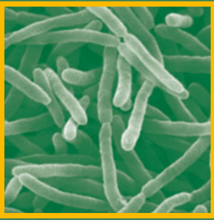
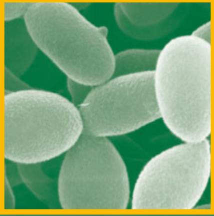
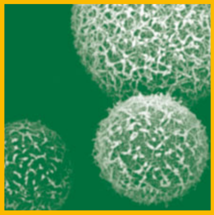


THE YEAST HANDBOOK





H. Ruth Ashbee
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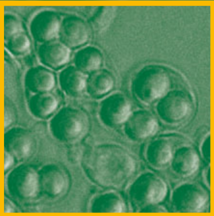
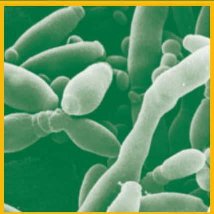


Pathogenic Yeasts



The Yeast Handbook

H. Ruth Ashbee • Elaine M. Bignell
Editors

Pathogenic Yeasts

Editors
Dr. H. Ruth Ashbee
Mycology Reference Centre
Dept. Microbiology
Leeds General Infirmary
United Kingdom LS1 3EX
h.r.ashbee@leeds.ac.uk

Dr. Elaine M. Bignell
Imperial College London
Div. Investigative Science
South Kensington Campus
London
United Kingdom SW7 2AZ
e.bignell@imperial.ac.uk

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Preface

Mycological studies of yeasts are entering a new phase, with the sequencing of several yeast genomes informing our understanding of their ability to cause disease and interact with the host. Parallel to this advancement, the ongoing use of traditional methods in many clinical mycology laboratories continues to provide information to diagnose and treat patients.

The aim of this volume is not to provide comprehensive coverage of all aspects of pathogenic yeasts, but rather to focus on certain topics and to review the current knowledge in those areas. Each chapter has been written by relevant internationally recognised experts and is self-contained, although necessarily there is some cross referencing between chapters. Broadly speaking the content can be subdivided into four sections: *Candida albicans*, *Cryptococcus neoformans*, other pathogenic yeasts (including *Malassezia*, and emerging yeast pathogens) and finally clinical laboratory considerations such as diagnosis and antifungal susceptibility.

Genomic advances are reviewed in the opening chapter by Butler and Fitzpatrick who summarise the state-of-the-art in comparative fungal genomics and the impact that bioinformatic analysis, coupled with advanced technologies, has already had upon our understanding of fungal pathogenesis and evolution of virulence. *Candida albicans*, arguably the best-characterised of the pathogenic yeasts, continues to provide a beacon for fungal molecular genetics and to exemplify the power that genomic approaches can bring to bear upon study the of eukaryotic pathogenetics. Moran *et al*. subsequently describe, and have implemented to great effect, the multilocus sequence typing methodology to decipher the epidemiology of infectious *Candida* species in their latest works reviewed in Chap. 2. The spectrum of *Candida* disease is addressed, in combination with the most comprehensively referenced overview available, of experimental modelling of *Candida* infection by MacCallum in Chap. 3. Cell-wall mediated virulence, another well-studied aspect of *Candida albicans* can be considered from both the host and pathogen perspective (Chap. 4, Munro) as the cell wall represents the immediate interface between the two and is partially responsible for prompting immunogenic responses in the infected host. The cell wall contains antigenic carbohydrate moieties and

proteins, which are central to disease, a further aspect of secreted protein activity in the infectious arena being facilitation of tissue invasion (Chap. 5, Naglik).

Yeast biofilms are important in many types of infection and recent advances in understanding the genetic control of their development have provided insight into their role in host-pathogen interactions, resistance to antifungals and cell–cell communication, all of which are discussed by Ramage and colleagues in Chap. 6.

Chapter 7 (Bicanic and Harrison) discusses the spectrum of disease that cryptococcal species cause and review recent studies, which have shown that effective regimens for treating cryptococcosis and managing the associated complications can impact the morbidity and mortality significantly and result in improved outcomes. Chapter 8 (Mitchell and Litvintseva) details the methods used to type *Cryptococcus* and the studies on its epidemiology, which have lead to a better understanding of the sub-groups within the genus and its phylogeny. This foray into the clinical implications of crytpococcal disease is followed by a review of cryptococcal virulence factors by Mühlschlegel and colleagues in Chap. 9, which interrogates the mechanisms employed by this organism to withstand the extraordinary stresses likely to be encountered during long periods of latent infection.

The third section of the book looks at various other pathogenic yeasts. Chapter 10, by Ashbee and Scheynius reviews the genus *Malassezia* and particularly the interesting advances that have been made recently with regard to its role in atopic dermatitis and interaction with the host.

New and emerging yeasts are reviewed in Chap. 11 (Mathews and Ashbee), looking at their microbiology, epidemiology, clinical manifestations, diagnosis, and, where data is available, their antifungal susceptibility patterns and therapeutic approaches to treating such infections and outcome.

Besides causing disease in humans, many yeasts are also significant pathogens in animals. Chapter 12 (Cabanes) covers the role of *Candida, Cryptococcus,* and *Malassezia* in disease processes in a range of animals.

The diagnosis of yeast infections is very important in clinical mycology laboratories and the introduction of new techniques is changing the way that many yeasts are identified. In Chap. 13, Barton reviews both the more traditional phenotypic and biochemical methods and also discusses the role of molecular and non culture-based diagnostic modalities. The final chapters of the book focus on antifungal susceptibility, therapy and antifungal resistance. Chapter 14, by Cuenca-Estrella and Rodriguez-Tudela, describes the various methods to determine yeast antifungal susceptibility and discusses the recent updates that have taken place in standardisation. In addition, it includes reviews of therapy for treatment of *Candida* and cryptococcal infections. Chapter 15 (Sanglard) provides an up-to-the-minute review of the molecular basis of antifungal resistance.

It is the hope of the editors and contributing authors that this book will provide a thorough and authoritative review of many aspects of the pathogenic yeasts. Because of the size of the book, we had to choose specific topics to include, but we hope that we have also managed to retain sufficient scope to be of interest to a wide range of colleagues. Given the parallels between clinical and investigative

mycological research, and the benefits that cross-fertilization between these fields has brought in recent years, perhaps the overriding strength of this publication is its accessibility to readers originating from, or attempting to better understand, either or both of these areas.

June 2009 *Ruth Ashbee and Elaine Bignell*

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Chapter 1
Comparative Genomic Analysis of Pathogenic Yeasts and the Evolution of Virulence

David A. Fitzpatrick and Geraldine Butler

Abstract The increased availability of molecular data has had a major impact on phylogenetic studies in general, and on the study of fungal phylogeny in particular. To date, more than 60 fungal genomes have been completely sequenced, ranging from the Chytridiomycota to the Ascomycota. There have been several attempts to reconstruct aspects of the fungal Tree of Life, using a variety of approaches (Fitzpatrick et al. 2006; James et al. 2006; Kuramae et al. 2006; Robbertse et al. 2006; Marcet-Houben and Gabaldon 2009).

Because the use of single genes to infer phylogenetic relationships can generate a number of different topologies, it has become increasingly common to use several genes, often concatenating information. A very thorough analysis was carried out by James et al. (2006), who used six genes from 200 species. This analysis supports a monophyletic origin for the Ascomycota, Basidiomycota, and Glomeromycota. The study also addressed the relationship of the Microsporidia, intracellular animal parasites whose phylogenetic origin has long been controversial. James et al. (2006) place the Microsporidia on the earliest diverging fungal branch.

