REVIEW

Folia Microbiol. **51** (1), 3–20 (2006) **http://www.biomed.cas.cz/mbu/folia/**

Biology of the Pathogenic Yeast *Candida glabrata*

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Received 21 February 2005
Revised version 19 September 2005

ABSTRACT. The yeasts, being favorite eukaryotic microorganisms used in food industry and biotechnologies for production of biomass and various substances, are also used as model organisms in genetic manipulation, molecular and biological research. In this respect, *Saccharomyces cerevisiae* is the best-known species but current situation in medicine and industry requires the use of other species. Here we summarize the basic taxonomic, morphological, physiological, genetic, *etc.* information about the pathogenic yeast *Candida glabrata* that is evolutionarily very closely related to baker's yeast.

Abbreviations

ABC	ATP binding cassette	mtDNA	mitochondrial DNA
MDR	multidrug resistance	PCR	polymerase chain reaction
MFS	major facilitator superfamily	*rho*$^-$/*rho*^{0}	partial deletion in/absence of mtDNA

CONTENTS

1 TAXONOMY AND EVOLUTION

The genus *Candida* consists of a heterogeneous group of yeast species that usually exist in two forms: yeast (simple cells) and hyphae (mycelium, pseudomycelium). In the case of *C. glabrata* (Berlese 1895) the natural occurrence of hyphae still has not been described. *C. glabrata* displays pseudohyphal growth only during nitrogen starvation (Csank and Haynes 2000). For this reason, the species previously named *Cryptococcus glabratus* (Anderson 1917) was reclassified in 1938 as *Torulopsis glabrata* (Lodder and DeVries 1938; Kocková-Kratochvílová 1990). Later it was demonstrated that the absence of hyphae is not an adequate argument for this separation from other *Candida* species (Odds *et al.* 1997).

The genus *Candida* belongs to mitosporic fungi. They do not exhibit sexual cycle but some species have a teleomorphic form. On this basis, they are classified as Ascomycetes or Basidiomycetes. So far, the teleomorphic forms were described for ten *Candida* species, but not for *C. glabrata* (Barnett *et al.* 1983; Meyer *et al.* 1984; Kurtzman and Fell 1998). Another comparative marker previously used for classification of yeasts also involved the ratio of sugars to fatty acids in the cell wall, but in the case of *C. glabrata* this attribute was also insufficient (Brondz and Olsen 1990).

Barns *et al.* (1991) compared the evolutionary relationship within the genus *Candida* on the basis of homology of 18S rRNA sequence and concluded that *C. glabrata* is evolutionarily more related to *S. cerevisiae* than to another pathogenic fungus – *C. albicans*. Contrary to other *Candida* species, *C. glabrata* is a "*petite*-positive" yeast (Marmiroli *et al.* 1985; Chen and Clark-Walker 2000).

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C. glabrata displays exclusively a haploid and asexual life cycle in contrast to sexually active *S. cerevisiae* that can shift in its life cycle between diploid and haploid forms of cells (Wong *et al.* 2002). All of these information was stressed by genetic analysis which resulted from a complete sequence analysis of the *C. glabrata* genome in Dujon *et al.* (2004). The abundance of C–G and A–T pairs, the number of genes, pseudogenes and gene duplications, the similarity between genes for functional RNA, the similarity and synteny of protein-family-coding genes, the total redundancy of DNA and other properties in four yeast species (*C. glabrata*, *Debaryomyces hansenii*, *Kluyveromyces lactis* and *Yarrowia lipolytica*) were recently compared with *S. cerevisiae* (Dujon *et al.* 2004).

The phylogenetic tree of the best-known yeast species involving *C. glabrata* based on the genome sequence analysis is shown on Fig. 1 (Sherman *et al.* 2004; **http://cbi.labri.fr/Genolevures/**). At present the following is the taxonomic classification of *C. glabrata* (**http://www.doctorfungus.org**):

Kingdom:	Fungi	Subclass:	Hemiascomycetes
Division:	Ascomycota	Order:	Saccharomycetales
Subdivision:	Ascomycotina	Family:	Saccharomycetaceae
Class:	Ascomycetes	Genus:	*Candida*

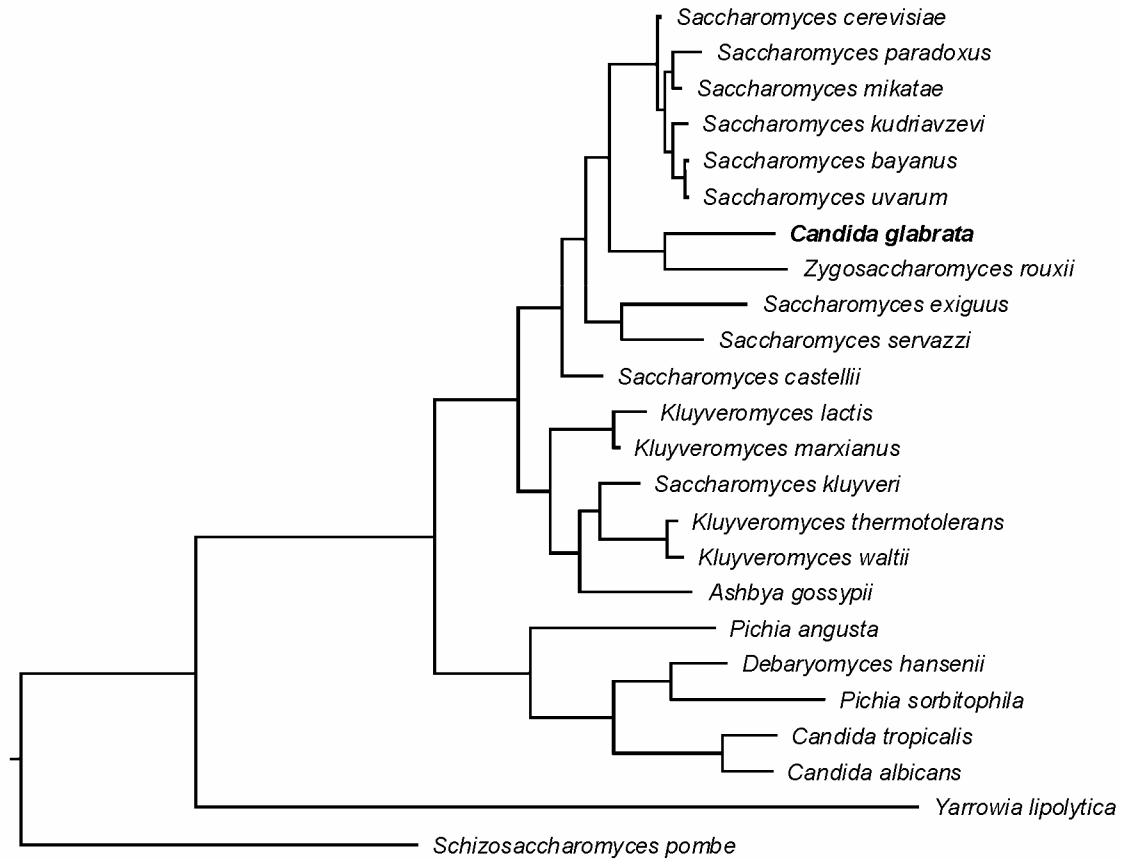

Fig. 1. The phylogenetic tree of some yeast species (**http://cbi.labri.fr/Genolevures/**).

2 MORPHOLOGY, PHYSIOLOGY AND LIFE CYCLE

The blastoconidia of *C. glabrata* are oval, 1–4 μm in diameter, exhibiting no differences between the commensal and pathogenic forms of this yeast species (**http://www.doctorfungus.org**). *C. glabrata* colonies are pasty, white to cream in color and glistening. On cornmeal Tween 80 agar and at 25 °C after 3 d, it produces clusters of tiny, oval, budding blastospores. The absence of pseudohyphae is very typical (Kocková-Kratochvílová 1990; Larone 1995; **http://www.doctorfungus.org**). The *C. glabrata* vaginal isolates display capsules (**http://www.cytopathology.org/NIH/**).

