (25µm in length) and its colonies appear pink with fuzzy texture on CHROMagar medium.

Another pathogenic *Candida* species is *C. tropicalis* that is isolated from patients with leukemia as well as from asymptomatic individuals. On CHROMagar medium its colonies appear from steel-blue to dark gray and can also exhibit a brown or purple halo. *C. parapsilosis* also causes Candidemia, especially in neonatal intensive care units (Bendel, 2003 & Chapman, 2003) as well as in patients with intravenous catheter and prosthetic devices (Kurtzman *et al.*, 2011). On CHROMagar, the colonies of this species appear from ivory to pink and lavender and some are wrinkled. In Paper I, we introduce a collection of approximately 200 clinical isolates of *C. glabrata*.

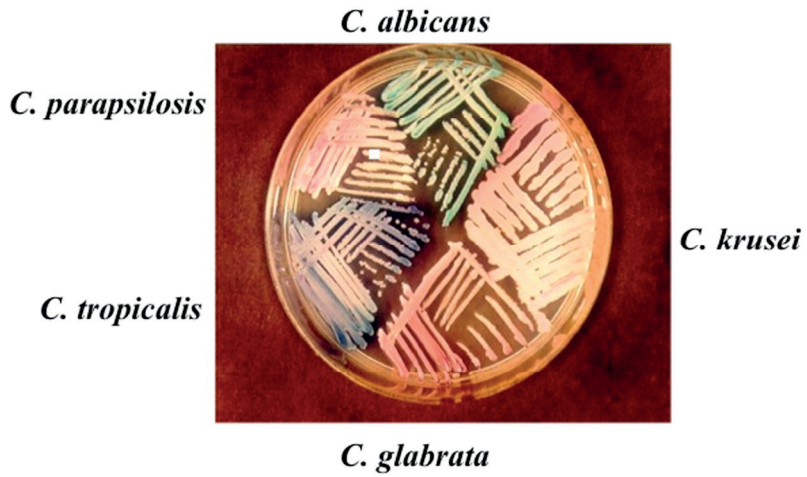


Figure 2: CHROMagar media with different colony colors of 5 different *Candida* species: *C. albicans*, *C. krusei*, *C. glabrata*, *C. tropicalis* and *C. parapsilosis* (Yücesoy & Marol, 2003).

3. Phylogeny of *Candida* species

Most *Candida* species including *C. albicans* belong to one monophyletic group, whereas *C. glabrata* is more related to *S. cerevisiae* than to the other *Candida* species (Kurtzman *et al.*, 1998; Susan *et al.*, 1991). The phylogenetic relationships are shown in Figure 1. The phylogenetic divergence between *S. cerevisiae* and *C. glabrata* is slightly larger than the distance between humans and fish (Dujon *et al.*, 2004). Kurtzman, (2003) has found that *C. glabrata* with three environmental species *Kluyveromyces delphensis*, *Candida castellii* and *Kluyveromyces bacillisporus* belong to *Nakaseomyces* genus. However, two more pathogenic species have recently been added to *Nakaseomyces* clade, *Candida nivariensis* and *Candida bracarensis* (Alcoba-Florez *et al.*, 2005; Correia *et al.*, 2006).

In *Nakaseomyces* genus, *C. glabrata*, two recently identified pathogenic species and *K. delphensis* are classified as one group namely, “glabrata group” (Gabaldón *et al.*, 2013). Certainly, *C. glabrata* is the predominant pathogen among *Nakaseomyces*. The ability of *C. glabrata* to become pathogenic is probably related to gene expansion of some gene families which have been reported as virulence genes involved in cell adhesion such as the *EPA* genes (Cormack *et al.*, 1999).

Although *C. albicans* and *C. glabrata* are phylogenetically separated by several nonpathogenic species, they share some common traits, such as genome instability (Diogo *et al.*, 2009; Polakova *et al.*, 2009; see also Paper I).

4. Clinical significance of *Candida* infections

Pathogenic fungi can be classified to different categories depending on the type of infection: (I) superficial infections (e.g. dermatophytes) involve the exposed parts of the body (skin and hair), (II) systemic infections, the pathogen is spread through the blood and causes severe infections which can be even fatal (e.g. histoplasmosis caused by *Histoplasma capsulatum*). Candidiasis (infections caused by yeast *Candida* species) are both superficial (mucosal surfaces of the mouth and vagina) and systemic diseases and occur as a result of immunosuppression (Moran *et al.*, 2011). *Candida* species are opportunistic organisms, they are usually found as a part of normal microflora of the body (e.g. oral cavity, digestive system and vagina). They are asymptomatic in the healthy individuals, but for not well known reasons and under specific circumstances, like in immunocompromised patients, they cause diseases that can be fatal.

Although several species such as *C. glabrata*, *C. prapsilosis*, *C. tropicalis* and *C. krusei* have been isolated from patients with Candidiasis, *C. albicans* remains the most common cause of these infections (MacFarlane *et al.*, 1990). Patients with low immunity may have Candidiasis even before or while being hospitalized (Scott *et al.*, 1996). Moreover, the risk of *Candida* species infection increases with duration of the hospital stay (Olaechea *et al.*, 2004). Until recently, *C. albicans* was thought to be the main cause behind *Candida* infections. However, with widespread use of antimycotic therapy together with use of immunosuppressive medicine, the infections with non-albicans *Candida* species and especially with *C. glabrata* have increased significantly during last decades (Komshian *et al.*, 1989). Due to the increase of the *C. glabrata* infections number and their high mortality rate, the molecular mechanisms behind its virulence deserve more concern and studies. Nowadays, *C. glabrata* has become of great interest to researchers also due to its rapid development of antifungal drug resistance, which may be related to its haploid nature (Jong *et al.*, 2007 & Fidel *et al.*, 1999). So far, little is known regarding the virulence of *C. glabrata* and host defense against this organism. To develop more effective antifungal drugs we need to understand the mechanisms behind the pathogenesis of *C. glabrata* infections.

Predisposing factors for *Candida* infections

Many factors are contributing to *Candida* infections. (1) Antibiotic over-use, which kills the healthful bacteria and allow the overgrowth of *Candida*. (2) Poor nutrition (e.g. elderly patients) and unbalanced diet with high levels of sugar. (3) Low immunity as a result of chemotherapeutic agents, HIV infection and steroid drugs usage will increase the probability of *Candida* infections. (4) The gender-specific likelihood of infection with specific pathogens may be related to certain anatomic and biochemical features (e.g., vaginal pH) or to neuroendocrine differences (Charles *et al.*, 1989). (5) Moreover, vaginal Candidiasis is more common among women using oral contraceptives compared to those that do not (Oriol *et al.*, 1972). (6) Age factor: it has been reported that the colonization of the oral cavity by *Candida* species increases with increasing age as the natural suppression of yeast carriage in the oral cavity breaks down in old individuals (Lockhart *et al.*, 1999). In addition, there are variable changes involved with aging process which making the older patient more potential for fungal infections. With aging the salivary pH gradually changes and becomes slightly more acidic compared with that in younger individuals leading to increase oral colonization of *C. glabrata* (Lundgren *et al.*, 1996). It has been found when there is hyposalivation (associated with aging process), the presence of protein in the oral cavity will be reduced and consequently lacking of the substance with antimicrobial activity, like lysozyme (Hof & Mikus, 2013). Low immunological response associated with aging has also been reported as predisposing factor to infections (Hof & Mikus, 2013). Moreover, in case of using medical devices (e.g. denture), *C. glabrata* has great tendency to attach to their surface which can protect yeast cells from high pH, flushing and antimicrobial activities of saliva (Brandtzaeg *et al.*, 1995; Situ *et al.*, 2000). In Paper I, most of the presented *C. glabrata* isolates originate from systemic infections, from patients at Danish hospitals.

Table 1: Predisposing factors for *Candida* infections.