The analysis of Fitzpatrick et al. (2006) used information from 4,805 single-gene families from 42 fully sequenced fungal genomes. A robust phylogeny was generated, supporting the major phyla (Zygomycota, Basidiomycota, and Ascomycota)

D.A. Fitzpatrick
Genome Evolution Laboratory, Department of Biology, National University of Ireland Maynooth, Maynooth, Co. Kildare, Ireland

G. Butler (✉)
UCD School of Biomolecular and Biomedical Science, Conway Institute, University College Dublin, Belfield, Dublin 4, Ireland

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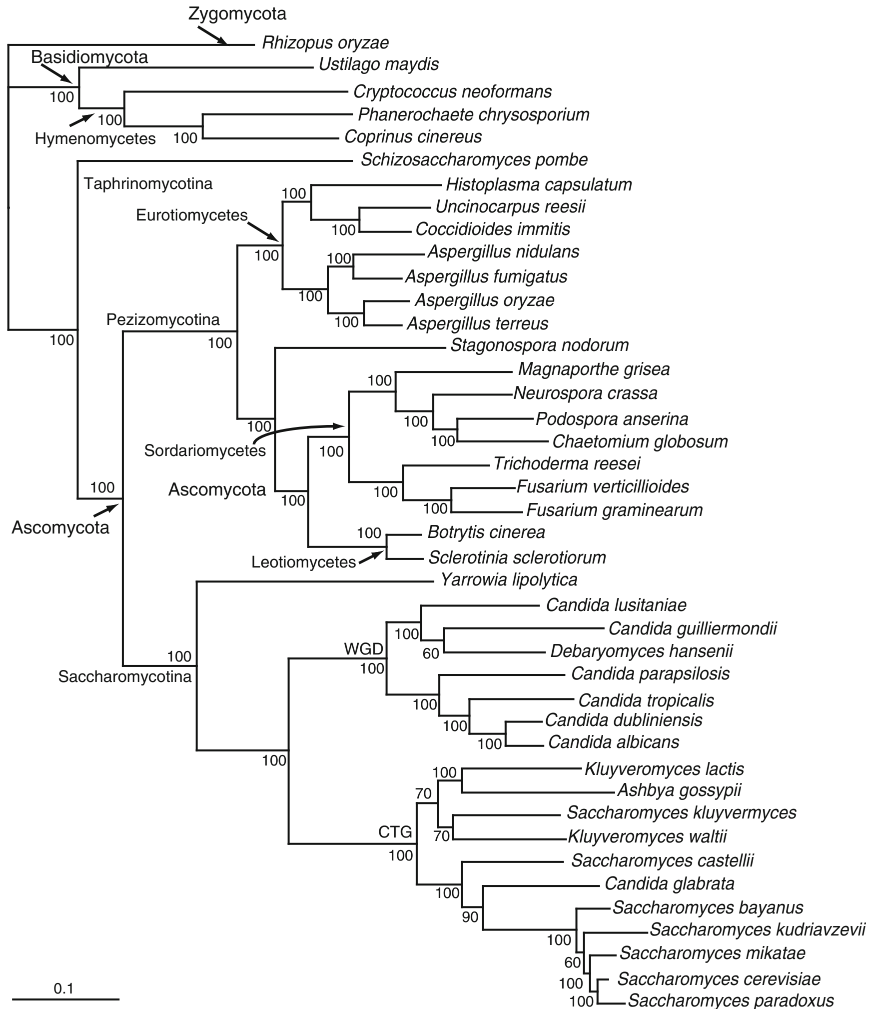

Fig. 1.1 Phylogenetic relation of fungal species. A maximum likelihood phylogeny was constructed using a concatenated alignment of 153 fungal genes from 42 species. Taken from Fitzpatrick et al. (2006)

(Fig. 1.1). The subphyla within the Ascomycota (Taphrinomycotina, Pezizomycotina, and Saccharomycotina) are strongly supported. At the time the analysis was performed few basidiomycete sequences were available, but the monophyletic origin of the Hymenomycetes is clear. The overall structure of the fungal tree is supported by several additional phylogenomic analyzes (Kuramae et al. 2006; Robbertse et al. 2006; Marcet-Houben and Gabaldon 2009).

The majority of fungi associated with human disease are ascomycetes, from either the subphyla Pezizomycotina (e.g., Aspergilli) or Saccharomycotina (e.g.,

Candida). This may explain why most available genome sequences are from these groups.

1.1 Comparative Genome Analysis

Several recent studies have shown how powerful comparative genome analysis can be, particularly in relation to the identification of lineage-specific or species-specific pathways. Cornell et al. (2007) analyzed genomes from 34 fungal species, mostly ascomycetes and basidiomycetes, in an attempt to identify signals of specialization and diversification. They first determined the level of gene duplication, as this may allow the acquisition of new functions. Among the Ascomycota, the level of gene duplication is higher in the Pezizomycotina (and in particular the Aspergilli) than it is among the Saccharomycotina. A lower level of duplication in some of the Pezizomycotina (for example, *Neurospora crassa*) is related to the activity of RIP (repeated induced point mutation; a mechanism that removes duplicated sequences) (Galagan and Selker 2004).

In general, the genes that are over-represented in the Pezizomycotina include cytochrome P450 proteins, which are involved in degradation of toxins and secondary metabolism and are associated with adaptation to environmental stress (Deng et al. 2007). There is also an increase in protein families involved in transport of sugars and small molecules such as drugs, and in proteins required for the metabolism of different carbon sources. In contrast, fewer duplications are identified only in the Saccharomycotina. However, there is a large expansion in Pir proteins, a motif associated with cell wall proteins (Kapteyn et al. 1996). Other motifs associated with cell wall synthesis are also increased in the Saccharomycotina including glucan synthases and mannosyltranferases. Domains associated with chitin synthesis are more prevalent in the Pezizomycotina.

Comparative analysis of the 34 genomes revealed substantial differences in fatty acid degradation, which could be correlated to the activity of organelles. In mammals, β-oxidation occurs in both the mitochondria and the peroxisomes, whereas in *Saccharomyces cerevisiae* it is restricted to the peroxisomes (Kunau et al. 1995; Hiltunen et al. 2003). Early steps in the reaction require acetyl-CoA dehydrogenase (in the mitochondria) and acyl-CoA oxidase (in the peroxisome). Cornell et al. (2007) used the presence or absence of these genes to suggest that nonperoxisomal β-oxidation was lost from the ascomycetes after the diversion from *Yarrowia lipolytica*. Peroxisomal β-oxidation has also been lost independently in some of the Pezizomycotina.

The analysis of Cornell et al. (2007) presents an excellent resource for subsequent analysis addressing the difference between pathogenic and nonpathogenic species within the phyla (for example, comparisons between *S. cerevisiae* and *Candida glabrata*, or among pathogenic and non-pathogenic Aspergilli and *Candida*). The data is publicly available in the *e-Fungi* database (http://www.cs.man.ac.uk/~cornell/eFungi/index.html).

1.2 Analysis of Basidiomycete Genomes

Within the basidiomycetes, *Cryptococcus neoformans* is the best-characterized human pathogen. It is a haploid basidiomycete yeast that commonly infects the central nervous system of immunocompromised patients (Hull and Heitman 2002). *Cr. neoformans* is classified into three major serotypes, *Cr. neoformans* var. *grubii* (serotype A), *Cr. neoformans* var. *neoformans* (serotype D), and a hybrid (serotype A/D). Serotypes B and C have recently been categorized as a separate species, *Cr. gattii* (Kwon-Chung et al. 2002). A *Cr. neoformans* serotype D isolate was sequenced in 2005 (Loftus et al. 2005), and the sequence of a serotype A isolate and two *Cr. gattii* isolates are currently in progress (http://www.broad.mit.edu/). The two *Cr. neoformans* have approximately 10–15% difference at the nucleotide level.