2.1 Metabolism of sugars

C. glabrata assimilates only glucose and trehalose, representing thus a limited number of sugars in comparison with *S. cerevisiae* or *C. albicans* (Hazen 1995). In connection with this fact, glucose repression known in species that assimilate sucrose, melibiose, maltose, lactose, *etc.* was not observed. However, the *CgSNF1* gene (sucrose non-fermenting, serine/threonine protein kinase), homologous to its ortholog playing an important role in regulation of fermentation and respiration in *S. cerevisiae*, has been isolated (Gancedo 1998). Its deletion in *C. glabrata* caused the loss of trehalose assimilation (Petter and Kwon-Chung 1996). The metabolism of trehalose is interesting not only from the point of view of energetic processes, but also of the role that trehalose plays in the environmental stress conditions (starvation, fluctuation of osmotic pressure, heat-shock) (Attfield 1987; Mackenzie *et al.* 1988).

Considering the fact that *C. glabrata* is an opportunistic pathogen its regressive evolution connected with the loss of 29 genes involved in the metabolism of galactose and other sugars, nitrogen, sulfur and phosphate is remarkable (Dujon *et al.* 2004). According to genome databases the genome of *C. glabrata* does not contain several homologues of *S. cerevisiae* genes, *e.g.*, *GAL1*, *GAL3*, *GAL7*, *GAL10*, *GAL80*, *SUC2*, *HEX2*, *MEL1* (**http://www.ncbi.nlm.nih.gov/entrez/query.fcgi**; **http://cbi.labri.fr/Genolevures/**).

2.2 Non-essentiality of mitochondrial DNA

C. glabrata belongs to the "*petite*-positive" yeast species (Marmiroli *et al.* 1985; Chen and Clark-Walker 2000). A partial deletion (*rho*$^-$) or even complete loss of mitochondrial genome (*rho*^{0}) is not lethal for this yeast. On the other hand, in its pathogenic life style it can even profit from dysfunctional mitochondria. The "petite" mutants were found to be more resistant to several antimycotics than the corresponding wild-type strains (Defontaine *et al.* 1999; Sanglard *et al.* 1999; Bouchara *et al.* 2000).

So far, no studies appeared dealing with point mutations or recombination of mitochondrial genes in *C. glabrata*. Although the biogenesis and function of mitochondria depend on a proper cooperation between mitochondrial and nuclear genomes only limited information appeared concerning *C. glabrata*. However, such cooperation has been clearly demonstrated in the biosynthesis of RNAase P (Shu and Martin 1991).

2.3 Mating types

C. glabrata is an asexual yeast in spite of the fact that genetic analyses revealed the presence of genes potentially responsible for conjugation and mating type switching (Lachke *et al.* 2000; Srikantha *et al.* 2003). Fig. 2 shows that *C. glabrata* contains three mating type loci with a configuration similar to that in

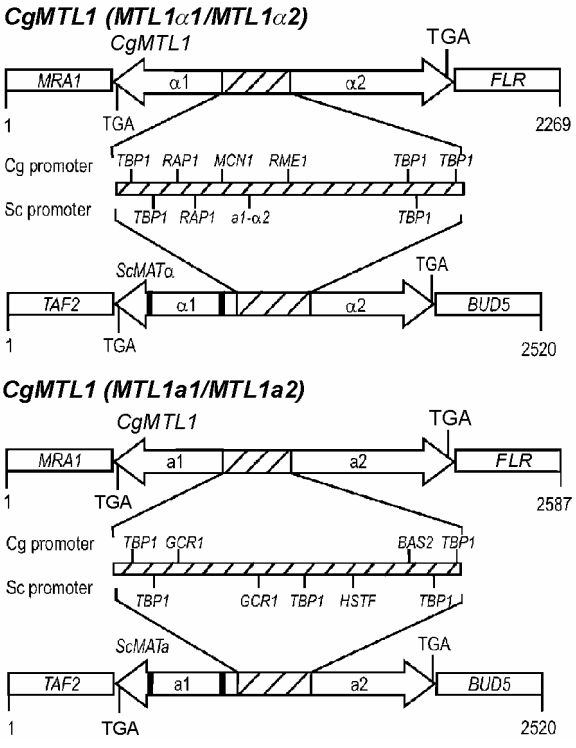

Fig. 2. Comparison of the *MAT* locus of *S. cerevisiae* (*top*) with the *MTL1* locus of *C. glabrata* (*bottom*) (Srikantha *et al.* 2003).

the *S. cerevisiae* mating type cassette system. This suggests that cassette switching may also occur at the expressed locus *MTL1* (mating type-like locus) in *C. glabrata*, which is homologous to the *MAT* locus in *S. cerevisiae*. Two silent loci *MTL2* and *MTL3*, homologous to *S. cerevisiae HMR* and *MHL*, and other genes apparently involved in conjugation (Fig. 3), karyogamy, meiosis and sporulation were also identified and

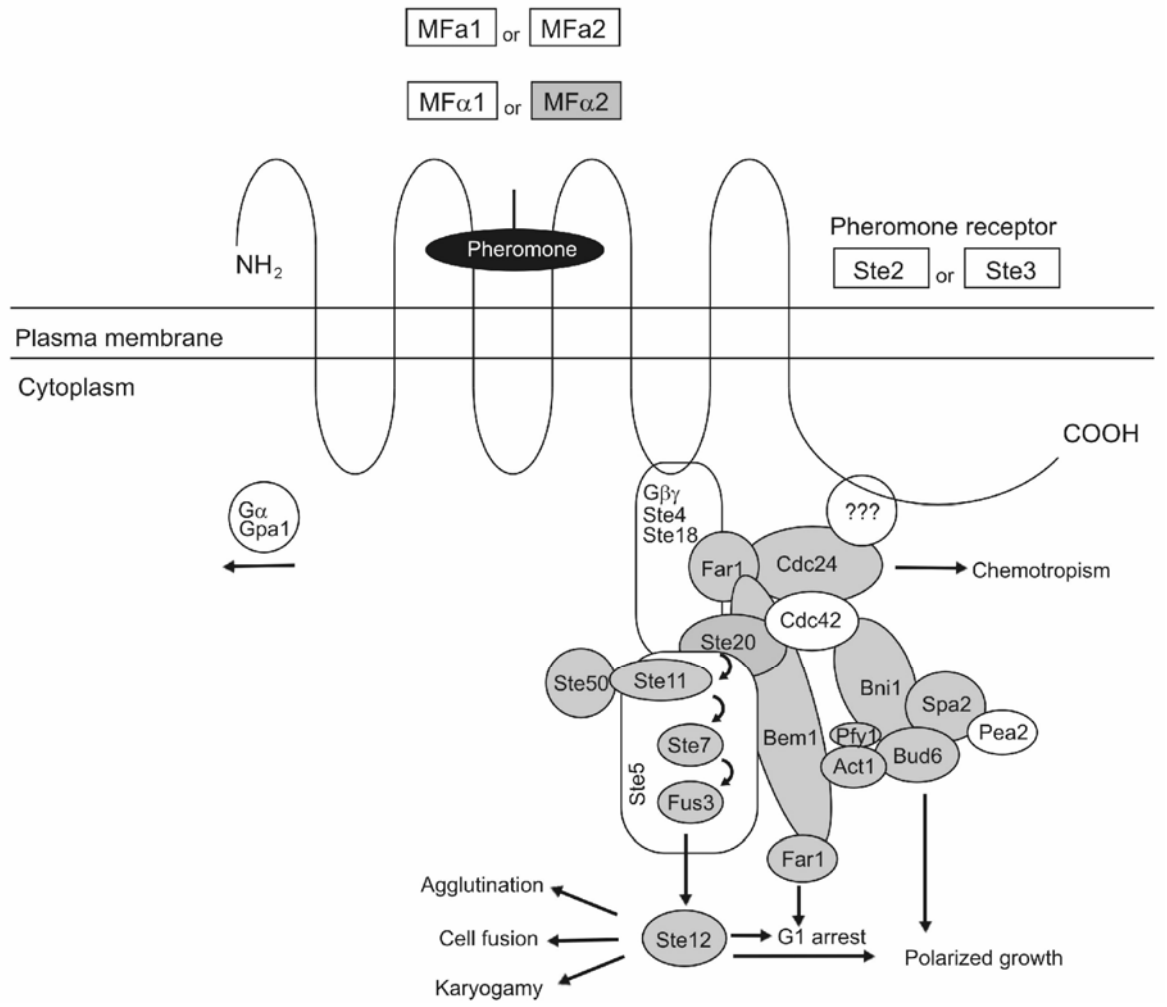

Fig. 3. The pheromone signaling pathway in *S. cerevisiae*; *grey* – the protein homologues identified in *C. glabrata* (adapted from Sheu *et al.* 2001, Wong *et al.* 2003 and Calcagno *et al.* 2004).