Route or source	Pathological conditions	Physiological state with high susceptibility to infection
Antibiotics Skin problems (e.g. burn or wounds) Devices (e.g. Indwelling catheter & denture) Oral contraceptive and vaginal pills Immunosuppressive Radiation	AIDS Cancer Organ transplantation Diabetes mellitus and other hormonal diseases	Neonate and elderly Neonates Pregnancy and menopause

5. Biology of *Candida glabrata*

C. glabrata is not polymorphic and under the standard condition, it grows as budding yeast (blastoconidia) which is oval 1-4 μm in diameter and exhibiting no difference between the commensal and pathogenic forms (Fig. 3). On Sabouraud dextrose agar media, *C. glabrata* appears as glistening, smooth and creamy colored colonies which are similar to those of other *Candida* species. Compared to *S. cerevisiae* and *C. albicans* which assimilate a number of sugars, *C. glabrata* assimilates only glucose and trehalose (Hazen, 1995). As it has been reported, *C. glabrata* has lost various genes involved in galactose metabolism (Dujon *et al.*, 2004) after its “regressive” evolution. The *SNF1* (sucrose non-fermenting, serine/therionine protein kinase), which has a role in regulation of respiration and fermentation in *S. cerevisiae*, has also been isolated from *C. glabrata*. As it has been reported the deletion of this gene, *SNF1*, caused loss of trehalose assimilation (Petter & Kwon-Chung, 1996). Recently it has been reported that *C. glabrata* is an efficient alcohol producer even in the presence of glucose (Hagman *et al.*, 2013). On CHROMagar as mentioned early, *C. glabrata* has pink to purple colonies.

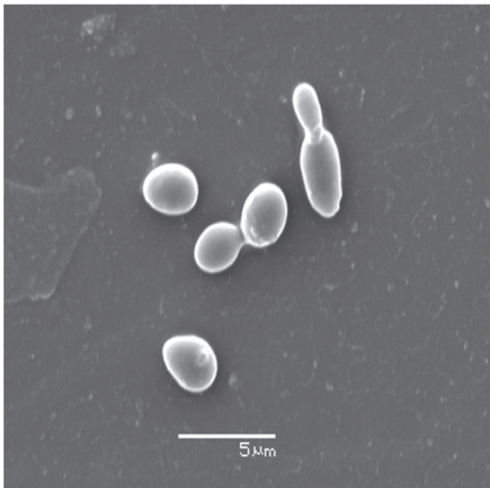


Figure 3: *C. glabrata* yeast under electron microscope (2-3 μm in diameter size). The picture also shows a daughter cell emerging through the budding process.

6. Mating in fungi

Fungi reproduce sexually and asexually. However, several factors such as nutrition, light, pH and humidity may induce the sexual reproduction. Unlike the vegetative cells, the reproductive units, like spores, are more resistant to environmental stress such as extreme temperatures, aggressive solvents and starvation (Staben *et al.*, 1994). It has been shown that *C. albicans* is diploid but no sexual reproduction has been observed for this species (Olaiya *et al.*, 1979, Riggsby *et al.*, 1982). This yeast reproduces asexually by budding or by hyphal formation. *C. albicans* has a highly regulated special sexual cycle where the opposite mating types mate efficiently producing a tetraploid, which after chromosomal loss, gradually get reduced to the diploid form (Hull *et al.*, 2000). The chromosome loss resulting in diploid state can occur efficiently in tetraploid strains if they are grown at 37°C on a pre-sporulation (pre-spo) medium with 10% glucose (Bennett & Johnson, 2003). The genome of *C. albicans* contains a cluster of genes *MTLa* and *MTL α* (mating type-like) homologous to the sexual cycle regulators *MATa* and *MAT α* of *S. cerevisiae*. However, *C. albicans* has additional regulator $\alpha 2$, encoded by the *MTLa* gene (Hull & Johnson 1999).

In *S. cerevisiae*, the genes of the *MAT* locus can be either $a1$ or α type ($\alpha 1$ and $\alpha 2$) and their expression give rise to three cell types, (a , α and a/α). (a) cells carry *MATa*, and (α) cells carry *MAT α* , while the a/α cell is produced by mating between an a cell and an α cell (Johnson, 1995; Herskowitz *et al.*, 1992). It is usually a diploid cell that has both *MATa* and *MAT α* and is able to undergo meiosis and spore formation (Hull *et al.*, 2000). *S. cerevisiae* genome encodes *HML*, carrying *MAT α* genetic information and *HMR* which harbors *MATa* genetic information and both of them are transcriptionally silenced. *S. cerevisiae* genome has also pheromone receptor genes, *STE2* and *STE3* that encode respectively α -factor and a -factor receptors (Herskowitz, 1988; Herskowitz *et al.*, 1992).

The *C. glabrata* genome carries many genes that could be involved in mating and meiosis. The situation is similar to the sexual species *Kluyveromyces delphensis*, which is the closest known sexually active relative. However, *C. glabrata* is apparently asexual and it is in this sense similar to *C. albicans* (this yeast has recently been shown to have a peculiar form of sex). It has conserved mating machinery but no sexual stage in its life cycle has been discovered so far (Wong *et al.*, 2003). Like *S. cerevisiae*, *C. glabrata* encodes three main mating type loci (*MTL1*, *MTL2* and *MTL3*) (Butler *et al.*, 2004, Srikantha *et al.*, 2003), in addition, the genome of *C. glabrata* encodes other *S. cerevisiae*

homologous genes, including *IME1* and *SMK1* that have no known specific function but are rather likely involved in mating or meiosis, as well as a pheromone production (*MFALPHA2*) (Wong *et al.*, 2003).

In *C. glabrata* the pheromone receptor genes *STE2* and *STE3* are expressed in both cell types (Muller *et al.*, 2008). The *C. glabrata* genome also encodes HO endonuclease which has a function in gene conversion that underlies mating type switching in *S. cerevisiae* (Butler *et al.*, 2004). Their recognition sites present at the *MTL1* locus and mating type switching at *MTL1* have been confirmed at the site of infection in the patients (Brockert *et al.*, 2003).

Table 2: Selected genes that regulate the sexual cycle of *S. cerevisiae* and perhaps of *C. glabrata* (See the text for references).

<i>S. cerevisiae</i>	Function	<i>C. glabrata</i>	Function
<i>MATa</i> ($\alpha 1$) <i>MATα</i> ($\alpha 1$ and $\alpha 2$)	Control sexual mating	<i>MTL1</i> (<i>a/alpha</i>)	Determines mating type
<i>HMRa</i>	Silent cassette	<i>MTL2a</i>	<i>MTLa</i> dependent splicing of $\alpha 1$ transcript
<i>HMLalpha</i>	Silent cassette	<i>MTL3alpha</i>	Silent locus
<i>STE2</i>	Specific pheromone receptor for α - factor	<i>STE2</i>	Pheromone receptor for α - factor, but it is not cell specific and expressed in both cell types
<i>STE3</i>	Specific pheromone receptor for α - factor	<i>STE3</i>	Pheromone receptor for α - factor, but it is not cell specific and expressed in both cell types
HO	Mating type switching	HO	Mating type switching

7. Genome evolution in *C. glabrata* and related species

7.1 Genome overview

The *C. glabrata* CBS 138 strain genome has been sequenced and available since 2004 (<http://cbi.labri.fr/Genolevures/>) (Dujon *et al.*, 2004). This strain has 13 chromosomes (Fig. 4); and this number proved to be higher than 8-12 determined by Kaufmann *et al.* (1989). The total size of the genome is 12.3 Mb. In comparison to *S. cerevisiae*, which has app. 6000 protein coding genes, and 38.3% of G+C content, *C. glabrata* genome has 5283 coding sequences, with the average gene size being 493 codons and 38.8% of G+C content. There is high sequence identity and high degree of gene synteny in both yeast species. *C. glabrata* has 207 genes coding for tRNAs in contrast to 274 genes in *S. cerevisiae*. Compared to *C. albicans* which has a diploid genome and therefore has about 32Mb of the nuclear DNA, the genome of *C. glabrata* is haploid (Magee *et al.*, 2002). Compared to *S. cerevisiae* that has a single intrachromosomal locus of rDNA, *C. glabrata* has two intrachromosomal rDNA repeat loci that are found in subtelomeric regions (Dujon *et al.*, 2004).