The formation of a polysaccharide capsule is a major virulence characteristic in *Cr. neoformans* (Bose et al. 2003). Analysis of the genome sequence led to the identification of 20 new genes required for capsule formation, several of which are confined to basidiomycetes (Loftus et al. 2005). Of 11 families that are specific to *Cr. neoformans*, two are involved in capsule formation (Loftus et al. 2005). Comparison of the serotype A and serotype D genomes revealed that a large region (approximately 14 kb) called an “Identity Island” was transferred from var. *grubii* (serotype A) to var. *neoformans* (serotype D), replacing the equivalent region in var. *neoformans* (Kavanaugh et al. 2006). This results in duplication of some genes in var. *neoformans* (such as the enolase gene), which may provide a selective advantage in rich media.

Mating-type in *Cr. neoformans* is strongly correlated with virulence (Kwon-Chung et al. 1992). *Cr. neoformans* has a bipolar mating structure, with a single mating-type locus (*MAT*), containing either a or alpha idiomorphs (Lengeler et al. 2000). The *MAT* locus is extremely large (encompassing approximately 6% of the chromosome), and arose by fusion of two unlinked gene clusters (Loftus et al. 2005; Fraser et al. 2007). Mating between *MAT*a and *MAT*α cells takes place in the laboratory, but in the wild the vast majority of isolates have the *MAT*α idiomorph (Heitman 2006). *MAT*α cells are more virulent than *MAT*a, and it is highly likely that sexual reproduction is required to allow propagation (Hsueh and Heitman 2008). Same-sex mating between serotype D α isolates was demonstrated in the lab (Lin et al. 2005), and the identification of αADα hybrids suggested that similar mating occurred in nature (Lin et al. 2007). Lin et al. (2009) have recently shown that approximately 8% of natural isolates of *Cr. neoformans* serotype A are α/α diploids, the majority arising from mating of identical cells. Diploids may affect virulence by increasing fitness, or by producing more resistant spores (Lin et al. 2009).

The basidiomycete *Malassezia globosa* is normally found on human skin, but it is also closely associated with dandruff (Chen and Hill 2005). The genome sequence was reported in 2007, together with a 1X coverage of a related species, *M. restricta* (Xu et al. 2007). *M. globosa* cannot grow in the absence of lipids which, analysis of the genome sequence suggests, is due to the absence of a fatty

acid synthase gene. *M. globosa* is closely related to the maize pathogen *Ustilago maydis,* yet there are substantial differences at the genome level that are likely to be related to host adaptation. *U. maydis* encodes many more carbohydrate-hydrolyzing enzymes than *M. globosa*. In contrast, the *M. globosa* genome is enriched for phospholipases, lipases, aspartyl proteases, and sphingomyelinases. Many of these families have been associated with virulence in *Candida albicans*, an ascomycetous human pathogen that also colonizes skin. Expression of lipase in *M. globosa* occurs on human scalp, which is likely to be a important pathogenic factor (Juntachai et al. 2008). The genomes of other fungal species that inhabit human skin are underway, in particular the ascomycete dermatophyte *Microsporum gypseum* (http://www.broad.mit.edu). A comparative analysis of all species is likely to lead to the identification of novel virulence factors required for skin colonization.

1.3 Analysis of Ascomycete Genomes

1.3.1 The Aspergilli

Aspergillus fumigatus is a major opportunistic pathogen that causes invasive disease, particularly in immunocompromised individuals. It is also a primary pathogen of the airways. Comparative genome analysis of the Aspergilli is a particularly powerful approach because of the availability of genome sequences from a wide variety of species. The genomes of one isolate of *A. fumigatus* and the sexual model organism *A. nidulans* was reported in 2005 (Galagan et al. 2005; Nierman et al. 2005), together with the sequence of *A. oryzae*, a fungus used for the production of traditional fermented foods in Japan (Machida et al. 2005). A second clinical isolate of *A. fumigatus* was sequenced in 2008, together with the genomes of a close sexual relative *Neosartorya fischeri* and a more distantly related asexual species *A. clavatus*, associated with disease in sheep and cows fed with infected grain (Fedorova et al. 2008.) Genome sequences from *A. terreus,* a second opportunistic pathogen (http://www.broad.mit.edu), *A. flavus*, a weak pathogen of animals and plants (Yu et al. 2008), and *A. niger*, an industrial species (Pel et al. 2007) provide an unprecedented resource for comparative analysis.

Initial comparisons revealed that gene order, or synteny, is generally conserved among *A. nidulans, A. fumigatus,* and *A. oryzae*, except at the sub-telomeric regions (Galagan et al. 2005). These regions tend to contain secondary metabolite clusters that are associated with adaptation and virulence. One of the most interesting hypotheses to emerge from the genome comparisons was the suggestion that *A. fumigatus* and *A. oryzae*, long believed to be asexual, may contain a fully sexual cycle. This was based on the identification of mating-type genes that determine sexual compatibility, and was subsequently supported by the identification of isolates of opposite mating type in the *A. fumigatus* population (Paoletti et al. 2005). In early 2009, the genome predictions were validated when the existence of a sexual cycle was biologically verified (O'Gorman et al. 2008). Similar

predictions from genomic analysis of the parasite *Giardia* were also recently supported by experimental evidence (reviewed in (Logsdon 2008)), which may have implications for analysis of other apparently asexual organisms.

A more recent analysis of two *A. fumigatus* isolates identified regions of segmental duplication in the clinical isolate that may have resulted from selective pressures in the host (Fedorova et al. 2008). There is a considerable level of polymorphism in the gene sequences between the two isolates, with amino acids sequence identities of homologous proteins ranging from 100% down to just 37%. Some of the most dramatic differences are in *het* (heterokaryon incompatibility) genes, which are likely to be involved in a pathway activated by a fusion of genetically incompatible individuals. Fedorova et al. (2008) identified > 800 genes that are specific to *A. fumigatus* (relative to *N. fischeri* and *A. clavatus*). These are enriched for genes involved in carbohydrate metabolism, secondary metabolism, and detoxification. In general, lineage-specific genes are more frequently found in sub-telomeric regions, and the *A. fumigatus*-specific genes in particular are located in 13 blocks, or "genomic islands" towards the telomeres. A metabolite cluster required for the synthesis of a mycotoxin fumigaclavine is located in one of the islands. A detailed analysis indicates the species-specific genes originate from gene duplication and divergence (Fedorova et al. 2008), rather than from Horizontal Gene Transfer (HGT), as suggested earlier (Galagan et al. 2005; Machida et al. 2005).

In general, proteins known to be associated with virulence in *Aspergillus* have a low rate of evolution (Nierman et al. 2005). However, Fedorova et al. (2008) found evidence of accelerated evolution in four genes (*pabaA, fos*-1, *pes*1, and *pksP*), involved in oxidative stress or nutrient availability (such as biosynthesis of folate). These may be of particular importance in interaction with the host.

1.3.2 Candida Genomes

Among the Saccharomycotina, *Candida* species are most closely associated with pathogenesis in human hosts. The definition of *Candida,* however, is not very specific, as it includes relatively distantly related species such as *C. glabrata* and *C. albicans*. We will use *Candida* to refer to the monophyletic clade containing *C. albicans*, which all share the characteristic that the CUG codon encodes serine rather than leucine (Fig. 1.1). Reassignment of CUG is an ancient event (Massey et al. 2003), which apparently allowed genome remodeling and adaptation to stress, and may have contributed to virulence (Silva et al. 2007).