compared with their homologues in *S. cerevisiae* (Wong *et al.* 2003). The presence of some of them may be explained by their function in other cell processes (*e.g.*, Ste12p – virulence, invasive growth, synthesis of the cell wall, pseudohyphae formation during nitrogen starvation, Ste20p – virulence, integrity of the cell wall, adaptation to hypertonic stress) (Calcagno *et al.* 2003, 2004). On the other hand, genome sequencing uncovered another 31 genes (including *IME1*, *FUS3*, *SMK1*) with homologous function only in the sexual cycle in *S. cerevisiae* without any known additional function. Wong *et al.* (2003) inferred that probably *C. glabrata* has an undiscovered sexual stage in its life cycle, similar to that recently proposed for *C. albicans*. The two *Candida* species represent two distantly related yeast lineages that have independently become both pathogenic and "asexual", or they may have a cryptic sexual cycle (Gow 2002). It is less possible that all of the 31 identified genes are pseudogenes. Moreover, *C. glabrata* orthologues *MFα2*, *MATα1* and *MATα2* were documented to be functional genes (Wong *et al.* 2003). However, some orthologues of genes playing a central role in mating were not found (*e.g.*, *STE2/STE3*, *MFA1*). Regarding meiotic recombination, the *SPO11* homo-logous gene was identified but its possible role is connected rather with DNA repair during mitosis (**`http://cbi.labri.fr/Genolevures/`**). In spite of the absence of a sexual cycle in *C. glabrata* an artificial parasexual replacement of chromosomes after fusion of spheroplasts of haploid parents followed by UV-induced segregation of chromosomes has been demonstrated (Whelan and Kwon-Chung 1987).

2.4 Killer toxins

The glycoprotein toxins of *S. cerevisiae* cause destruction of the cytoplasmic membrane and result in an efflux of potassium ions, AMP and ATP that inhibit cell growth and induce death of damaged cells. The action of toxins requires an energized status of sensitive cells. On the other hand, the action of toxins produced by *C. glabrata* is independent of the energized state of sensitive cells to induce efflux of potassium ions and AMP (Bussey and Skipper 1975, 1976; Skipper and Bussey 1977). The incidence of killer activity among other *Candida* species has been recently reported in *C. apis*, *C. bombicola*, *C. fructus*, *C. krusei*, *C. sorbosa* (Abramches *et al.* 1997) and *C. maltosa* (Buzzini and Martini 2001).

3 GENETICS

The genetic system of *C. glabrata* consists of chromosomes in nuclear and mitochondrial DNA. Natural occurrence of plasmids, *e.g.*, 2 μm, typical of *S. cerevisiae*, has not been reported so far.

3.1 Chromosomal DNA

C. glabrata has 13 chromosomes (sizes of ≈0.5–1.4 Mb; Fig. 4) (Dujon 1992; Dujon *et al.* 2004; **`http://cbi.labri.fr/Genolevures/`**); this number is higher than the 8–12 chromosomes determined by Kaufman and Merz (1989). The total size of chromosomal DNA is 12.3 Mb. The rDNA forms two distinct loci in subtelomeric regions of chromosomes 12 (L) and 13 (M). Approximately, 200 genes for tRNA and 5300 protein coding genes are borne on the chromosomes. In comparison with *S. cerevisiae* (6 chromosomes, total size of 12.1 Mb, approximately 6000 protein coding genes) these differences are not very significant. In general, low redundancy and high degree of regressive evolution are typical of this yeast that is probably associated with the adaptation to pathogenic life style (Dujon *et al.* 2004).

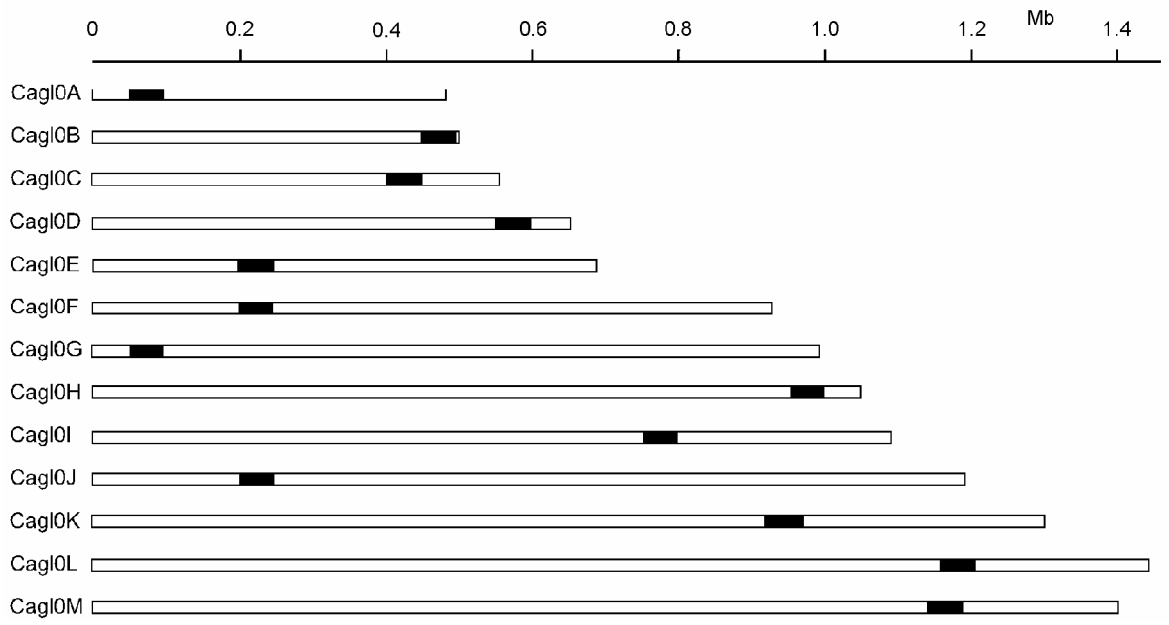

Fig. 4. The chromosomes of *C. glabrata* (**`http://cbi.labri.fr/Genolevures/`**); the *black regions* correspond to centromeres.

Each *C. glabrata* chromosome contains genes, one centromere (*CEN*) in the subterminal or terminal chromosome region, telomeres (*TEL*) and autonomously replicating sequences (*ARS*) that are evolutionarily conserved. Replication of telomeres is controlled by the Rap1p protein which was isolated by complementation of the *rap1* mutation in the *S. cerevisiae* mutant strain. The homology between *Sc*Rap1p and *Cg*Rap1p lies in the range of 65–78 %. Relatively high homology was also found between telomere sequences, *CgTEL*: 5′-CTG **TGG** G**GT** C**TG GGT** G-3′, *ScTEL*: 5′-**TGG** T**GT** G**TG GGT** G-3′ (**bold** indicates the binding site for Rap1p in *C. glabrata* and *S. cerevisiae*, respectively), occurring only once in both yeast species (Cohn *et al.* 1998; Haw *et al.* 2001).

Some differences were found in the homology between centromeres. Although the structures of centromeres are very similar in both yeast species, the centromere from *C. glabrata* does not function in the *S. cerevisiae* mutants (Kitada *et al.* 1997). Three elements, important for the function of the centromere were disclosed and compared to those in *S. cerevisiae* (*ScCDEI – CgCDEI*, *ScCDEII – CgCDEII*, *ScCDEIII – CgCDEIII*). The *CgCDE* elements consist of conserved sequences of nucleotides (Table I). Preliminary analysis based on construction of chimeric centromeres revealed that species-specific elements *CgCDEII* and *CgCDEIII* are essential for the proper function of the centromere (Kitada *et al.* 1996, 1997).