The mitochondrial genome of *C. glabrata* is circular and composed of 20 Kb. The protein coding genes compared to those of *S. cerevisiae* is conserved, i.e. three subunits of ATP synthase (*ATP6*, *ATP8*, and *ATP9*), Apocytochrome b (*COB*), three subunits of cytochrome oxidase (*COX1*, *COX2*, *COX3*) (Koszul *et al.*, 2003) and ribosomal protein (*VARI*) (Ainley *et al.*, 1985). However, the genome of *C. glabrata* encodes the large (*LSU*) and the small subunit (*SSU*) of Ribosomal RNAs, RNA component of RNase P (Shu & Martin 1991) and set of 23 tRNA where the tRNA- recognizing CGN codon (Arg) is missing compared to *S. cerevisiae* (Clark-Walker *et al.*, 1985). Nosek and Fukuhara found that the genome of *C. glabrata* does not encode subunits of NADH (Nicotinamide adenine dinucleotide) ubiquinone oxidoreductae (complex I) in contrast to other *Candida* species (Nosek & Fukuhara, 1994).

The chromosome number of yeast species in this clade varies between 8 chromosomes in *C. castellii* (Gordon *et al.*, 2011) to 15 in *N. bacillisporus* (Gabaldón *et al.*, 2013). The chromosomal number of “glabrata group” species is less variable, 10-13 chromosomes. The degree of gene order is more conserved among species in “glabrata group” with higher protein identity level (77-88%), compared to 53% between *C.*

castellii and *N. bacillisporus* (Gabaldón *et al.*, 2013). In Paper II I discussed the comparative genomic aspects among *C. glabrata* and its closest relatives.

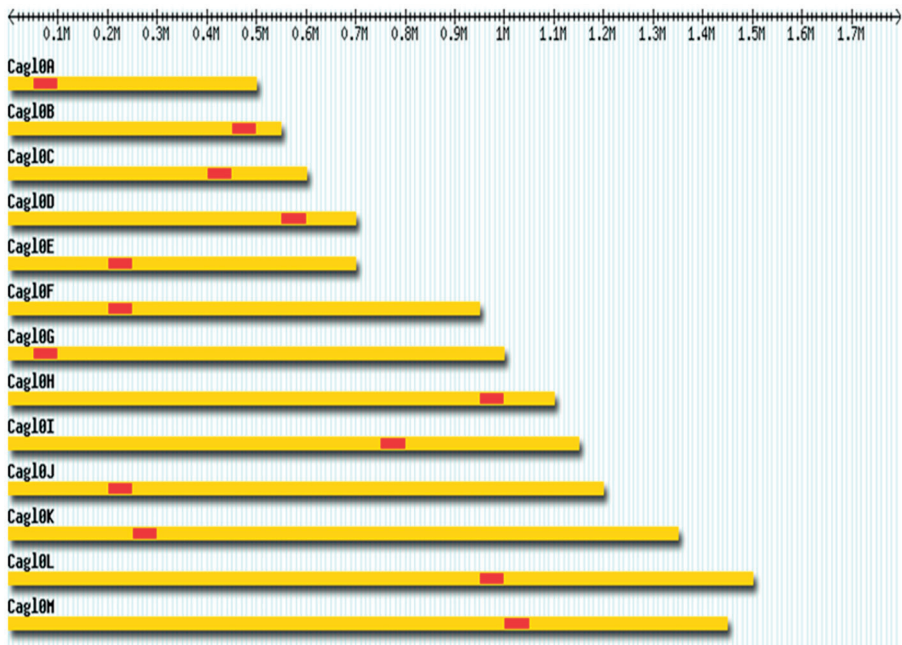


Figure 4: The genome of *Candida glabrata* CBS 138 showing 13 chromosomes marked from A to M according to *C. glabrata* nomenclature. Red stripes symbolize the position of the centromere. (<http://cbi.labri.fr/Genolevures>).

7.2 Evolution and gene loss

After the whole genome duplication (WGD), *C. glabrata* has undergone a faster gene loss than *S. cerevisiae*, e.g. *C. glabrata* has lost genes involved in galactose, phosphate, and nitrogen and sulfur metabolism (Dujon, 2004). *C. glabrata* adaptation in human as a commensal and as a pathogen might be related to this gene loss (Dujon *et al.*, 2004; Dujon, 2010; Ehrlich *et al.*, 2008). If the gene has no important function in the organism, it will become a pseudogene and finally disappear from the genome (Wolfe, 2006). For example, *C. glabrata* has lost genes for galactose metabolism (*GAL1*, *GAL7*, and *GAL10*), which is consistent with the fact that it is unlikely to find galactose in the human host. The pathway of nicotinic acid biosynthesis (*BNA* genes) has also been lost in *C. glabrata* (Domergue *et al.*, 2005).

The loss of active transposons has also been observed in many yeast genomes. Both active classes I, non-long terminal repeat (LTR) and class II (propagating without RNA intermediate) have been described for *C. albicans*. 34 families of LTR-retrotransposon (Ty elements) have been identified in *C. albicans* and three of them have been found as active members (Goodwin *et al.*, 2000). In contrast, *C. glabrata* does not possess repetitive elements like transposons. Only one mutated full length copy of Ty3 element has been found in this yeast species.

7.3 Genome dynamics

In eukaryotes, the number and rough organisation of chromosomes are well preserved within isolates of the same species. Changes in the genomic organization are rare and often associated with pathological events (e.g. cancer). In contrast for some pathogens, aneuploidy and genome rearrangements are reported and are assumed to be advantageous for survival in the host. For example in *Leishmania*, a great variation in karyotypes, aneuploidy and deletion are associated with changes in the drug resistance and virulence (Beverley, 1991; Ubeda *et al.*, 2008). Genome rearrangements such as chromosome length polymorphism and variation in the chromosome copy number have been described for pathogenic yeast *C. albicans* (Diogo *et al.*, 2009) and have been reported to be associated with azole resistance (Coste *et al.*, 2007). The second most prevalent yeast human pathogen *C. glabrata* has dynamic genome which is rapidly changing during the period of infection (Shin *et al.*, 2007). Different karyotypes of *C. glabrata* have been observed in the same patient over the period of infection (Paper I). In *C. glabrata*, chromosomal rearrangements, translocations, chromosome fusions and inter-chromosomal duplications lead to variable karyotypes and acquisition of drug resistance (Paper I; Polakova *et al.*, 2009). The chromosomal translocations and copy number variation within tandem gene repeats has also been detected in *C. glabrata* genome (Muller *et al.*, 2009). Moreover, subtelomeric silencing in *C. glabrata* is induced by Sir complex (Sir2, Sir3, and Sir4), and it has been reported that the polymorphism Sir3 is associated with more adherence of *C. glabrata* to epithelial cells (Martinez-Jimenez *et al.*, 2013), whereas the deletion of *HST1* (homologue of *SIR2*) is associated with increased fluconazole resistance and decrease in the susceptibility of *C. glabrata* to stress conditions like hydrogen peroxide (Orta-Zavalza *et al.*, 2013). In Paper II I discussed the relationship between the genome dynamics and virulence.