The *Candida* clade includes a number of species that are strongly associated with disease, such as *C. albicans*, *C. tropicalis,* and *C. parapsilosis*, that together account for the majority of *Candida* infections (Pfaller and Diekema 2007). Other species, such as *C. lusitaniae* and *C. guilliermondii* are much weaker pathogens, whereas species such as *Lodderomyces elongisporus*, *Debaryomyces hansenii,* and *Pichia stipitis* are rarely, if ever, associated with disease.

The *C. albicans* genome (isolate SC5314) was first reported in 2004 (Jones et al. 2004), and subsequently revised and updated (Braun et al. 2005; van het Hoog et al. 2007). An initial comparison with *S. cerevisiae* suggested that the *C. albicans* genome is enriched for secreted aspartyl proteinases, lipases and high-affinity iron transporters required for obtaining nutrients from the environment (Braun et al. 2005). Subsequent analysis identified the *TLO* family of transcription factors that are specifically enriched in *C. albicans*, and may play a role in virulence (van het Hoog et al. 2007).

Comparative genomic analysis of *Candida* species was greatly advanced by sequencing of a second *C. albicans* isolate (WO-1), together with the genomes of *C. tropicalis, C. parapsilosis, L. elongisporus, C. guilliermondii,* and *C. lusitaniae* (Butler et al, 2009). The genome of *C. dubliniensis*, a weak pathogen and close relative of *C. albicans*, was also sequenced (Jackson et al., submitted). Comparison of the two *C. albicans* isolates, which belong to different population clades (Tavanti et al. 2005), showed that whereas the overall rate of nucleotide polymorphism is similar, there are more extended regions of homozygosity in *C. albicans* WO-1. Similar stretches of homozygosity were observed in the diploid genomes of *C. tropicalis* and *L. elongisporus*, suggesting they may have arisen from passage through a sexual cycle, or through break-induced replication. In contrast, the level of polymorphism in the *C. parapsilosis* genome is more than 70-fold lower than in its close relative *L. elongisporus*. *C. parapsilosis* is a more frequent cause of disease than any of its close relatives (Lockhart et al. 2008). It is possible that a bottleneck occurred in a virulent ancestor of the *C. parapsilosis* population, correlated with the loss of a mating partner (Logue et al. 2005).

1.3.3 Evolution of Gene Families Associated with Virulence

Previous analysis has suggested that some gene families are associated with virulence, particularly in *C. albicans* (Naglik et al. 2004). Comparative analysis of the *Candida* genomes identified three families in particular that are enriched in the strongly pathogenic species *C. albicans*, *C. tropicalis,* and *C. parapsilosis* - the Hyr/Iff family, Als adhesins and the Pga30-like family (Butler et al. 2009). All three families have high numbers of gene duplications and are often found in clusters.

The Hyr/Iff family was originally identified in *C. albicans*, among a set of proteins with shared motifs in their N-terminal domains (d'Enfert et al. 2005). Most family members contain a potential GPI-anchor site, suggesting they are components of the cell wall. The best-characterized member of the family is Hyr1, a gene induced during hyphal development, but not required for the hyphal response (Bailey et al. 1996). Expression of *HYR1* is regulated by the major regulators of the hyphal response, Rfg1, Nrg1, and Tup1 (Kadosh and Johnson 2001; Garcia-Sanchez et al. 2005; Kadosh and Johnson 2005). Iff11, a member of the family with no predicted GPI-anchor, is O-glycosylated and secreted (Bates et al. 2007). A knockout mutant is attenuated for virulence in a mouse model, and

probably affects cell wall organization (Bates et al. 2007). Hyr/Iff-like proteins are found in all members of the *Candida* clade, but are not present in *S. cerevisiae* or its relatives. There are particularly large numbers in *C. albicans* (11), *C. tropicalis* (18), and *C. parapsilosis* (17) (Butler et al, 2009). Families contain a large number of internal repeats, and are rapidly evolving. It is therefore likely that they play a role in host/pathogen recognition.

The Als (agglutinin-like sequence) family has been well characterized in *C. albicans* (Hoyer 2001; Hoyer et al. 2008). There are eight members in the genome, and all contain three general domains. These are an N-terminal domain, relatively poor in N-glycosylation sites, a Ser/Thr rich region likely to be heavily glycosylated, and a domain carrying a large number of internal repeats. The Als family resembles the *FLO* gene family from *S.* cerevisiae and the *EPA* family in *C. glabrata* (Cormack 2004; Kaur et al. 2005). *FLO* genes are required for cell-cell adhesion in *S. cerevisiae* (Guo et al. 2000), and the *EPA* family regulate adherence of *C. glabrata* to host cells (De Las Penas et al. 2003; Domergue et al. 2005). Expression of *EPA* genes is usually silenced, but expression is induced during urinary tract infections due to limitations in nicotinic acid (Domergue et al. 2005).

Als proteins are most likely localized at the cell surface (de Groot et al. 2004), and there is substantial evidence that the family function as adhesins in *C. albicans*. Expression of some family members in *S. cerevisiae* induces adhesion to endothelial and epithelial cells (Gaur and Klotz 1997; Fu et al. 1998), and expression of *ALS3* is required for adhesion of *C. albicans* to host cells (Phan et al. 2007). However, deleting *ALS4* reduces adherence to endothelial but not to epithelial cells (Zhao et al. 2005), and deleting *ALS1* has no effect on adherence of *C. albicans* to epithelial cells (Zhao et al. 2004), suggesting that family members may have different roles. *ALS* genes are also differentially expressed in model systems of disease (Green et al. 2004; Cheng et al. 2005).

ALS genes are also important for adherence to plastic surfaces, and for biofilm development. *ALS3* is expressed at early stages and *ALS1* at late stages of biofilm formation in continuous flow conditions (Nailis et al. 2009). Deletion of *ALS3* results in formation of fragile biofilms in vitro (Zhao et al. 2006), although biofilms formed *in vivo* are unaffected (Nobile et al. 2006). The transcription factor Bcr1 is required for expression of *ALS3*, and deletion of *BCR1* reduces biofilm development (Nobile and Mitchell 2005). Nobile et al. (2008) have suggested that Als1 and Als3 are complementary in function to Hwp1, a cell surface protein required for attachment to epithelial cells (Staab et al. 1999).

Somewhat surprisingly, Als3 has recently been shown to play a role in acquisition of iron from host ferritin (Almeida et al. 2008). The authors showed that only hyphal cells bind ferritin, and that *ALS3* is required for growth on ferritin as a sole iron source. Als3 is often described as the “king” of the family (Hoyer 2001), reflecting its biological importance. It will therefore be of great interest to investigate the role of related proteins in adhesion and iron acquisition in other *Candida* species.

Regulation of expression of *ALS* genes is complex. Expression of *ALS1* and *ALS3* requires Efg1 (Braun and Johnson 2000; Fu et al. 2002). Dissection of the *ALS3* promoter region identified two major regions, one that is required for

hyphal-specific regulation, and one that increases the level of expression (Argimon et al. 2007). Expression is repressed by Nrg1 and Tup1, and to a lesser extent by Rfg1. Expression requires Efg1, Tec1, and Bcr1, with Tec1 acting indirectly via Bcr1 (Argimon et al. 2007). It is therefore likely that a number of signaling pathways converge at the *ALS3* promoter, and possibly also affect expression of other members of the family.

ALS genes were identified in all sequenced members of the *Candida* clade, except for *C. lusitaniae* (Butler et al, 2009). There is a particular expansion in the *C. tropicalis* genome (to 16), and somewhat fewer members (5) in *C. parapsilosis*. It is not clear how important a role this family plays in virulence, as *L. elongisporus*, a relatively non-pathogenic species that is closely related to *C. parapsilosis*, also contains four members. There is also little evidence of differential expression of the family during biofilm development in *C. parapsilosis* (Rossignol et al. 2009). However, in *C. albicans* there is substantial allelic variation among the genes in the Als family, in particular in the length of the internal repeat domain (Lott et al. 1999; Zhao et al. 2003; Hoyer et al. 2008). Allele-specific expression may be important for adhesion, and may therefore vary between isolates.