Table I. Three elements of *C. glabrata* centromere (Kitada *et al.* 1996)

Element	Number of base pairs	Sequence
CgCDEI	8	(G/A)TCAC(G/A)TG
CgCDEII	78–87	AT reach region
CgCDEIII	26	TGT(T/A)T(T/A)TG(T/A)TTTCCGAA(A/C)(T/A)(T/A)(T/A)(T/A)AAAA

The *S. cerevisiae ARS* are AT-rich chromosome region (73–82 %), containing the consensus sequence 5′-(A/T)TTTAT(A/G)TTT(A/T)-3′ in one or more copies (Newlon 1988). In *C. glabrata* only one report deals with the isolation of AT-rich DNA region with *ARS* activity that originated from the vicinity of the *MT-IIa* gene (Mehra *et al.* 1992).

3.2 *Mitochondrial DNA*

The mitochondrial genome of *C. glabrata* belongs to the smallest one observed among hemiascomycetous yeasts; its size is 19–20 kb (Shu and Martin 1991; Koszul *et al.* 2003) (Fig. 5). This circular mtDNA contains 8 protein coding genes: *COB* (for the apocytochrome *b*), *ATP6*, *ATP8*, *ATP9* (for three subunits of ATP synthase), *COX1*, *COX2*, *COX3* (for 3 subunits of cytochrome *c* oxidase), and *VAR1* (for the ribosomal protein). The genes encoding functional RNAs were also identified: 23 tRNA-coding genes, rRNA for small (SSU) and large (LSU) subunit of mitochondrial ribosome and RNA subunit of mitochondrial RNAase P (Shu and Martin 1991). The *COX1* gene was found to contain 3 introns of type I, *CgCOX1.1*, *CgCOX1.2*, *CgCOX1.3*, each with an open reading frame. Like *S. cerevisiae*, *C. glabrata* mtDNA does not contain genes for NADH oxidoreductase, whereas such genes were found in other *Candida* species. All the genes are transcribed from the same strand of mtDNA, resulting in polycistronic transcripts (Clark-Walker and Sriprakash 1983). Fig. 6 illustrates differences between mtDNA of *S. cerevisiae* and *C. glabrata* (Koszul *et al.* 2003). Respiration-deficient *rho*$^{-}$/*rho*^{0} mutants of *C. glabrata* are viable. Some of *rho*$^{-}$ mutants of *C. glabrata* contain two circular DNAs with a size of 3 and 6 μm (O'Connor *et al.* 1976).

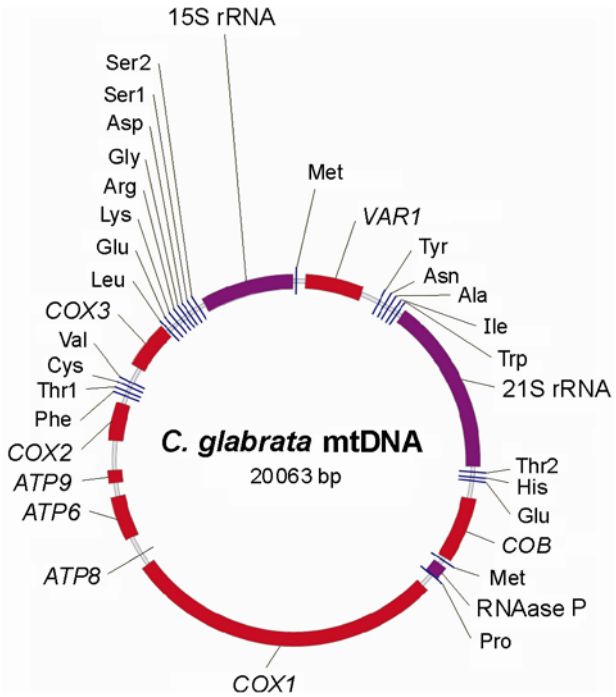

Fig. 5. The sequence-derived map of the mitochondrial genome of *C. glabrata*; the protein-coding and RNA-coding sequences are *red* and *violet*, respectively, the tRNA-coding sequences are *blue* (adapted from Koszul *et al.* 2003).

3.3 *Genetic analysis*

With regard to the asexual life cycle of *C. glabrata* the methods of classical genetics, based on the conjugation and sporulation of cells (*e.g.*, tetrad analysis, random spore analysis, complementation analysis, *etc.*), cannot be used for genetic analysis

here. Therefore, the application of molecular methods based on the technology of recombinant DNA is more popular. Gietz *et al.* (1992) developed a protocol for lithium acetate transformation of *C. glabrata* and the transformation by electroporation was adapted from the procedure originally used for transformation of *Cryptococcus neoformans* (Varma *et al.* 1992).

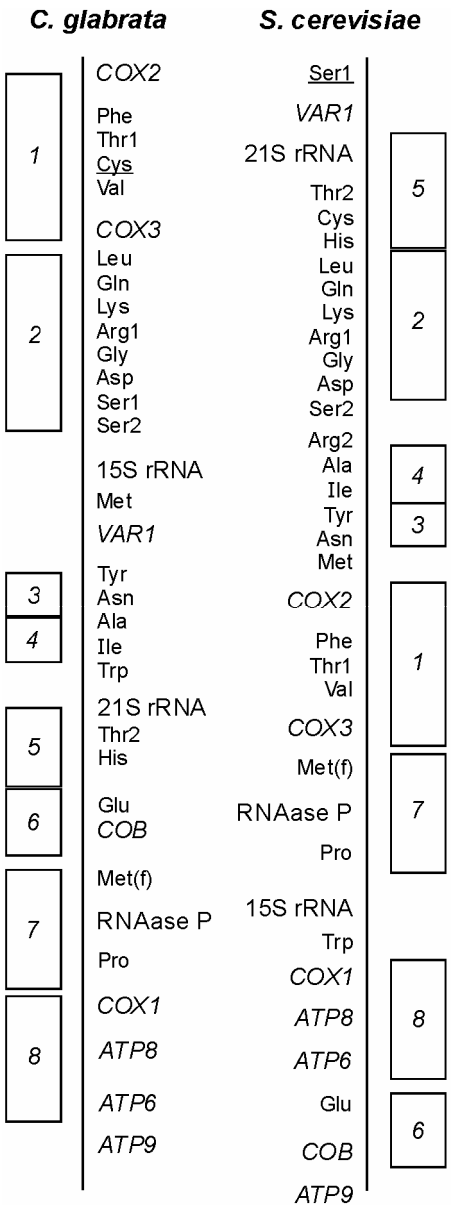

Fig. 6. The comparison of *C. glabrata* and *S. cerevisiae* mitochondrial genome maps; conserved gene series are represented by *numbered boxes* in each genome; underlined gene names represent the translocation of a single gene in an otherwise conserved block (adapted from Koszul *et al.* 2003).

Several transformation systems were developed for the study of gene function in *C. glabrata.* They are based on complementation of mutations occurring in the biosynthetic pathways of some nucleotides or amino acids (*ADE2*, *URA3*, *LEU2*, *HIS3*, *TRP1*) and can be used both with homologous and heterologous auxotrophic host strains (Mehra *et al.* 1992; Zhou *et al.* 1994; Kitada *et al.* 1995; Hanic-Joyce and Joyce 1998; Willins *et al.* 2002). Corresponding wild-type genes originating from both *S. cerevisiae* and *C. glabrata* can be used as selectable markers. In the case of the *ADE2* marker gene the prototrophic transformants of *C. glabrata* or *S. cerevisiae* are selectable on the basis of colony-color assay. The function of phosphoribosylaminoimidazole carboxylase ('AIR-carboxylase'; EC 4.1.1.21) in *ade2* mutants is lacking and resulting in pink color of yeast colonies on a complex medium due to the inability to synthesize adenine, while Ade^+ transformants are white (Hanic-Joyce and Joyce 1998). Dominant selectable markers, such as resistance to aureobasidin A (Okado *et al.* 1998) or kanamycin (Castaño *et al.* 2003), were also reported.