8. Ploidy and chromosomal instability

The genomic instability and gross chromosomal rearrangements are usually associated with pathological disorders. The chromosomal instability is change in chromosome number caused by failure in spindle checkpoint or error in chromosomal segregation leading to chromosome gain or loss (Draviam *et al.*, 2004). The genome rearrangement could be considered as a mechanism for the organism to adapt to new environment. In human, the genome instability is usually associated with inherited diseases and it has been found in 90% of solid tumor (Albertson *et al.*, 2003). In yeast like *S. cerevisiae*, the genome instability increases with the aging, which is almost like with the humans where the risk of the cancer is often increased with age (McMurray *et al.*, 2003). Torres *et al.* (2007) has reported that the haploid and diploid cells of *S. cerevisiae* with aneuploidy have growth defects and they are associated with delay in cell cycle progression with increased glucose uptake. In addition to growth defect, the aneuploidy yeast has been found to have abnormal phenotypes like formation of elongated buds (Nikitin *et al.*, 2008). However, the delay of cell growth has also been observed when the strains carrying extra chromosomes and it might be due to more proteins with imbalance in protein consumption (Torres *et al.*, 2007). This result has been confirmed by Jung *et al.* (2011) where they found that in the presence of the chromosomal rearrangement, the haploid strains have ability to degrade the protein and keep the cell in balance compared to the diploids which have shown a lower translation rate. Recently, they have reported that the evolved isogenic haploids of *S. cerevisiae* adapted faster than the diploid strains in different environments (Gerstein *et al.*, 2011). *C. albicans* yeast was considered to be an obligate diploid, but recently they have reported that this yeast has ability to form haploid that can mate to form diploids or it can undergo auto-diploidization (Hickman *et al.*, 2013). Moreover, upon exposure to stress, the diploids of *C. albicans* can mate and create tetraploids which eventually return to the diploid state as a result chromosomal loss (Forche *et al.*, 2008). *C. glabrata* and all other species in *Nakaceomyces* have haploid genome with size 10 to 12 Mb except *N. bacillisporus* which is diploid (Gabaldón *et al.*, 2013). In Paper III I present my studies on the haploid nature of *C. glabrata*.

9. The mechanisms of virulence in *C. glabrata*

It has been shown that *C. glabrata* has relatively lower virulence and pathogenicity in animal models compared to *C. albicans* (Bernardis *et al.*, 1990, Shakir *et al.*, 1983). In humans, infections with *C. glabrata* are frequently associated with other *Candida* species and commonly isolated from oral lesions with *C. albicans* (Vazquez *et al.*, 1999). These mixed infections can cause severe symptoms and are more difficult to treat (Redding *et al.*, 1999). The infections caused by *Candida* species are mediated by several virulence factors including adherence and biofilm formation, production of hydrolytic enzymes which facilitate tissue damaging and others. Several virulence factors (Table 3 & 4) are discussed in the following pages. In Paper IV I attempted to silence a few novel putative virulence genes.

Table 3: Selected genes playing a role in the virulence of *C. glabrata* and *S. cerevisiae* (See the text for references).

Organism	Family protein	Gene	Function
<i>C. glabrata</i>	Lectins	<i>EPA</i> gene family of 23 genes	Involved in adhesion to host cells and different surfaces
		<i>EPA1</i>	Involved in cell to cell adhesion and plastic surfaces adherence
		<i>EPA6</i> & <i>EPA7</i>	Adherence, colonization and biofilm formation
	Phospholipases	<i>PLB1</i>	Not known
	Proteinases	<i>YPS</i> gene family	Cell wall remodeling
		<i>YPS1</i> & <i>YPS7</i> <i>YPS2-6,8-11</i>	Cell wall integrity and interaction with host
	Yak 1p kinase	<i>YAK1</i>	Serine-therionine protein kinase which regulate <i>EPA6</i> expression
Hypervirulence Factor	<i>ACE2</i>	Host- <i>Candida</i> interaction and its deletion cause cell separation defect	
Transcription factor involved in filamentation and cell wall architecture	<i>STE12</i>	Filamentous growth on nitrogen starvation.	
<i>S. cerevisiae</i>	Transcription factor involved in filamentation and cell wall architecture	<i>STE12</i>	In the haploid cell, Ste12p regulate both invasive growth phenotype and mating, whereas in diploid cells, it plays a role in the filamentous growth on nitrogen starvation
	Proteinases	<i>YPS1 to 3, 6, and 7</i>	Cleave proteins and peptides C terminal to basic residues, Induced during cell wall remodeling

Table 4: Selected genes playing a role in the virulence of *C. albicans* (See the text for references).

Organism	Family protein	Gene	Function
<i>C. albicans</i>	Adhesins	<i>ALS</i> gene family (Agglutinin like sequence)	Adherence, involved in interaction of <i>C. albicans</i> with host tissue
		<i>HWP 1</i> (hyphae-specific protein)	Attachment of <i>Candida</i> to host tissue
	Secreted aspartyl proteinases	<i>SAP</i>	Associated with hypha formation, adhesion and phenotypic switching.
	Phospholipases	<i>PLB</i>	It is involved in the spread of the organism through the gastrointestinal tract and as well as in the blood

9.1 Adhesion and biofilm formation

The adherence is required for colonization and persistence of *Candida* species in the host and it could be important during the progression from colonization to infection (Cannon *et al.*, 1995). Compared to *C. albicans*, *C. glabrata* has lower capacity to adhere to oral epithelium. This low capacity of *C. glabrata* adherence to epithelium may be due to lack of hyphae formation (Villar *et al.*, 2004) which is important in adherence and persistence of *C. albicans* in the host (Odds, 1994).

At the molecular level, it has been reported that a hypha-specific protein encoded by *HWP1*, mediates covalent attachment of *C. albicans* to human epithelial cells (Staab *et al.*, 1999) and *ALS*, agglutinin like sequence, which encodes large cell-surface

glycoproteins that are required in the process of adhesion to host surfaces during the early stage of infection (Hoyer *et al.*, 1995 and 2001). It has been demonstrated that Als3p has an invasion-like properties in *C. albicans* (Phan *et al.*, 2007). *C. glabrata* genome encodes several EPA genes. This gene family is similar to *ASL* and *HWP1* of *C. albicans* and encodes glucan-cross-linked cell-wall proteins that bind asialo-lactosyl containing carbohydrates of host cell (Cormack *et al.*, 1999). EPA genes are unique to *C. glabrata* and are required for adaptation and adherence to the epithelial cell. However, in *S. cerevisiae*, *FLO*, coding for lectin-like protein, is considered to be a homologue of the *C. glabrata* EPA genes involved in binding to mannose chains on the surface of other cells (De las Penas *et al.*, 2003).

Moreover, *Candida* isolates can attach to the surfaces and their ability to form biofilm on the surfaces of devices is depending on their capacity of adherence. The biofilm are the structural cells community in which cells are encapsulated into extracellular matrix polysaccharides and are growing on different surfaces. Cells in the biofilm are more resistant to antifungal treatment. This resistance to antimicrobial agents can be achieved by delaying the penetration of the antimicrobial agent, change in the growth rate of biofilm organisms, or by physiological changes of growth mode of biofilm to overcome environmental stress (Donlan *et al.*, 2002). Infections associated with biofilm formation have great significance especially those formed on medical devices (e.g. indwelling intravascular catheters) due to high resistance of the microorganisms in the biofilm to antimicrobial agents which may increase the morbidity and mortality rates (Kumamoto *et al.*, 2002). Malfunction of the device (e.g. voice prostheses) can also happen and frequent changing of the device is necessary (Van der Mei *et al.*, 2000). In *C. albicans*, for instance, it has been reported that various gene families are involved in the adhesion and some of them are involved in biofilm formation (Sundstrom, 1999). *ALS1* expression, for example, is induced during biofilm formation (Chandra *et al.*, 2001). Moreover, it has been reported that *ALS3* has a crucial role in cell adhesion and its deletion strongly decreased the adhesion to epithelial cells (Wächtler *et al.*, 2012). In *C. glabrata*, it has been shown that *EPA6*, a new gene regulated by the kinase and by a biofilm signal, is the main adhesion element involved in biofilm (Iraqi *et al.*, 2005) as well as *EPA7* that has also been reported to be involved in *C. glabrata* biofilm formation.

9.2 Secreted hydrolases

Clearly, multiple virulent factors are involved in reducing the host defense system. In *C. albicans* several factors are involved in the pathogenicity and these are not well understood in *C. glabrata*. Secreted hydrolases (phospholipases and proteinases) are responsible for tissue damage and invasion of host immune responses. *C. albicans* phospholipase A, B, C, and D, lysophospholipase, and lysophospholipase-transacylase

are secreted and may take a part in the invasion of host cell tissues by hydrolyzing phospholipids of cell membranes into fatty acids, e.g. in Candidiosis (Niewerth et al., 2001). In addition, proteinases production is one of the virulence factors that is associated with infection by helping the pathogen to invade and colonize the host tissue and to evade the host immune response by degrading proteins involved in defense mechanisms (Lerner et al., 1993; Naglik et al., 2003).