The role of the GPI-anchored Pga30-like family is relatively unexplored in *C. albicans*. Family members are associated with the cell wall (de Groot et al. 2004; Castillo et al. 2008), and may be important for *de novo* construction (Castillo et al. 2006). There are 12 members in the *C. albicans* genome and 14 in *C. tropicalis*. However, the role of the family in virulence is not clear as both the strong pathogen *C. parapsilosis* and the relatively weak pathogen *L. elongisporus* have six members. Further investigation is required to elucidate the importance of individual family members.

Other families enriched in the more common pathogens (*C. albicans, C. tropicalis, C. parapsilosis, C. lusitaniae,* and *C. guilliermondii*) include ferric reductases, secreted lipases, oligopeptide transporters, and the cytochrome P450 family (Butler et al, 2009). Some of these families were identified in earlier analysis of the *C. albicans* genome (Braun et al. 2005). The lipase family, although relatively understudied, has been associated with virulence in *C. albicans* (Hube et al. 2000; Stehr et al. 2004). Recently, Gacser et al. (2007) have shown that deleting *LIP1* and *LIP2* in *C. parapsilosis* attenuates virulence in a mouse model, and also reduces biofilm formation. This family is, therefore, deserving of further analysis in the *Candida* clade. In addition, the Opt (oligopeptide transport) family is required for growth of *C. albicans* on proteins, and may be necessary for adaptation to host environments (Reuss and Morschhauser 2006).

1.3.4 Analysis of Synteny in Candida Genomes

To aid comparative analyzes in *Candida* species, we created the Candida Gene Order Browser (CGOB; http://cgob.ucd.ie/) (Fitzpatrick et al. in preparation). This incorporates all sequenced *Candida* genomes (*C. albicans*, *C. dubliniensis,*

C. tropicalis, C. parapsilosis, L. elongisporus, P. stipitis, C. guilliermondii, C. *lusitaniae,* and *D. hansenii*), together with *S. cerevisiae*, into a visual gene order browser. The browser is based on a tool developed for analyzes of *S. cerevisiae* and related species (YGOB; (Byrne and Wolfe 2005)).

CGOB combines homology and synteny (gene order) information in "pillars" and "tracks". Sets of homologous genes are stored in CGOB's pillars (Fig. 1.2). Genes from all the *Candida* species were integrated into homology pillars by identifying bi-directional best BlastP hits. We then systematically edited the browser by validating and manually refining each pillar. The syntenic context of each gene is calculated by the CGOB engine, and displayed in horizontal "tracks." The visual display is dynamic, and can be centered on any gene from any species. The annotations assigned in the original publications (*P. stipitis* (Jeffries et al. 2007)*, D. hansenii* (Dujon et al. 2004)*, C. albicans* (Braun et al. 2005)) or in prepublication (http://www.broad.mit.edu/, http:///www.sanger.ac.uk)) were used where possible. A different color palette is used to display chromosomal segments from each species. A change in color within a species indicates a break in gene

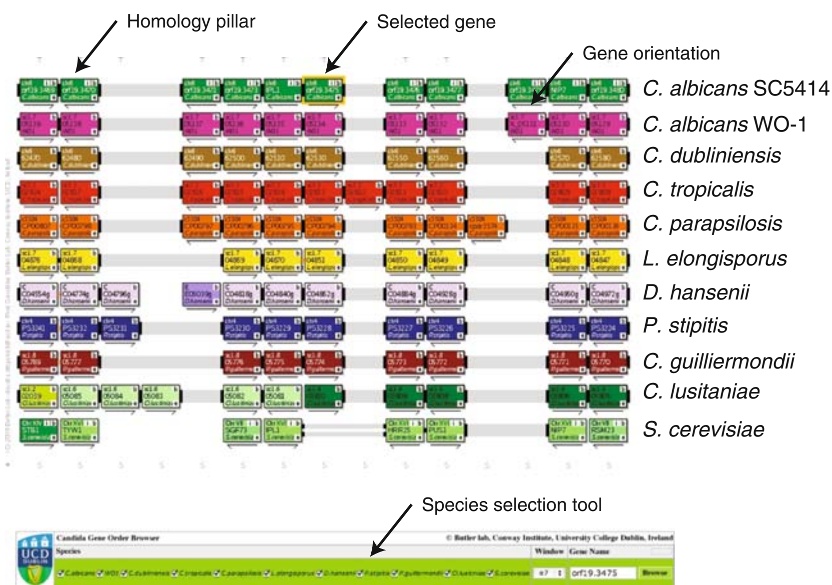

Fig. 1.2 Screenshots from CGOB. The species displayed are selected using the species selection tool at the bottom, and the targeted gene is entered in the search box. Each gene is represented by a box, and each chromosomal segment by a color. A change in chromosomal color (For example in *D. hansenii* and *C. lusitaniae*) indicates a break in synteny. Genes are joined by connectors; a solid bar links adjacent genes, two smaller bars link genes less than 5 loci apart, and an orange bar indicates an inversion. The connectors are extended in gray over regions where there are additional genes in other species. The targeted gene is highlighted with an orange box. The "i" buttons in the *C. albicans* and *S. cerevisiae* tracks link to the Candida Genome Database and the Saccharomyces Genome Database, respectively

order. Nearby genes are also joined by connectors (Fig. 1.2). The information is linked to both the Candida Genome Database (http://www.candidagenome.org) and the Yeast Database (http://www.yeastgenome.org).

CGOB can be used to help identify and confirm gene loss, and gene gain. For example, in the apparently sexual and homothallic yeast *L. elongisporus*, there is no evidence of sequences encoding the a-factor pheromone, or the a-factor receptor Ste3 (Butler et al, 2009). Examining the chromosomal region surrounding *STE3* in the *Candida* species reveals that gene order is generally conserved (Fig. 1.3). The genes surrounding *STE3* are conserved in all the species. However, orthologs of *orf19.2494* (to the left of *STE3* in *C. albicans*) and *orf19.2489* (to the right of *STE3*) are adjacent in *L. elongisporus*. It therefore appears that *STE3* has been lost from *L. elongisporus* only. The gene sequence is not found elsewhere in the genome.

Synteny analysis has been used to support the identification of gene gain and/or replacement by HGT. There are two incidences in the genome of *C. parapsilosis*; in one, a proline racemase gene has been acquired from a proteobacteria, and inserted adjacent to a neutral amino acid transporter (Fitzpatrick et al. 2008) (Fig. 1.4). The insertion occurs at a breakpoint in synteny between the *Candida* species, and may be associated with the presence of a tRNA. The biological consequence of acquiring a bacterial proline racemase is not known. However, HGT can have significant effects on metabolism, such as the ability of *S. cerevisiae* and related species to synthesize biotin (Hall et al. 2005; Hall and Dietrich 2007). In a second example, the ancestral PhzF (phenazine F) gene in *C. parapsilosis* was lost and replaced with a bacterial homolog (Fig. 1.5, Fitzpatrick et al. 2008). Again, the biological significance is not known.

CGOB is much more than a display tool; it can be used to gather information about lineage specific gene loss, gain or amplification, and to identify gene clusters associated with metabolic pathways. It will be used in the future to identify metabolic and genotypic differences between pathogenic and non-pathogenic *Candida* species.