The episomal vectors for *C. glabrata* transformation contain *ARS* for autonomous replication from *S. cerevisiae* (Zhou *et al.* 1994) or *C. glabrata* (Mehra *et al.* 1992) and *CEN* from *C. glabrata* to increase the stability of plasmids in the *C. glabrata* host strain (Kitada *et al.* 1996). It was observed that *CEN* from *C. glabrata* does not function in *S. cerevisiae* (Kitada *et al.* 1996). Similarly, *ori* from the 2-μm plasmid of *S. cerevisiae* was unable to support the autonomous plasmid replication in *C. glabrata* (Zhou *et al.* 1994) and, therefore, transformants formed only abortive colonies (Mehra *et al.* 1992). Surprisingly, the *COXII* gene fragment from mtDNA of *S. cerevisiae* can also serve as the origin of plasmid replication in *C. glabrata* (Hanic-Joyce and Joyce 1998). In general, vectors containing the centromere are single-copy and more stable than high-copy-number vectors lacking the centromere.

The integrative transformation of *C. glabrata* is based on *ARS* lacking plasmids or DNA fragments and complementation of *leu2*, *ura3* or *his3* mutations in the host strain. It can be homologous if the plasmid contains a sequence ≥100 bp in size homologous to the genomic DNA of *C. glabrata*, or nonhomologous (Zhou *et al.* 1994; Cormack and Falkow 1999). Recently, a bacterial transposon Tn*7* with kanamycin resistance as a selectable marker has been used for random mutagenesis of *C. glabrata* chromosomal genes. The fragments of genomic library of *C. glabrata* containing integrated Tn*7* were introduced into the yeast host genome by recombination using single crossing-over that caused duplication of mutant locus, or double crossing-over that resulted in substitution of the standard locus by a mutated one (Castaño *et al.* 2003).

C. glabrata is amenable to homologous or heterologous expression of bacterial or yeast genes (Macreadie *et al.* 1994). For regulated gene expression, a promoter fused with different genes of interest has been constructed. The level of expression was regulated by the addition of tetracycline. This system allows determination of the essentiality of gene products expressed under the control of *tetO*::*ScHOP1* promoter, since expression of fusion genes in the presence of tetracycline is inhibited (Nakayama *et al.* 1998). Another

regulatable gene expression from *C. glabrata MT-IIa* or *MT-IIb* promoters (metallothionein-coding genes) was reported in the presence of copper (Mehra *et al.* 1992; Thorvaldsen *et al.* 1995; Kitada *et al.* 1996; El Barkani *et al.* 2000; Willins *et al.* 2002).

The properties of some vectors used in *C. glabrata* transformation are summarized in Table II.

Table II. The vectors used for transformation of *C. glabrata*

Type of vector	Vector	Yeast sequence	Bacterial sequence	Reference
Integrative	pUAUR-1	*AUR-1*	*ori*, AMP^R	Okado *et al.* 1998
	pCGLEU	*ScLEU2*	*ori*, AMP^R, *lacZ*	Geber *et al.* 1995
	pDM4	*ScURA3*	*ori*, AMP^R, *lacZ*	*ditto*
	pCGDL7	*ScLEU2*	*ori*, AMP^R, *lacZ*	*ditto*
	pGDV1	*ScLEU2*	*ori*, AMP^R	Petter and Kwon-Chung 1996
	pAD1A1	Δ*CgADE2*	*ori*, AMP^R, *lacZ*	Hanic-Joyce and Joyce 1998
	Tn*7*	*ScURA3*	KAN^R	Castaño *et al.* 2003
Centromeric	pRS316	*ScARSH4*, *ScCEN6*, *ScURA3*	*ori*, AMP^R, *lacZ*	Zhou *et al.* 1994
	pGRB2.2K28	*CgARS*, *CgCEN*, *CgURA3*, P_{PGK1}–, *pptox*, *GFP*	*ori*, AMP^R	Eiden-Plach *et al.* 2004
	p112-8M	*CgARS*, *CgCEN*, *CgHIS3*, *ScURA3*	*ori*, AMP^R, *lacZ*, *P(LAC)*, *P(BLA)*	Kitada *et al.* 1996
	pCgACH-3	*CgARS*, *CgCEN*, *CgHIS3*	*ori*, AMP^R, *lacZ*, *P(LAC)*, *P(BLA)*	*ditto*
	pCgACT-14	*CgARS*, *CgCEN*, *CgTRP1*	*ori*, AMP^R, *lacZ*, *P(LAC)*, *P(BLA)*	*ditto*
	pCgACU5	*CgARS*, *CgCEN*, *CgURA3*	*ori*, AMP^R, *lacZ*, *P(LAC)*, *P(BLA)*	*ditto*
Episomal	pJH2-14	*CgADE2*, *ori (COXII)*	*ori*, AMP^R, *lacZ*	Hanic-Joyce and Joyce 1998
	pMIR4	*CgADE2*, *ori (COXII)*	*ori*, AMP^R, *lacZ*	*ditto*
	pBM2-9	*CgADE2*, *ori (COXII)*	*ori*, AMP^R, *lacZ*	*ditto*
Integrative	p97CGH	*CgHIS3*, *tetO*::*ScHOP1*-gene	*ori*, AMP^R	Nakayama *et al.* 1998
	p98CGH	*CgHIS3*, *tetO*::*ScHOP1*-gene	*ori*, AMP^R	*ditto*
	p99CGH	*CgHIS3*, *tetO*::*ScHOP1*-gene	*ori*, AMP^R	*ditto*

4 IDENTIFICATION

Correct and prompt identification of pathogenic fungi, causing mainly system infections and candidemia, plays an important role in the application of suitable therapy. For pathogen identification both microbiological and biochemical methods, as well as nowadays, immunological and molecular-biological methods are frequently used.

The cultivation on special media containing a chromogenic substrate allows to distinguish *C. glabrata* from other yeast species in a mixed culture derived from clinical samples on the basis of color and surface of growing colonies. For example on CHROMagar Candida plate colonies of *C. glabrata* are dark-pink (Fig. 7) (Odds and Bernaerts 1994; Bouchara *et al.* 1996; Powell *et al.* 1998; Willinger and Manafi 1999).

The biochemical identification is based on fermentative and enzyme reactions, and mainly on the fact that *C. glabrata* assimilates only trehalose and glucose (Piens *et al.* 2003). The classical tube method and more frequent commercial microtests are used: API *Candida* (*bioMérieux*), API20C Aux (*bioMérieux*), Auxacolor (*BioRad*), Fungichrom I (*International Microbio*), Fungifast I twin (*International Microbio*), Vitek Yeast Biochemical Card (*bioMérieux*), Vitek 2 ID-YST (*bioMérieux*), ID 32 C (*bioMérieux*), RapID Yeast Plus (*Innovative Diagnostic*), Mycotube (*Roche*), Rosco diagnostic tablet (*Eurobio*), Glabrata RTT (*Fumouze Diagnostics*). They consist of dehydrated media with a composition differing in the content of sugars or other substrates for enzymes. The Glabrata RTT (rapid trehalose test) system appears to be the most favorite, because of its cost-effectiveness, unpretentiousness for laboratory equipment and labor input (Freydiere *et al.* 2002). The results are available within a few minutes, whereas sensitivity and specificity of identification is estimated to lie between 94 and 98 % (Freydiere *et al.* 2002–2004; **http://www.lucron bioproducts.com/Vl/fumouze-glabrata.htm**). The identification of *C. glabrata* by other systems

mentioned above takes some hours or days; however, the accuracy increases with time (Schuffenecker *et al.* 1993; Fenn *et al.* 1994; Buchaille *et al.* 1998; Freydiere *et al.* 2001; Lopez *et al.* 2001; Huang *et al.* 2001).