9.2.1 Phospholipases

C. glabrata secretes phospholipase B (Kantarcioglu et al., 2002) but its role in the virulence has not been fully understood. There are three phospholipase B (*PLB*) genes encoded by the *C. glabrata* genome and they are orthologues to *PLB* of *S. cerevisiae*. The roles of *C. glabrata* *PLB1* and *PLB2* have been analyzed (Ghannoum et al., 2000) but their effect on virulence has not been reported yet. In contrast, *C. albicans* phospholipase B is well studied and its activity was shown to be involved in the spread of *C. albicans* through the gastrointestinal tract as well as in the blood (Ghannoum et al., 2000, Dolan et al., 2004).

It has also been demonstrated that the phospholipase production by *C. albicans* is correlated with the site of infection and isolates from the blood infection produce higher level of phospholipase than do isolates from wound and urine (Price et al., 1982). Ten genes encoding for lipases have been identified in *C. albicans*. Interestingly it has been shown that mutants were significantly less virulent in intravenous infected murine models (Marcos-Arias et al., 2011).

9.2.2 Proteinases secretion

The secreted aspartyl proteinases (*SAPs*) have been reported to be expressed during *C. albicans* mucosal and systemic infections, they degrade many human proteins, such as albumin, hemoglobin, keratin and secretory immunoglobulin A (Lerner et al., 1993; Hube et al., 1998). *C. albicans* genome encodes 10 *SAP* genes and the main role of these proteinases is to provide nutrition for the cells and evade the immune response allowing *Candida* isolates to survive, penetrate and cause infections on different host surfaces. *C. albicans* is polymorphic organism and exist in yeast or hyphal state and it has ability for phenotypic switching (Soll et al., 1992). *C. albicans* *SPA* gene family may be differentially expressed in the different forms. The expression of *C. albicans* *SAP* genes is complex as it is also connected to other putative virulence factors (hyphal formation, adhesion and phenotypic switching). Therefore, this enzyme family had a significant role in *C. albicans* virulence (Naglik et al., 2003).

In contrast to *C. albicans*, *C. glabrata* has specific cluster of 11 genes that encode a family of putative aspartyl proteases, *YPS* family genes. *YPS1* and *YPS7* are involved in cell wall remodeling while *YPS2-6*, 8-11, cluster of nine genes, are implicated in cell wall integrity, adherence to a host cells as well as survival in macrophages (Kaur *et al.*, 2007) and it has been shown that lacking of these *YPS* genes is associated with attenuated virulence. *C. glabrata* *YPS* genes family is closely related to the *YPS* (Yapsin) genes of *S. cerevisiae*, coding for five glycosylphosphatidylinositol (GPI)-linked aspartyl proteases involved in cell wall remodeling (Gagnon *et al.*, 2006; Dujon , 2010; Jawhara *et al.*, 2012).

9.2.3 Mannosylation of glycans

β -1,2-oligomannosides are cell wall molecules that are known to be associated with phosphopeptidomannans and phospholipomannans in *C. albicans* (Shibata *et al.*, 1985). These components are encoded by a family of nine genes, β -mannosyltransferase genes (*BMTs*) (Mille *et al.*, 2008). It has been reported that these cell wall components play an important role during *C. albicans* infections acting as adhesions (Fradin *et al.*, 2000) and modulating the host immune response (Jouault *et al.*, 2000). The *C. glabrata* genome also encodes homologous 7 (*BMT1-7*) genes and they are likely to be involved in mannosylation and virulence. The function of these genes is not well studied yet, but recently, it has been found that the colonization of *C. glabrata* to the human intestine was decreased after deletion of 5 clustered *BMT* genes *BMT2-BMT6* simultaneously (Jawhara *et al.*, 2012).

9.3 Phenotypic and morphological switching

The ability of *Candida* species to evade the immune system, avoid the drug therapy, invade different body tissues and be able to adjust rapidly to the physiological changes in the host suggests that this yeast has a phenotypic plasticity that helps in fast adaptation to the environmental changes in the host (Soll, 2002). It has been observed that the phenotypic switching in *C. albicans* occurs more frequently in deep mycosis compared to superficial infections (Jones *et al.*, 1994) and is more common to isolates from patients rather than to isolates from oral cavity of healthy people (Hellstein *et al.*, 1993). In *C. albicans*, there are two developmental programs that contribute to its phenotypic plasticity, the bud-hypha transition (Soll, 1986 & Gow, 1997) and high-frequency phenotypic switching (Soll 1992 & 1997). The transition to hypha may help the organism to penetrate the host tissue (Richardson, 1981) whereas, high-frequency phenotypic switching is involved in pathogenesis through secretion of aspartyl proteinases which are encoded by phase-specific genes (Soll, 1992 & Soll, 1996) and

genes involved in drug resistance regulation of drug resistance genes (Balan *et al.*, 1997). It has been found that 37°C and natural pH, which mimic the human environment, stimulate the growth of the yeast cell form (Odds, 1988). It has also been observed that the newly formed filamentous cells attach to the host cells better than the yeast cells (Cutler, 1991; Odds, 1988).

In *C. glabrata*, both phase-specific genes including a metallothionein and haemolysins genes are involved in high frequency phenotypic switching (Lachke *et al.*, 2000). The *HLP*, hemolysin-like protein gene as it has been reported by Luo *et al.*, (2004) was related to the hemolytic activity of *C. glabrata*. On plates containing copper sulphate, *C. glabrata* rapidly switches to different colonies colors (white, light brown and dark brown (Lachke *et al.*, 2000)) by increasing the rate of transcription of metallothionein genes. Most of these colonies are composed of budding cells and pseudohyphae. *C. glabrata* has also a reversible switching system between core and irregular wrinkled colony phenotypes as a result of pseudohyphae formation (Lachke *et al.*, 2002).

Unlike *C. albicans*, *C. glabrata* can only switch to pseudohyphal form (under nitrogen starvation conditions) (Csank *et al.*, 2000), and it has never been found in a filamentous form. The morphological switch in *S. cerevisiae* is regulated by the transcriptional regulator Ste12p which is activated by a mitogen activated protein kinase (MAPK) pathway (Calcagno *et al.*, 2003). In *S. cerevisiae* haploid strains, Ste12p regulates both invasive growth phenotype and the response to mating pheromone, whereas in diploid cells, it plays a role in the filamentous growth in response to nitrogen starvation (Gustin *et al.*, 1998, Roberts *et al.*, 2000; Gancedo, 2001).

In *C. glabrata*, the expression of the *ACE2* gene (transcriptional activator-encoding) has a critical role in the *Candida*-host interaction. Moreover, Inactivation of *ACE2* results in cell separation defect and overgrowth of cells in clumps and as result vascular occlusion at the time of infection (Kamran *et al.*, 2004). Moreover, *C. glabrata ace2* mutant is hypervirulent in mouse model (Kamran *et al.*, 2004). The deletion of the corresponding gene in *S. cerevisiae* results in reduction in virulence from the already low virulence of the parental strain (MacCallum *et al.*, 2006). On the contrary, *sdd1* mutants of *S. cerevisiae* have altered the cell wall composition and architecture like *C. glabrata ace2* mutants and have increased virulence in the animal model (Wheeler *et al.*, 2003).

10. Antifungal resistance mechanisms

The antifungal agents resistance can be defined as the antifungal agents are unable to eradicate the fungus from the host with persistence of the pathogen and consequently progression of infection despite of tolerable drug level. However, the clinical response does not only depend on the susceptibility of the fungus to the drug, rather it is a combination of several factors like drug interaction, host immunity, patient compliance and some other factors like biofilm formation on the medical devices (catheters and prosthetic valves) (White *et al.*, 1998). Some antifungal drugs and their mode of actions are described in Table 5 and figure 5.