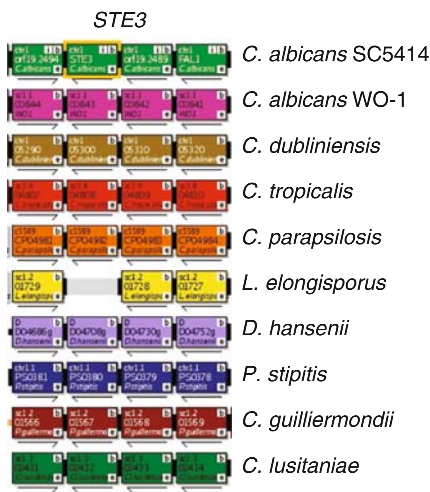

Fig. 1.3 Loss of *STE3* from *L. elongisporus*. The screenshot from CGOB shows the gene order surrounding the *STE3* gene in the *Candida* species. Solid blocks of color indicate that synteny is conserved. However, *L. elongisporus* is missing the *STE3* ortholog

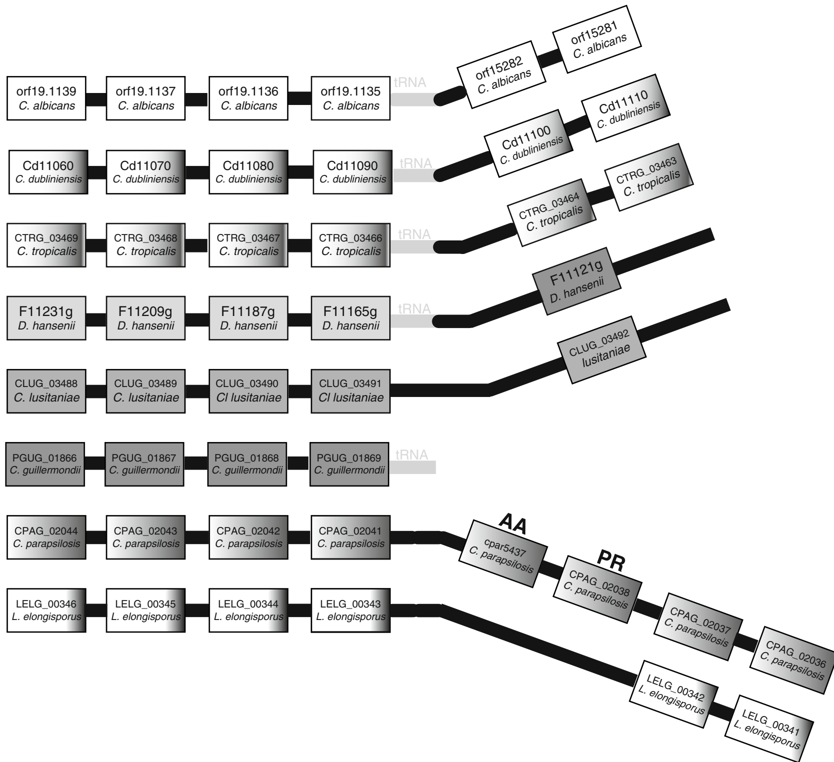

Fig. 1.4 Acquisition of a proline racemase gene in *C. parapsilosis*. The species and gene names are shown in each box, and orthologous genes are stacked in pillars. Synteny is conserved on the left-hand side; on the right hand side syneny between *C. parapsilosis* and *L. elongisporus* is conserved, except for an insertion of a proline racemase (PR) and a neutral amino acid transporter (AA) in *C. parapsilosis* (redrawn from Fitzpatrick et al. (2008))

1.4 Differential Gene Regulation and Evolution of Virulence

It is become increasingly clear that phenotypic variation is not only due to changes in protein coding sequences, but perhaps more importantly, to changes in gene regulation (Wray 2007). For example, transcriptional rewiring resulted in differences in the regulation of ribosomal genes, amino acid biosynthesis, galactose metabolism, and mating between *C. albicans* and *S. cerevisiae*, which may have contributed to adaptation to specific niches (Ihmels et al. 2005a, b; Martchenko et al. 2007; Tuch et al. 2008). In addition, variation in sporulation efficiency among natural isolates of *S. cerevisiae* is associated with nucleotide changes in three transcription factors, *IME1*, *RME1*, and *RSF1*, demonstrating that altered transcriptional regulation can have a major effect on phenotype (Gerke et al. 2009).

Recently, it has been suggested that differences in virulence within isolates of a single species may be attributed to differences in gene regulation (Thewes et al.

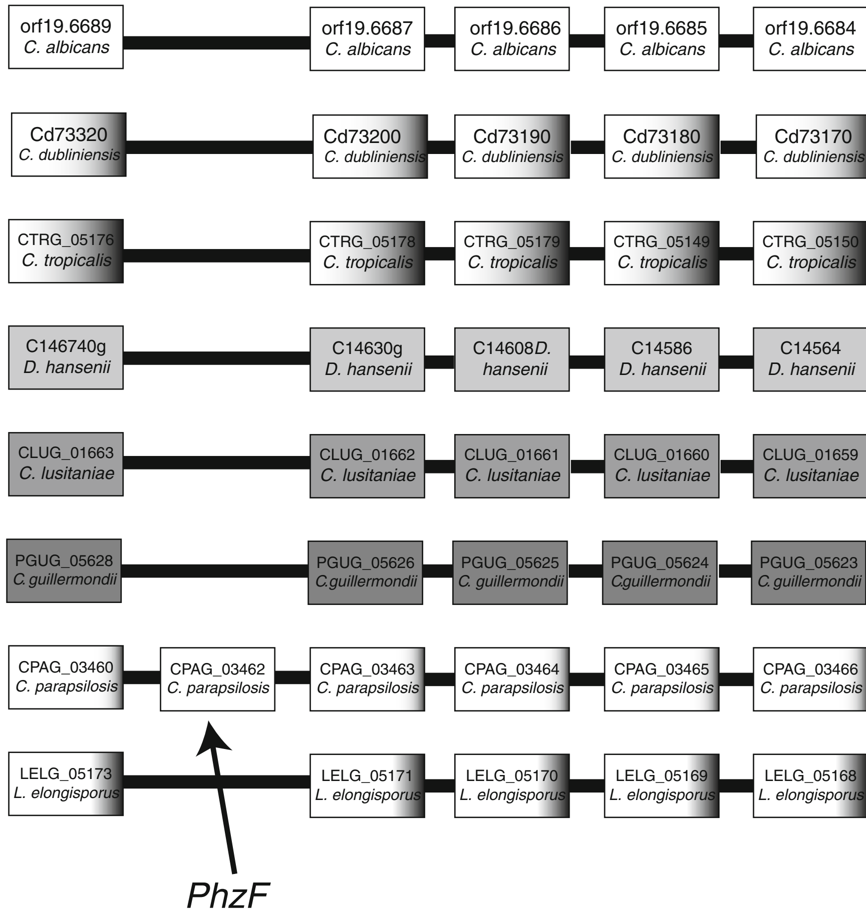

Fig. 1.5 Acquisition of a phenazine F gene in *C. parapsilosis*. The species and gene names are shown in each box, and orthologous genes are stacked in pillars. Synteny is conserved in the *Candida* species, apart from the insertion of a *PhzF* homolog in *C. parapsilosis*. Redrawn from Fitzpatrick et al. (2008)

2008). The authors compared the genomic and transcriptional profiles of two *C. albicans* isolates, one invasive and virulent isolate (SC5314), and one noninvasive and less virulent isolate (ATCC10231). No genomic differences were detected using comparative genome hybridization. However, the transcriptional profile of the two isolates differed substantially (Thewes et al. 2007, 2008). Seventy-nine genes were identified with higher expression in the non-invasive isolate, including genes associated with stress and nitrogen metabolism, and genes of unknown function. It is possible that expression of some genes is not compatible with invasion, and may therefore influence virulence. The genome sequence of *C. albicans* ATCC10231 is not yet available, so it is not possible to determine if

the changes in transcription profile are associated with nucleotide changes in transcription factor coding sequences, as reported in *S. cerevisiae* (Gerke et al. 2009). However, it is clear that comparison of isolates from a single species will provide a very fertile area for future research.