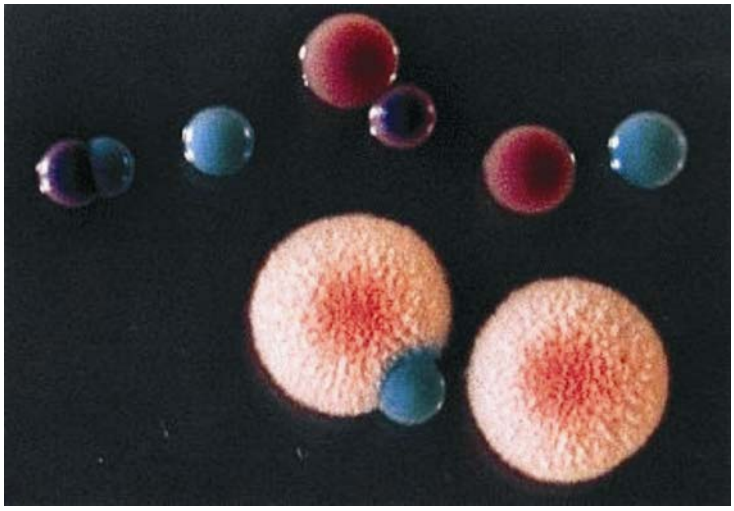

Fig. 7. The growth of *Candida* spp. on CHROMagar *Candida* plate; four *C. albicans* colonies are *green*, two *C. glabrata* colonies are *pink*, two *C. tropicalis* colonies are *bluish-purple* and the two large, *pale pink*, rough colonies are *C. krusei*; *m*agnification ×5.5 (Odds and Bernaerts 1994).

Among the physico-chemical methods, NMR spectroscopy allows species determination based on different content of labeled metabolites and cell components (Himmelreich *et al.* 2003). Another method, the Fourier transform infrared (FT-IR) spectroscopy (Freydiere *et al.* 2001) and Raman UV microspectroscopy, allow identification of yeast species without prior labeling of analyzed metabolites and cell components, and in the association with microscope also their cellular distribution (Maquelin *et al.* 2002). The accuracy of these methods is very high but requirements for laboratory equipment and necessity of experienced workers are their disadvantages.

Among the molecular-based methods, PCR followed by multiplex hybridization is frequently used. It allows the identification of different pathogenic yeast species directly in tissue samples. The method is based on the amplification of specific regions of chromosomal DNA (*ITS1* and *ITS2* – internally transcribed spacer region, between 5.8S–18S, and 18S–28S rRNA genes, respectively; 18S gene; *TOPII* gene – topoisomerase II) and hybridization of amplicons with known DNA probes derived from the most frequent pathogens. The amplicons that do not hybridize are analyzed by DNA sequencing. Some advantages of these methods are the following: high sensitivity (requirement of only ≈20 cells for isolation of sufficient amount of DNA for PCR), direct work with tissue or blood sample that does not require prior isolation of the pathogen by cultivation, the time for identification of pathogen is reduced to 2 d, and almost 100 % of correct identification (Niesters *et al.* 1993; Maiwald *et al.* 1994; McCullough *et al.* 1998; Hendolin *et al.* 2000; Luo and Mitchell 2002; Selvargan *et al.* 2003; Kanbe *et al.* 2003; Hsu *et al.* 2003; Willinger *et al.* 2003; Massonet *et al.* 2004). The PCR products can be detected by agarose gel electrophoresis (PCR-AGE) or using color-marked primer followed by colorimetric enzyme immunoassay (PCR-EIA) (Ahmad *et al.* 2004; Coignard *et al.* 2004). Pulsed-field gel electrophoresis (PFGE), random amplification of polymorphic DNA (RAPD), restriction enzyme analysis (REA) can also be used, especially in epidemiological research, when more isolates of one species or from one patient are isolated (Lehmann *et al.* 1992; Arif *et al.* 1996; Plachý *et al.* 2005).

Along with species identification, the determination of pathogen susceptibility to different antimycotics is also helpful for a proper medical treatment (**`http://www.doctorfungus.org`**). The broth microdilution, E-test and disk diffusion methods can be used for testing the susceptibility to fluconazole (Barry *et al.* 2002). These methods were also used for itraconazole, ketoconazole, voriconazole, ravuconazole – inhibitors of sterol biosynthesis (Espinel-Ingroff *et al.* 1996; Pfaller *et al.* 2001*a*, 2003*a*), caspofungin – inhibitor of glucan synthesis (Pfaller *et al.* 2001*b*), amphotericin B – changing membrane permeability (Espinel-Ingroff *et al.* 1996) and flucytosine – inhibitor of DNA and RNA synthesis (Espinel-Ingroff *et al.* 1996). Nowadays, there are many commercial susceptibility tests for determination of minimal inhibitory concentrations of drugs such as *Candi*fast, ATB Fungus, Diff Test, Mycostandard, Mycototal (Druetta *et al.* 1993),

Fungitest, ASTY, Sensititre, YeastOne and others. For the precise determination of the yeast susceptibilities to azole antimycotics the NCCLS microdilution method is highly recommended (Espinel-Ingroff *et al.* 1998).

5 PATHOGENICITY

Candida spp. are found as a normal part of microflora of human mucosal surfaces. *C. glabrata*, like *C. albicans*, is considered to be a human commensal. It can also cause vaginitis, oropharyngeal infections, thrush, urogenital tract infections and can be isolated even from feces. Moreover, in immunocompromised patients they cause very dangerous system infections and candidemia (Beck-Sague and Jawris 1993; Krčméry *et al.* 1998; Vazquez *et al.* 1998; Fidel *et al.* 1999; Nucci and Colombo 2002). In general, *Candida* spp. are considered to be opportunistic nosocomial pathogens (Nguyen *et al.* 1996; Berrouane *et al.* 1999) with a high morbidity and mortality (Fidel *et al.* 1999).

The international surveillance of bloodstream infections due to *Candida* spp. (SENTRY – *Antimicrobial Purveillance Program*) detected species differences in clinical isolates originating from the United States, Canada, Latin America, and Europe in 1997–99. It was found that *C. albicans* is still the main cause of bloodstream infections (55 %), followed by *C. glabrata* (15 %), *C. parapsilosis* (15 %), *C. tropicalis* (9 %), and by other *Candida* spp. (6 %) (Pfaller *et al.* 2001*a*). A similar species distribution has been found after monitoring the situation in Scandinavia (Sandven *et al.* 1998). On the other hand, the epidemiological study of candidemia in Czechia revealed a lower occurrence of infections caused by *C. glabrata* (Buchta *et al.* 1998; Hamal *et al.* 2003)

C. glabrata is often isolated from intravenous catheters, feces, urine, and body surfaces of oncological and HIV patients, from patients with transplantates, diabetes mellitus, burns, and neonates (Aisner *et al.* 1976; Hickey *et al.* 1983; Wingard *et al.* 1993; Rangel-Frausto *et al.* 1999; Kao *et al.* 1999). It usually enters the host body through the urogenital tract, lungs and other mucosal surfaces and induces formation of purulent infiltrates (Hazen 1995).

The high danger results from the lower sensitivity of *C. glabrata* to commonly used azoles (Van Den Bossche *et al.* 1992; Cross *et al.* 2000; Pfaller *et al.* 2004). Indeed, it is often isolated as a drug-resistant yeast, replacing other *Candida* species in patients receiving azole treatment (Pfaller *et al.* 1999). The biochemical mechanism of this resistance is more often associated with the decrease of antimycotic concentration inside the cell than with the changes in lanosterol 14α-demethylase (sterol 14-demethylase; EC 1.14.13.70) – the target molecule of azoles. Such intracellular decrease of antimycotic concentration is usually caused by the MDR phenomenon, when the active efflux of antifungal substances from the cell plays the main role (Parkinson *et al.* 1995). The comparison of clinical isolates before and after azole therapy using DNA restriction-fragment-length polymorphism (RFLP) analysis led to the conclusion that the drug resistance is the result of the selection of resistant clones from a primarily mixed population and not due to mutations (Hitchcock *et al.* 1993). So, the excessive use of azole antifungals, mainly in prophylactic therapy, later results in superinfection caused by *C. glabrata* (Hitchcock *et al.* 1993; Newman *et al.* 1994; Leverdiere *et al.* 2000).