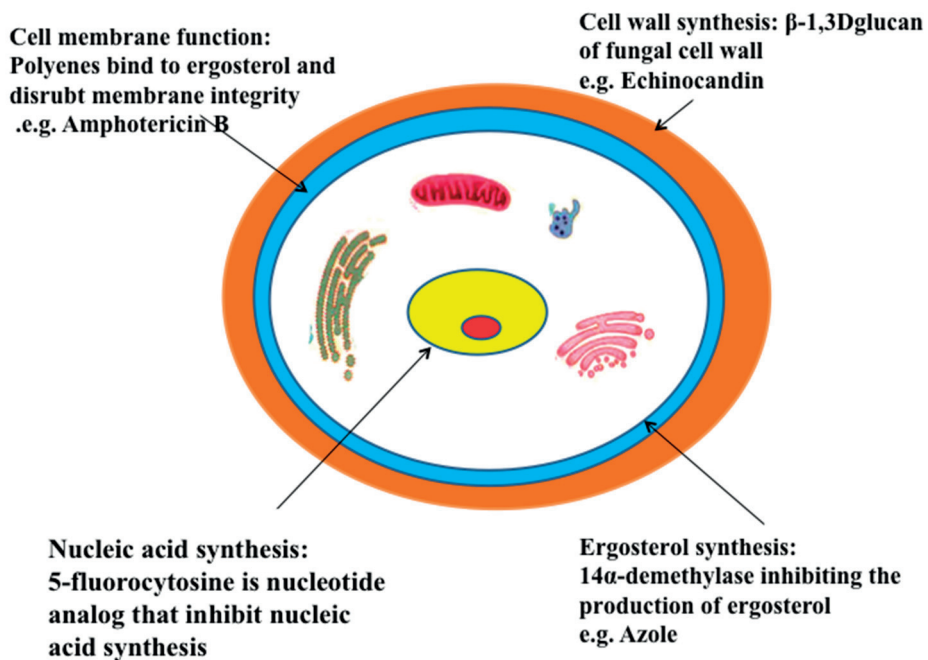


Figure 5: Antimycotics and mechanism of action.

Table 5: Summary of common antifungal agents, mode of action and mechanism of drug resistance (See the text for references).

Antifungal agent Family	Site of action and target genes	Mechanism of drug resistance (more details in Tables 6 & 7)
Azoles derivatives (e.g. Fluconazole)	Inhibition of Ergosterol biosynthesis 14 α - demethylase (<i>ERG11</i>) Sterol desaturase (<i>ERG3</i>)	1-Genetic mutation which alter the drug binding. 2-Upregulation of efflux pump.
Echinocandins (e.g. Caspofungin)	Incomplete cell wall synthesis by inhibition of β -1,3-glucan synthase (<i>FKSI</i>)	Gene mutations
Allylamines (e.g. Trbinafine)	Ergosterol biosynthesis Squalene epoxidase	Accumulation of Squalene with disruption of cellular organization
Nucleic acid inhibitors (e.g. Flucytosine)	flucytosine enters the cell through an energy-dependent cytosine permease and inhibit DNA and RNA synthesis.	Alterations in the 5-fluorouracil metabolic pathway
Polyenes (e.g. Amphotericin B)	Ergosterol binding and change in the permeability of the cytoplasmic membrane (<i>ERG3</i>)	Gene mutation

The resistance can be classified to primary or innate resistance when the pathogen is naturally resistant to drugs, i.e. prior exposure to drug (e.g. *C. krusei* is known to be resistant to fluconazole) (Wingard *et al.*, 1991; Hakki *et al.*, 2006) and secondary when the isolate develops the resistance during the treatment period. These resistant yeasts have ability to cause serious fungal diseases that are in fact more difficult to treat (Bastide *et al.*, 1989; Just *et al.*, 1989).

There are three major groups of antifungal agents that have their antifungal activities through inhibition of ergosterol biosynthesis, azoles, polyenes and allylamines. Among all antifungal agents, azoles are the most frequently used for treatment of mycoses. Fluconazole, azoles antifungal, has a good solubility in water and stability in gastrointestinal tract that gives the advantage to be used orally. Ergosterol is the main component of the cell fungal membrane and serves as a regulator of the membrane fluidity and integrity (Nozawa & Morita, 1986). Cytochrome P-450 dependent 14 α -dimethylzymosterol is considered to be one of the main azole targets in fungi (Hitchcock *et al.*, 1990) and its inhibition leads to accumulation of sterol precursors (14 α -methylated sterols) and formation of cell membrane with altered structure and function. In mammals, it has been reported that the synthesis of cholesterol can also be blocked at stage of 14 α -demethylation but it requires higher dose than that for fungi to have the same effect (Vanden *et al.*, 1987, Vanden *et al.*, 1982). Some of genes involved in multidrug resistance are described in table 6 & 7 and discussed in the following text. Some of these genes also appear in my studies as presented in Paper III.

Table 6: Selected genes of *C. albicans* and *S. cerevisiae* involved in drug resistance (See the text for references).

Organism	Gene	Function
<i>S. cerevisiae</i>	<i>PDR5</i>	ABC transporter involved in multidrug resistance.
	<i>ERG11</i>	Encodes lanosterol 14- α demethylase,
	<i>SNQ2</i>	ABC transporter involved in multidrug resistance.
	<i>AUS1</i>	ATP-binding cassette family, involved in uptake of sterols and anaerobic growth.
	<i>PDR1</i>	Zinc cluster protein, recruiting other zinc cluster proteins to pleiotropic drug response elements (PDREs) to fine tunes the regulation of multidrug resistance genes.
<i>C. albicans</i>	<i>CDR1</i> – <i>CDR5</i>	ABC transporter involved in multidrug resistance
	<i>ERG11</i>	Encodes lanosterol 14- α demethylase
	<i>FLU1</i>	Encoding a major facilitator superfamily
	<i>MDR1</i>	Encoding a major facilitator superfamily

Table 7: Selected genes of *C. glabrata* involved in drug resistance (See the text for references).

Organism	Gene	Function
<i>C. glabrata</i>	<i>CDR1</i>	ABC transporter involved in multidrug resistance
	<i>CDR2(PDH1)</i>	ABC transporter involved in multidrug resistance
	<i>ERG11</i>	Encodes lanosterol 14- α demethylase
	<i>SNQ2</i>	ABC transporter involved in multidrug resistance
	<i>MDR1</i>	Encoding a major facilitator superfamily
	<i>AUS1</i>	ATP-binding cassette family, involved in uptake of sterols
	<i>PDR1</i>	Zinc cluster protein , recruiting other zinc cluster proteins to pleiotropic drug response elements (PDREs) to fine tune the regulation of multidrug resistance genes

In *C. albicans*, several mechanisms have been reported to be involved in azole resistance and they include impaired drug uptake caused by (I) target modification and alteration in the cell wall or plasma membrane, alteration in the affinity of lanosterol 14 α -demethylase encoded by the *ERG11* gene, (II) active efflux mechanism: ATP-binding cassette (ABC) transporter family (*CDR1* and *CDR2*) or major facilitator superfamily, *MDR1* (multidrug resistance) and *FLU1* (fluconazole resistance). Overexpression of *CDR1* (*Candida* drug resistance), *CDR2* and *MDR1* has been found in many cases of azole resistance and deletion of corresponding genes resulted in hypersensitivity to and accumulation of antifungal agent (Sanglard *et al.*, 2002). It has been reported that a number of different mutations in the *ERG11* gene resulting in amino acid substitutions may contribute to *C. albicans* azole resistance by alteration in the affinity of azole derivatives to Erg11p (Favre *et al.*, 1999; Franz *et al.*, 1998). A combination of the resistance mechanisms is a common feature in the development of azole resistance and it is hard to find the role of a single mechanism in azole susceptibility in *C. albicans* (Sanglard *et al.*, 1998). In addition, mutation in the *ERG3* that participates in the ergosterol biosynthesis pathway has been shown to be involved in azole resistance (Vanden Bossche *et al.*, 1998; White *et al.*, 1998; Ghonnoom & Rice, 1999; Casalnuovo *et al.*, 2004; Chau *et al.*, 2005). Alteration of the sterol biosynthesis pathway by deletion of *ERG3* can cause fluconazole resistance in *C. albicans* (Kakeya *et*

al., 2003). Similarly, *C. glabrata* genome rapidly develops secondary resistance to azoles by overexpression of ABC transporters, *CDR1* and *CDR2* (also known as *PDH1*) (Miyazaki *et al.*, 1998; Sanglard *et al.*, 1999; Sanglard *et al.*, 2001), or by up regulation of *ERG11* (Henry *et al.*, 2000).