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Chapter 2
Molecular Epidemiology of *Candida* Species

Gary P. Moran, Brenda A. McManus, David C. Coleman, and Derek J. Sullivan

Abstract *Candida* species have increased in importance as opportunistic pathogens over the last 25 years. *C. albicans* is still the major fungal pathogen of humans, however during this period, other previously obscure *Candida* species have emerged as significant pathogens. This increase in infections has created the need for reliable, informative and discriminatory techniques for strain typing in *Candida* species and several molecular techniques have been evaluated for this purpose. In the post-genome era, analysis of sequence polymorphisms has become the method of choice for strain typing in *C. albicans* and multi-locus sequence typing (MLST) has become the standard tool for this purpose. This chapter summarises the main developments in this area in recent years, describing the impact of MLST on our understanding of the epidemiology and population structure of *Candida* species. The potential impact of high throughput, post-Sanger sequencing technologies on the field is also discussed.

2.1 Introduction

Candida albicans is the major fungal pathogen of humans. A normal resident of the oral-gastrointestinal tract, *C. albicans* is an opportunistic pathogen and infection is generally restricted to those with impaired defences or specific immunodeficiencies (Wenzel 1995). Although *C. albicans* is responsible for the majority of yeast infections in humans, several other *Candida* species have also been associated with disease, including *C. dubliniensis*, *C. tropicalis*, *C. parapsilosis*, *C. glabrata* and *C. krusei* (Moran et al. 2002). These species are recovered less frequently from the oral-gastrointestinal tract in healthy individuals and are generally considered less pathogenic than *C. albicans*. However, in the compromised host, these species

G.P. Moran (✉), B.A. McManus, D.C. Coleman, and D.J. Sullivan
Microbiology Research Unit, Division of Oral Biosciences, Dublin Dental School & Hospital, Trinity College Dublin, University of Dublin, Dublin 2, Republic of Ireland
e-mail: gpmoran@dental.tcd.ie

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have become significant pathogens and distinct differences in their epidemiology have been shown due to their unique biology, host specificities and anti-fungal drug susceptibilities (Moran et al. 2002).

Candida infections are generally endogenous in origin, and prior colonisation with the organism is often regarded as one of the major risk factors for candidiasis (Pfaller 1995; Pfaller and Diekema 2007). Colonisation rates are higher in individuals whose mucosal immunity is impaired due to old age, diabetes mellitus or smoking (Lockhart et al. 1998; Manfredi et al. 2002). The balance between colonisation and overt infection is delicate and even discreet changes in the host's normal commensal flora can lead to mucosal infection (Lockhart et al. 1998; Vargas and Joly 2002). Mucosal infection, in the form of pseudomembraneous candidosis (thrush) also commonly occurs when oral or vaginal immunity fails to keep the endogenous yeast population in check. More severe, invasive forms of infection can occur when neutrophil function is impaired or counts are lowered due to immunosuppressive therapy or cancer (Pfaller 1995).

2.1.1 Epidemiology of Candidosis

Candidal carriage is ubiquitous in the human population and superficial infection of the oral mucosa is a common sequela of underlying immunodeficiencies. Oral candidosis is a frequent complication of cancer chemotherapy, diabetes, broad-spectrum antibiotic use and HIV-infection (Manfredi et al. 2002; Sangeorzan et al. 1994; Vargas and Joly 2002). The HIV pandemic resulted in a huge increase in the incidence of mucosal *Candida* infection during the 1990s. However, since the introduction of highly active anti-retroviral therapy (HAART) in the late 1990s, the incidence of oral candidosis in the HIV-infected population has dropped dramatically (Cauda et al. 1999). *C. albicans* is by far the most common cause of mucosal yeast infection, being the sole species recovered from up to 70% of HIV-infected individuals and up to 90% of cases of *Candida* vaginitis (Coleman et al. 1993; Sobel 2007). Other *Candida* species can be recovered alone or co-isolated with *C. albicans* from sites of mucosal infection (Coleman et al. 1995). The significance of non-*C. albicans Candida* species in oral specimens is disputed by some researchers, who have associated their isolation with asymptomatic carriage (Ruhnke 2006). However, some studies have directly implicated non-*C. albicans Candida* species, such as *C. dubliniensis* and *C. glabrata,* with overt symptoms of oral candidosis and have also associated these species with alternative clinical presentations such as erythematous candidosis (Fidel et al. 1999; Sullivan et al. 1993). The emergence of some non-*C. albicans Candida* species in the HIV-infected population in the 1990s may have been a direct result of the widespread use of fluconazole, as *C. glabrata* and *C. krusei* tend to exhibit intrinsic resistance to this agent (Warnock et al. 1988; Wingard et al. 1991).

The epidemiology of invasive *Candida* infection has changed dramatically in the last 30 years (Pfaller 1995). The incidence of these infections has steadily increased

since the 1980s, largely due to the increasing population of immunocompromised patients in our hospitals (Banerjee et al. 1991; Martin et al. 2003). Widespread use of cytotoxic therapies to treat cancer and the use of immunosuppressive drugs in organ transplantation have greatly increased the number of neutropenic patients in intensive care units. Risk factors for infection include cancer, extremes of age, prior colonisation and the presence of intravenous catheters (Pittet et al. 1994; Wenzel 1995). Recently, Martin et al. (2003) analysed the rate of sepsis in hospitals in the USA from 1979 to 2000 and found that the rate of sepsis due to fungal organisms increased by 207%. More recent data based on figures compiled from National Hospital Discharge Survey (NHDS) statistics in the USA indicate a levelling off in the incidence of nosocomial fungal infection, with an incidence of 22–29 infections per 100,000 population in the period 1996–2003 (Pfaller and Diekema 2007). Similar incidences have been reported in Europe and Canada (Pfaller and Diekema 2007). Data indicate that the distribution of species responsible for invasive infection has also shifted during this period. Most reports indicate that the recovery of non-*C. albicans Candida* species from blood cultures has increased relative to *C. albicans* (Nguyen et al. 1996; Pfaller and Diekema 2004). *C. albicans* now only accounts for 50–60% of all species recovered from blood cultures, with *C. glabrata, C. parapsilosis* and *C. tropicalis* making up for the majority of the remaining species (Pfaller and Diekema 2007). The reasons for this shift are unclear, but this may be partly due to the reduced susceptibility of these species to fluconazole, commonly used throughout the 1990s, or to the increase in the numbers of immunocompromised patients susceptible to infection with less virulent species of *Candida* (Moran et al. 2002). *C. glabrata* has a high propensity to develop resistance to azole anti-fungals, whereas *C. krusei* is inherently resistant to fluconazole (Fidel et al. 1999; Samaranayake and Samaranayake 1994). However, increased reporting of infection caused by non-*C. albicans Candida* species may also be the result of recent improvements in isolation and identification methods for *Candida* species.

The distribution of species recovered from blood culture also changes with geography, particularly with regard to *C. parapsilosis*, which is reported as the second most commonly isolated *Candida* species in Latin America and Europe, whereas in North America, *C. glabrata* is the second most significant species (Table 2.1). *C. parapsilosis* is the species most commonly recovered from the hands of health care workers and can often produce a mucoid biofilm, features

Table 2.1 Geographic variations in the recovery of *Candida* species from blood culture

Species	Location[a]			
	USA (%)	Europe (%)	Latin America (%)	Asia-Pacific (%)
C. albicans	51	60	50	56
C. glabrata	22	10	7	10
C. parapsilosis	14	12	16	16
C. tropicalis	7	9	20	14
C. krusei	2	5	2	2

[a]Data taken from Pfaller et al. (2006)

that may account for its high prevalence in catheter-related infections (Levin et al. 1998). *C. parapsilosis* is also particularly associated with infection in neonatal intensive care units (Levy et al. 1998).