The virulence factors of *C. glabrata* are still poorly understood. The absence of hyphae, the absence of specific antigens related to those known in *C. albicans* and the slight adherence to endothelial and epithelial cells are the facts that do not support the ability of *C. glabrata* to cause infection (Hasenclever and Mitchel 1960; Fidel *et al.* 1999). The potential virulence factors may be phospholipases that facilitate infiltration through the phospholipid barrier of the epithelial cells (Ghannoum 2000) or adherence to thrombocytes that facilitates the dissemination of the yeast in the bloodstream (Robert *et al.* 2000). *C. glabrata* also exhibited higher relative cell-surface hydrophobicity and adhesion to acrylic denture surfaces than *C. albicans* (Luo and Samaranayake 2002); indeed, Weig *et al.* (2004) detected by *in silico* analysis the presence of some potential adhesins, biofilm-forming proteins, phospholipases and proteinases. Recently, the *HLP* gene and its gene product that causes type α and β hemolysis have been identified in clinical isolates of *C. glabrata* (Luo *et al.* 2004).

Azoles belong to the most frequently used antifungal compounds for treatment of mycoses. In comparison with other azoles, especially fluconazol has some advantages, *e.g.*, good solubility in water, stability in the gastrointestinal tract that allows its peroral use, possibility of parenteral administration, low toxicity and selective attack on yeast cells that contain ergosterol in their cytoplasmic membranes. The nitrogen in the azole molecule reacts with the iron in the heme of lanosterol 14α-demethylase, an essential enzyme of the biosynthetic pathway of ergosterol. An important disadvantage of fluconazole and other azoles is their fungistatic effect (Van Den Bossche *et al.* 1983; Yoshida and Aoyama 1987).

C. glabrata is able to take up cholesterol or other ergosterol precursors from the host when the biosynthetic pathway of ergosterol is blocked (after deletion of the *ERG9* gene for squalene synthase) (Na-

kayama *et al.* 2000). This apparently increases its viability and decreases the success of antifungal therapy. Similar observations were also done later during studies dealing with both regulation of expression of the *ERG11* gene (lanosterol 14α-demethylase) (Nakayama *et al.* 2001) and transposon mutagenesis of the *ERG1* gene (squalene epoxidase; EC 1.14.99.7) (Tsai *et al.* 2004). *In vivo* experiments on the mouse have shown that *C. glabrata* accumulates abnormal precursors of ergosterol that are different from those accumulated in *S. cerevisiae* and *C. albicans* after fluconazole treatment (Kelly *et al.* 1993, 1995). On the other hand, all three yeast species exhibited similarity with respect to mutations in the *ERG3* gene ($\Delta^{5,6}$-desaturase; EC 1.14.19.1) that suppress the mutation in the *ERG11* gene and protect cells from toxic ergosterol precursors (Geber *et al.* 1995). These observations indicate that ergosterol biosynthetic genes and their products are not the best targets for antifungal therapy by azoles.

In this respect, promising seems to be a combination of azole or morpholine antifungals with calcineurin inhibitors (Ca^{2+}/calmodulin-dependent serine/threonine protein phosphatase) since these substances exhibited a synergistic fungicidal effect *in vitro* (Onyewu *et al.* 2002). Calcineurin is the key enzyme of the calcium signaling pathway. Signalization by calcium controls many cellular processes essential for life, *e.g.*, morphogenesis, cell cycle, response to pheromones. The calcium is compartmentalized in several organelles (vacuoles, mitochondria, endoplasmatic reticulum) or present in a high concentration outside the cell. Its influx into the cell or excessive release from cell organelles is influenced by the presence of azole antifungals (Edlind *et al.* 2002).

6 MULTIDRUG RESISTANCE

Under *in vitro* conditions a high-frequency azole resistance (HFAR) has been described in *C. glabrata*. This phenotype is developed after treatment of yeast with subinhibitory concentration of azoles and is associated with overexpression of genes for ABC-type transporters (Sanglard *et al.* 1999, 2001).

The wild-type strain of *C. glabrata* exposed to fluconazole concentration of 50 μg/mL developed resistance to this drug and other azoles at a surprisingly high frequency and, at the same time, upregulation of the *CgCDR1* gene (homologue of the *CDR1* gene in *C. albicans* and the *PDR5* gene in *S. cerevisiae*) has been observed (Sanglard *et al.* 1999). The *CgCDR1* deletion mutant could still develop a high frequency azole resistance in a medium containing fluconazole at a concentration of 5 μg/mL due to a distinct ABC-type transporter gene similar to *CgCDR1*, called *CgCDR2* (synonym *PDH1*; Miyazaki *et al.* 1998). In MDR of *C. glabrata* the *CgCDR1* gene plays the main role (Wada *et al.* 2002).

The mechanism of high-frequency azole resistance is tightly coupled with a functional state of mitochondria. It was shown that the loss of mitochondrial DNA results in an increased expression of some genes involved in resistance to antimycotics (Defontaine *et al.* 1999; Sanglard *et al.* 1999; Bouchara *et al.* 2000). Along with ABC-type transporter encoding genes another candidate is the *ERG11* gene encoding lanosterol 14α-demethylase that is directly connected to fluconazole resistance (Marichal *et al.* 1997; Sanglard *et al.* 1998; Henry *et al.* 2000). The mechanism of this type of resistance relates to: (*i*) increase of concentration of target protein Erg11p in the cell, (*ii*) decrease of Erg11p affinity to azole antifungals, or (*iii*) changes in the biosynthetic pathway of ergosterol. It is also possible that the loss of functional mitochondria induces overproduction of ABC-type transporters different from *Cg*Cdr1p and *Cg*Cdr2p which can be supported by the finding that *rho*^{0} mutants obtained after ethidium-bromide mutagenesis and defective in both the *CgCDR1* and *CgCDR2* genes were still resistant to azoles (Sanglard *et al.* 2001). It was also supposed that functional mitochondria are responsible for conversion of nontoxic ergosterol precursors to toxic ones, so the loss of mitochondrial functions is the result of selection pressure caused by an excessive azole use (Kontoyiannis 2000).

Kaur *et al.* (2004) identified (by transposon mutagenesis based on random insertion of Tn*7* to the genome) other genes that play a certain role in the control of *C. glabrata* susceptibility to fluconazol. Some mutants obtained that were more susceptible to fluconazole than the wild-type strain contained mutations in genes coding for ABC-type transporters (*CgCDR1*, *CgPDR16*), calcium transporters (*CgCCH1*, *CgMID1*), coactivators of RNA polymerase II (*CgSTP20*, *CgSRB8*, *CgRGR1*, *CgNUT1*, *CgRTG2*), components of nuclear ubiquitin complex (*CgCDC34*, *CgCDC53*), ATPases (*CgSTV1*, *CgVMA13*) and other genes (*CgYNR047w*, *CgECM7*, *CgPOM152*, *CgINP53*). Mutants that displayed higher resistance to antimycotics contained mutations in genes involved in the biogenesis of ribosomes (*CgDBP3*, *CgRPL16a*, *CgNOP8*), biogenesis of mitochondria (*CgSUV3*, *CgMRPL4*, *CgSHE9*), or other genes (*CgSIN3*, *CgSDA1*, *CgPGS1*, *CgYCR041w*).

The promoter regions of the *CgCDR1* and *CgCDR2* genes contain sequences homologous to pleiotropic drug responsive element (PDRE) that are required for Pdr1p and Pdr3p binding to promoters of their target genes in *S. cerevisiae*. Therefore, the regulation of expression of these genes in *C. glabrata* can be similar to that in *S. cerevisiae* (Miyazaki *et al.* 1998; Sanglard *et al.* 1999; Izumikawa *et al.* 2003).