It has been shown that the expression level of *CDR1* and *CDR2* is increased in azole-resistance *C. glabrata* clinical isolates (Miyazaki *et al.*, 1998; Bennett *et al.*, 2004; Sanglard *et al.*, 2001; Sanglard *et al.*, 1999). Moreover, it has also been reported that the expression of *CgCDR1* and *CgCDR2* genes is increased in association with increased azole resistance as a result of mitochondrial loss of *C. glabrata* (Brun *et al.*, 2003).

Evolutionarily, *C. glabrata* is closely related to *S. cerevisiae*, and recent studies have identified a Pdr1p orthologue, pleiotropic drug resistance transcriptional factor, (CgPdr1p) in *C. glabrata* that regulates drug efflux pumps and controls multidrug resistance in *S. cerevisiae* by up regulation of *CDR1* and *PDH1* (*CDR2*) expression (Vermitsky & Edlind, 2004; Tsai *et al.*, 2006; Vermitsky *et al.*, 2006). This mechanism as it has been reported might be conserved between *C. glabrata* and *S. cerevisiae* (Izumikawa *et al.*, 2003; Vermitsky *et al.*, 2004) as gain of function mutation in *CgPDR1* gene enhances both antifungal resistance and virulence (Ferrari *et al.*, 2009). Four families of ABC transporters have been reported in *S. cerevisiae*, *MDR*, *CFTR*, *YEF* and *PDR* (Higgins, 1992).

The genome of *C. glabrata* also carries *CgAUS1*, sterol transporter gene that might lower the susceptibility of this yeast to fluconazole (Nakayama *et al.*, 2007; Ferrari *et al.*, 2009). It has been reported that in *S. cerevisiae*, both Pdr1p and its paralogue Pdr3p, the zinc-cluster transcription factors, confer resistance to azole resistance and other toxins through transcriptional activation of ABC transporter genes including *Pdr5*, *Snq2* and *Yor1*, as well as phospholipid-transfer genes such as *Pdr16* (Balzi *et al.*, 1987; Balzi *et al.*, 1994; Meyers *et al.*, 1992; Katzmann *et al.*, 1994). One study (Thakur *et al.*, 2008) has shown that Pdr1p orthologues in *S. cerevisiae* and *C. glabrata* could bind directly to xenobiotics and activate genes encoding drug efflux pumps and Gal11p (also known as MED15), mediator co-activator subunit, that play a specific role in xenobiotic-dependent gene activation and *MDR1* in *S. cerevisiae* and *C. glabrata*.

Thakur *et al.*, 2008, have also found that xenobiotic-dependent expression of the *PDR5* and *PDR16* genes was specifically and strongly decreased in the *gal11* deletion mutant. The *MDR1* homologue of *C. glabrata* has been found to confer to azole resistance when expressed in *S. cerevisiae*, and could be implicated in azole resistance in this pathogen (Sanglard *et al.*, 1999). Other factors like ploidy and degree of mutations dominance may play role in the rate of evolution of fluconazole resistance in *S. cerevisiae*, where haploids are expected to evolve resistance faster than diploids.

However, when a haploid strain of *S. cerevisiae* is exposed to high concentration of fluconazole, the recessive mutation in the *ERG3* gene will strongly appear under these

conditions, while in stepwise increase in fluconazole concentration, mutations in *PDR1* and *PDR3* are favored (Anderson *et al.*, 2003) which suggests that the ploidy and the degree of dominance are essential factors in the development of antifungal drug resistance.

Other antifungal drugs include polyenes like Amphotericin B; this antifungal drug has a broad spectrum antifungal activity gives the advantage to use it cases of invasive *Candida* infections. This fungicidal drug (Amphotericin B) acts by inserting into the fungal membrane and generating pores resulting in loss of transmembrane potential and impaired cellular function. In *S. cerevisiae* it has been shown that the mutations in the genes of ergosterol synthesis, *ERG4*, *ERG6*, and *ERG3*, are associated with accumulation of sterol intermediates, and these mutants are more resistant to polyenes (Arthington *et al.*, 1991). In *C. albicans* and *C. glabrata*, the defect in the *ERG3* gene is found to be associated with the lack of ergosterol and increased resistance to polyenes (Kelly *et al.*, 1996; Nolte *et al.*, 1997).

Caspofungin (the first clinically used echinocandin) inhibits the synthesis of β -1,3D glucan of the fungal cell wall (is not present within the mammalian cells). This inhibition results in cytological and ultrastructural changes as pseudohyphae, thickened cell wall and buds with failure to separate from the mother cells (Traxler *et al.*, 1977). The target for echinocandins is glucan synthase, which in *S. cerevisiae* is encoded by *FKS1* and *RHO1* genes. In addition, *S. cerevisiae* genome encodes *FKS2*, homologous to *FKS1*. It has been reported that mutations in the *FKS1* gene are associated with high-level of in vitro resistance to echinocandins (Kurtz, 1997; Kurtz *et al.*, 1997). In *C. albicans*, it has recently reported that the reduced susceptibility to caspofungin is mediated by mutation in *FKS1* (Balashov *et al.*, 2006) that has been observed in clinical as well as laboratory strains. It has also been reported, that overexpression of ABC transporter, Cdr2p, confers caspofungin resistance (Bachmann *et al.*, 2002; Schuetzer *et al.*, 2003).

Although the echinocandins are well tolerated, yet they have a few side effects and exhibit drug-drug interaction, for instance, if caspofungin is co-administrated with rifampicin, the dose should be increased because rifampicin is a liver enzyme-inducer, and it increases the rate of metabolism of other drugs by promoting the up regulation of hepatic cytochrome P450 enzymes.

Flucytosine is an antifungal drug that is converted in the cell by cytosine permease into 5-fluorouracil. 5-fluorouracil inhibits the protein synthesis within the fungal cell by inhibition of DNA and RNA synthesis. The resistance to flucytosine is mediated by mutation in cytosine deaminase or uracil phosphoribosyl transferase genes.

Another factor which may increase the rate of *Candida* infections is the host status. The phagocytic cells, neutrophils and mononuclear phagocytes, have a crucial role in innate immunity (Zelante *et al.*, 2007) and the neutrophil has an important role in the initial

host response against *Candida* by damaging the hyphae through the oxidative and non-oxidative mechanisms. Therefore, in neutropenia patients, the rate of *Candida* infection is very high and these patients are at risk to develop invasive *Candida* infections (Shoham & Levitz 2005). In Paper IV I studied interactions between my yeast mutants and human macrophages.

11. RNA interference

RNA interference is a gene silencing pathway that regulates the gene expression in many eukaryotes at both transcriptional and post- transcriptional levels. This phenomenon was first observed in plants and has been established as silencing trigger in *Caenorhabditis elegans* (Fire *et al.* 1998). It was initially recognized as host defense mechanism that protects the organism from invading viruses and random integration of mobile elements of the host genome (Waterhouse *et al.*, 2001). In human, the RNAi pathway has been successfully used to silence many genes, for example those relevant to viral diseases, such as HIV (Dave and Pomerantz, 2004).

Friedman *et al.* (2009) has reported that RNAi regulates more than 60% of mammalian genes. This ancient mechanism is also present in protozoa and most fungi and involved in regulation of several cellular and physiological processes through small interfering RNAs (siRNAs) or microRNAs (miRNA) (Bartel, 2004). The microRNA (miRNA) is a class of endogenous small non-protein coding RNA that can down regulate the gene expression in plant and animals either by degradation of mRNA or by blocking the protein translation (Bartel, 2004). Another class of RNAi is small interfering (siRNA) mediated pathway, where the double strands RNA cleaved into 20-23 nucleotides by Dicer, RNase III protein (Malone & Hannon, 2009). The siRNA strand subsequently binds to RNA-induced silencing complex (RISC) where recognized by argonaute (Ago), small RNA-binding protein and is further degraded (Figure 6).