Table III. Occurrence of the *C. glabrata* genes involved in the control of drug resistance[a,b]

No.	*S. cerevisiae* gene	*C. glabrata* gene	Size of the gene product (AA) *S.c./C.g.*	Identity, %	Function	Chromosome *S.c./C.g.*
1	*PDR5*	CAGL0M01760g	1511/1499	73	ABC	XV/M
2	*SNQ2*	CAGL0I04862g	1501/1507	72	ABC	IV/I
3	*YOR1*	CAGL0G00242g	1477/1477	73	ABC	VII/G
4	*YCF1*	CAGL0L06402g	1515/1535	71	ABC	IV/L
5	*PDR12*	CAGL0M07293g	1511/1515	77	ABC	XV/M
6	*PDR15*	CAGL0F02717g	1529/1542	75	ABC	IV/F
7	*PDR16*	CAGL0J07436g	361/344	71	ABC	XIV/J
8	*PDR17*	CAGL0J08074g	350/347	69	ABC	XIV/J
9	*YBT1*	CAGL0C03289g	1661/1648	68	ABC	XII/C
		CAGL0E03982g	1661/1659	68	ABC	XII/E
10	*BPT1*	CAGL0E03355g	1559/1535	57	ABC	XII/E
11	*STE6*	CAGL0K00363g	1290/1227	54	ABC	XI/K
12	*ROD1*	CAGL0A02046g	837/776	58	ABC	XV/A
		CAGL0K10714g	837/705	62	ABC	XV/K
13	*AUS1*	CAGL0F01419g	1394/1398	71	ABC	XV/F
14	*MDL1*	CAGL0E00385g	695/608	59	ABC	XII/E
15	*MDL2*	CAGL0A01133g	773/801	70	ABC	XVI/A
16	*ADP1*	CAGL0L07744g	1049/1055	67	ABC	III/L
17	*YOL075C*	CAGL0I08019g	1294/1285	66	ABC	XV/I
18	*ATR1-like*	CAGL0M03003g	542/558	65	MFS2	XIII/M
19	*ATR1*	CAGL0B02343g	542/537	64	MFS2	XII/B
20	*AZR1*	CAGL0B02079g	613/624	60	MFS2	VII/B
21	*YOR378w*	CAGL0L02519g	515/529	37	MFS2	XV/L
22	*VBA1*	CAGL0J01375g	562/564	66	MFS2	XIII/J
23	*DTR1*	CAGL0M06281g	572/542	63	MFS1	II/M
24	*TPO1*	CAGL0E03674g	586/577	70	MFS1	XII/E
		CAGL0G03927g	586/567	73	MFS1	XII/G
25	*TPO2*	CAGL0I10384g	614/630	74	MFS1	VII/I
26	*TPO4*	CAGL0L10912g	659/651	71	MFS1	XV/L
27	*FLR1*	CAGL0H06017g	548/577	65	MFS1	II/H
		CAGL0H06039g	548/589	66	MFS1	II/H
28	*QDR2*	CAGL0G08624g	542/583	69	MFS1	IX/G
29	*AQR1*	CAGL0J09944g	586/592	64	MFS1	XIV/J
30	*YHR048w*	CAGL0J00363g	514/498	63	MFS1	VII/J
31	*YJR124C*	CAGL0H00440g	448/445	69	other MFS	X/H
32	*SNG1*	CAGL0B03949g	547/632	39	other MFS	VII/B
		CAGL0G09273g	547/545	49	other MFS	VII/B
33	*FEN2*	CAGL0L08052g	512/484	72	allantoate permease	III/L
34	*PDR1*	CAGL0A00451g	1068/1107	59	TF (Zn_2Cys_6)	VII/A
35	*YRM1*	CAGL0L04400g	786/987	34	TF (Zn_2Cys_6)	XV/L
		CAGL0L04576g	786/865	39	TF (Zn_2Cys_6)	XV/L
36	*STB5*	CAGL0I02552g	743/1005	43	TF (Zn_2Cys_6)	VIII/I
37	*MSN2*	CAGL0F05995g	704/597	30	TF (Zn_2Cys_6)	XIII/F
38	*MSN4*	CAGL0M13189g	630/541	26	TF (Zn_2Cys_6)	XI/M
39	*RDS1*	CAGL0D02486g	832/878	27	TF (Zn_2Cys_6)	III/D
40	*RDS2*	CAGL0M02651g	446/462	61	TF (Zn_2Cys_6)	XVI/M
41	*RDS3*	CAGL0K08074g	107/107	62	pre mRNA splicing	XVI/K
42	*WAR1*	CAGL0H04367g	944/945	37	TF (Zn_2Cys_6)	XII/H
43	*YAP1*	CAGL0H04631g	650/588	63	TF (bZip)	XIII/H
44	*YAP2*	CAGL0F03069g	409/486	36	TF (bZip)	IV/F
45	*YAP4*	CAGL0H08173g	295/88	76	TF (bZip)	XV/H
46	*YAP6*	CAGL0M08800g	383/263	27	TF (bZip)	IV/M
47	*NGG1*	CAGL0E00693g	702/829	47	general TF	IV/E

[a]According to **http://alpha10.bioch.virginia.edu/fasta/**; **http://www.ncbi.nlm.nih.gov/entrez/query.fcgi**, **http://www.yeastgenome.org/**; **http://cbi.labri.fr/Genolevures/** .

[b]MDR genes – *C. glabrata* (*C.g.*) *vs. S. cerevisiae* (*S.c.*), ABC – ATP-binding cassette protein, MFS – protein of major facilitator superfamily, TF – transcription factor, AA – number of amino acids.

In silico analysis using genome databases, FASTA and BLASTP program (**http://alpha10.bioch.virginia.edu/fasta/**; **http://www.ncbi.nlm.nih.gov/entrez/query.fcgi**; **http://www.yeastgenome.org/**; **http://cbi.labri.fr/Genolevures/**) allowed us to summarize the occurrence of *C. glabrata* genes involved in the control of drug resistance (Table III). This analysis was done by comparison of amino acid sequences of homologous gene products taking into account the known *S. cerevisiae* MDR associated proteins with the identity of 27–76 %. These proteins belong to some protein groups and protein families (*see* Table III) compared with reviews and papers by Nelissen *et al.* (1997) and Stolz and Sauer (1999) for MFS-type transporters, by Bettina-Bauer *et al.* (1999) and Dean *et al.* (1994) for ABC-type transporters, and Moye-Rowley *et al.* (2003) and Kren *et al.* (2003) for transcription factors. The size of many orthologues was very similar but some relatively large differences (in number of amino acids) were also found, *e.g.*,

*Sc*Rod1p – 837 *vs*. *Cg*Rod1p (CAGL0K10714g) – 705
*Sc*Yap1p – 650 *vs*. *Cg*Yap1p – 588
*Sc*Yap4p – 295 *vs*. *Cg*Yap4p – only 88
*Sc*Stb5p – 743 *vs*. *Cg*Stb5p – 1005
*Sc*Msn4p – 630 *vs*. *Cg*Msn4p – 541.
*Sc*Yrm1p – 786 *vs*. *Cg*Yrm1p – 987
*Sc*Yap2p – 409 *vs*. *Cg*Yap2p – 486
*Sc*Yap6p – 383 *vs*. *Cg*Yap6p – 263
*Sc*Msn2p – 704 *vs*. *Cg*Msn2p – 597

The Ngg1p homologue that negatively regulates MDR in *S. cerevisiae* was also found. Homologues of other well documented or potential resistance genes involved in the control of drug resistance in *S. cerevisiae* were not found. On the other hand, one cannot rule out that *C. glabrata* could possess other resistance genes for which orthologues are not present in the *S. cerevisiae* genome.

7 CONCLUSION

The incidence of nosocomial infections caused by the highly resistant pathogenic fungus *C. glabrata* has a still growing trend. Therefore, it is necessary to focus our attention on this problem from the viewpoint of both medicine and fundamental research. The mechanisms of pathogenicity and resistance to azole antifungals in *C. glabrata* are still poorly understood. Its pathogenicity can bring a certain risk but, on the other hand, the knowledge acquired from studies of pathogenic yeast species can help to improve the antifungal therapy of mycoses by application of new antimycotics or introduction of new strategies to combat these infectious diseases. The haploid and asexual character of *C. glabrata* and its ability to express the *S. cerevisiae* genes support the use of this microorganism for the study of molecular mechanisms of drug resistance and its reversal in pathogenic yeast species.

This work was partially supported by grants from the *Agency for Support of Science and Technology* (APVT-51-000502, APVT-20-000604) and from the *Slovak Grant Agency* (VEGA 1/0019/03, VEGA 1/3250/06).

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