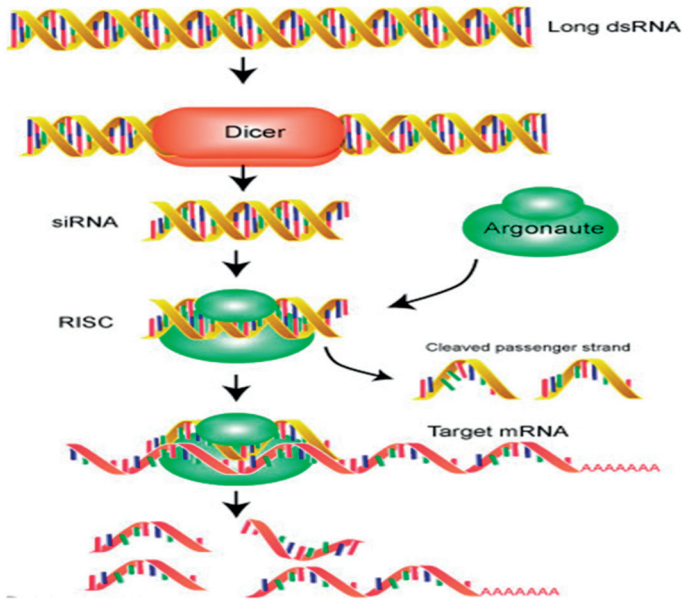


Figure 6: The mechanism of RNAi. Long dsRNA gets cleaved by Dicer into 21-23 nt long small siRNA. The siRNA gets assembled in RISC where Argonaute cleaves sense strand of siRNA and antisense strand guides the complex to homologous mRNA which gets cleaved and translation is stopped (http://www.rnaiweb.com/RNAi/What_is_RNAi).

RNAi pathway is conserved among the budding yeasts such as *Saccharomyces castelli*, *Kluyveromyces polysporus* and *C. albicans*, but it has been lost in several budding yeast lineages, like *S. cerevisiae* and *C. glabrata* (Drinnenberg *et al.* 2009). Although *S. cerevisiae* lacks RNAi pathway, it has been reconstituted in this yeast by introducing the *Dcr1* and *Ago* genes from its close relative *S. castelli* (Drinnenberg *et al.* 2009). Recently, it has been shown that the human RNAi, (Dicer and *Ago2*) can also be reconstituted in *S. cerevisiae* in the presence of TRBP (trans-activation response RNA-binding protein) (Suk *et al.*, 2011). In contrary, Wang *et al.* (2013) have reported that the RNAi pathway could be restored in *S. cerevisiae* by introducing of *S. castelli* Dicer and human *Ago2* genes but not human Dicer gene even in the presence of TRBP.

It has also been found that Hsp90 has a regulatory role in the reconstituted RNAi in *S. cerevisiae* and it enhances the combination between dsRNA and Argonaute (Wang *et al.* 2013). Hsp90 is highly conserved in eukaryotes and it catalyzes the ATP hydrolysis during formation of RISC (Iwasaki *et al.*, 2010). RNAi may represent an

ideal tool to study essential yeast genes which can otherwise not be deleted to study the resulting phenotypes. In Paper IV I present the development of the first RNAi tool to study *C. glabrata*.

12. Summary of papers

Paper I

Small chromosomes among Danish *Candida glabrata* isolates originated through different mechanisms.

192 strains of the pathogenic yeast *Candida glabrata* have been isolated from patients at Danish hospitals. Regardless the hospitals and the years of isolation, we observed that these strains were closely related but exhibited large karyotype polymorphism. A few strains had extra chromosomes, which were smaller than 0.5 Mb. Our analysis showed that there were two mechanisms which could participate in their origin, a segmental duplication or a segmental translocation. Apparently, under certain conditions *C. glabrata* can increase its fitness by genomic rearrangements including appearance of small chromosomes.

Paper II

Genome Structure and Dynamics of the Yeast Pathogen *Candida glabrata*.

In this review we focused on how *C. glabrata* can become a successful opportunistic pathogen. The genome dynamics including variation in the number and the size of chromosomes with intra- and inter-chromosomal segmental duplications have been observed among clinical isolates of *C. glabrata*. Therefore, further genome sequencing of pathogenic isolates would be very fruitful and it would most likely shed some light on new virulence genes as well as helps to understand the mechanisms behind the yeast genome dynamics. Recently our Cornell collaborators have determined the genome structure of a dozen of our isolates (unpublished results).

Paper III

The haploid nature of *Candida glabrata* is advantageous under harsh conditions.

C. glabrata has so far only been found as a haploid and asexual yeast. Could its haploid nature and frequent genome rearrangements be an advantage for *C. glabrata* to survive in different environments? To address this question, the competitions between haploid and artificially created diploid strains of *C. glabrata* were studied *in vivo* (in a fly and mouse models) and *in vitro* (flask experiments) under normal and stress conditions (fluconazole, high temperature). Our competition studies in experimental populations suggest that the haploid nature often provides an advantage under harsh conditions likely because haploid cells can express recessive mutations which could under certain conditions be advantageous for the mutant lineages.

Paper IV

Development of RNAi tools to study putative virulence genes in the yeast *Candida glabrata*

Similar to *Saccharomyces cerevisiae*, *C. glabrata* genome has lost the RNAi pathway. In this paper we successfully reintroduced the RNAi system into *C. glabrata* by cloning both Dicer and Argonaute genes from *Saccharomyces castellii*. We could observe that RNAi has efficiently silenced the expression of both the *URA3* and *ADE2* genes. Our results have also shown that the silencing effect with hairpin constructs is stronger than the antisense. We took advantage of this concept to study more putative virulence genes which could not be deleted, and their expression was silenced by the introduction of the *S. castellii* *DCR1* and *AGO1* genes. The transformants were further studied *in vivo*, in human macrophages, and the survival rate of one silenced mutant decreased suggesting that the RNAi efficiently works in *C. glabrata* and could be used in the future to study further essential genes.

Conclusion

The yeast *Candida glabrata* has during the last decades become one of the main causes of mucosal and systemic infections. Many virulence genes that have been found associated with pathogenicity of *C. albicans* are not studied in *C. glabrata*, many species-specific virulence factors still need to be discovered. Apparently, *C. glabrata* can remodel its genome to adapt to different environments (section 7.3). Moreover, this yeast has intrinsic resistance to azole antifungal drugs (section 10). Although some researchers claim that the *C. glabrata* genome is comparatively stable (section 7.3), this is in contrast to our results (Paper I) and many other works which showed that this yeast has a very dynamic genome. In Paper I we showed that *C. glabrata* has greatly rearranged chromosomes. Approximately 200 clinical isolates were analyzed for genome structure and stability and we observed that the isolated strains of *C. glabrata* display enormous genomic plasticity (Paper I). The chromosomal polymorphism was observed among different isolated strains. Even the strains which were isolated from the same patient have different karyotypes. Segmental duplications, segmental translocations and novel chromosomes have also been observed among the clinical isolates.

Other species related to *C. glabrata* have recently been emerging as pathogens (section 7.1). We discussed in Paper II the comparative genomics aspects of related species. We also discussed how the genome rearrangements contribute to the virulence of *C. glabrata* and how it increases the fitness under harsh conditions.

C. glabrata is an asexual yeast and only haploid isolates have been observed so far (section 6 & 8). This haploid nature is likely an advantage for this yeast to survive in human body. In Paper III we showed that the haploid nature of *C. glabrata* increased the fitness under stress condition which is likely because in haploids the mutations more clearly result in phenotypes which might be advantageous when the environmental conditions fluctuate a lot.

C. glabrata genome has several genes with unknown function. Some of these genes could play a role in virulence. Therefore, one part of my thesis was to study the putative virulence genes which might be involved in the pathogenicity of this yeast. In Paper IV we attempted to silence a few genes with a newly developed approach (RNAi) (section 11). Some of these uncharacterized genes were identified in Paper III. Our results showed that developed RNAi tool is an excellent approach for further studies of other genes which could be essential for this yeast.

Although several studies have been done to understand the molecular mechanisms behind the virulence of *C. glabrata*, only future genome sequencing of various isolates could help to learn more about the genome dynamics.

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