2.2 Molecular Epidemiology

One of the goals of molecular epidemiology is to devise reliable, reproducible and informative methods to differentiate between unrelated isolates of the same species for purposes of epidemiological surveillance (Soll 2000). By distinguishing isolates based on phenotypic or molecular properties, one can identify those isolates that are highly likely to be epidemiologically related. These data can allow microbiologists to locate the source of infecting isolates in nosocomial outbreaks, in recurrent infections and inform us on the population structure of the organism in question. Prior to the widespread use of molecular techniques, mycologists relied on phenotypic properties such as morphology, carbohydrate utilisation patterns and serotyping to distinguish between isolates of *C. albicans* (Pfaller et al. 1990). The use of these techniques was hampered by their poor discriminatory power and the inherent phenotypic instability of *C. albicans*. During the 1990s, molecular techniques began to take precedence over phenotypic tests due to their greater discriminatory power. Several molecular typing methods have been applied to *C. albicans,* including multi-locus enzyme electrophoresis (MLEE), Restriction enzyme analysis (REA), karyotype analysis and randomly amplified polymorphic DNA (RAPD) analysis (Sullivan and Coleman 2002). However, of all of the techniques used during this period, Southern hybridisation of genomic DNA with sequences corresponding to dispersed, repetitive elements in the *Candida* genome proved to be the most discriminatory and reliable.

2.2.1 DNA Fingerprinting with Dispersed, Repetitive Elements

Southern hybridisation with the dispersed repetitive element Ca3 has proven to be one of the most informative typing methods available for epidemiological analysis of *C. albicans* (Schmid et al. 1990; Soll 2000). One of the advantages of Ca3 fingerprinting is the ability to digitally compare fingerprint patterns, which allows quantitative analysis of the genetic relationships between isolates (Schmid et al. 1990). However, the drawbacks of DNA fingerprint analysis include the laborious nature of generating the fingerprints and the difficulty of comparing fingerprint data between laboratories. Population studies with the Ca3 probe have identified five major genetic groups, referred to as ‘clades’, in the *C. albicans* population (Pujol et al. 2002). These clades have been termed I, II, III, SA and E and exhibit different geographic specificities and phenotypic traits. Isolates from clades SA and E are recovered predominately from South Africa and Europe, respectively. However,

strains from clade I predominate in all geographical areas (Pujol et al. 2002). Isolates from clade I also exhibit reduced susceptibility to the anti-fungal agent 5-fluorocytosine (5FC). Pujol et al. (2004) found that 73% of clade I isolates were resistant or less susceptible to flucytosine (MIC $\geq 0.5\,\mu g\ ml^{-1}$) whereas only 2% of non-clade I isolates exhibit reduced susceptibility (Pujol et al. 2004). The mechanism of 5FC resistance in this population has been linked to a point mutation (C301T) in the *FUR1* gene encoding phosphoribosyltransfersase (Dodgson et al. 2004). Isolates heterozygous at this locus exhibit reduced susceptibility while those exhibiting high-level resistance are homozygous for this substitution. In parallel to the work of Pujol et al. (2004), Schmid et al. (1999) also identified a group of closely related *C. albicans* isolates that predominate in all geographic areas and could be associated with all forms of disease. Schmid et al. (1999) argued that this group represents a general purpose genotype (GPG) of *C. albicans* that are especially successful at colonising the human host (Schmid et al. 1999). Evidence for how these genetic differences could contribute to virulence was provided by examination of *ALS7* allelic variation (Zhang et al. 2003). *ALS7* is a member of a gene family encoding a group of cell wall proteins called the agglutinin-like sequences (Als) with roles in adhesion (see Chap. 4). The majority of isolates within the GPG cluster had between 14 and 17 copies of a tandem repeat located within the open reading frame, and that these alleles were much less common in strains outside of the cluster. Variation in the number of tandem repeat copies has been associated with changes in Als protein adhesive properties (Oh et al. 2005).

Ca3 fingerprinting has also been used to resolve questions about the source and spread of infecting *C. albicans* isolates. Most individuals harbour their own unique strain of *C. albicans* and commensal isolates and infecting isolates are often genetically indistinguishable (Schmid et al. 1990; Schroppel et al. 1994; Vargas and Joly 2002). Some individuals, particularly HIV-infected patients, may be colonised by more than one strain of *C. albicans* (Vargas and Joly 2002). In cases of recurrent oral or vaginal candidosis, Ca3 fingerprinting has shown that the same strain often persists through different episodes of infection, however replacement of the original strain or the emergence of a closely related genetic variant of the original strain is not uncommon (Lockhart et al. 1996; Schroppel et al. 1994). The latter phenomenon has been termed 'substrain shuffling' or 'microevolution' and was initially identified in isolates recovered from recurrent vaginal infections (Lockhart et al. 1996). In this study, a fragment of the Ca3 probe, termed C1, was shown to be useful in distinguishing between closely related isolates. DNA fingerprinting has also provided evidence for transmission of *C. albicans* strains between sexual partners (Schroppel et al. 1994). Nosocomial transmission of *C. albicans* strains between patients in intensive care units has also been investigated by Ca3 fingerprinting (Marco et al. 1999; Taylor et al. 2003). However, evidence suggests that most infections are endogenous and that transmission of strains from healthcare workers to patients is less common.

Similar repetitive elements have been isolated from other *Candida* species and have been used to generate fingerprint patterns. A *C. dubliniensis* specific probe, Cd25, was described by Joly et al. (1999) that could discriminate two distinct

groups of *C. dubliniensis* isolates, termed Cd25 group I and II. The majority of Cd25 group I isolates (67.6%) were recovered from human immunodeficiency virus (HIV)-infected individuals, whereas the majority of Cd25 group II isolates (70.4%) were from HIV-negative individuals. Subsequent analysis identified a third distinct clade of *C. dubliniensis* isolates (Cd25 group III) recovered from patients in Saudi Arabia and Egypt (Al Mosaid et al. 2005). Interestingly, this clade of *C. dubliniensis* isolates were found to be resistant to 5FC, although the mechanism is so far unknown. David Soll and colleagues have also developed fingerprinting probes for *C. glabrata* (Lockhart et al. 1997) *C. tropicalis* (Joly et al. 1996) and *C. parapsilosis* (Enger et al. 2001).

2.2.2 Multi-Locus Sequence Typing (MLST) of *C. albicans*

In recent years, DNA sequencing has become more affordable and widely available, which has made typing methods that involve characterising DNA sequence polymorphisms more accessible. The most widely used of these techniques is MLST, initially developed for typing pathogenic bacteria (Maiden et al. 1998). The chief advantage of this technology is that data can be stored in databases and is readily accessible by researchers in other locations (Bougnoux et al. 2004). The nature of DNA sequence analysis means that the data are reproducible and unambiguous. MLST is a highly discriminatory method that relies on the analysis of nucleotide sequence polymorphisms within the sequences of PCR-generated fragments (400–500 bp) of 6–8 housekeeping genes (loci) (Odds and Jacobsen, 2008). An outline of the procedure is shown in Fig. 2.1. In haploid organisms, the sequences obtained at each locus are assigned as discreet alleles, and for each isolate the combination of alleles define an allelic profile, or sequence type (ST). While there are four possible variations at each polymorphic locus in haploid species, diploidy presents 10 possible combinations of the bases ATG and C due to potential heterozygosity, thus increasing the potential number of alleles at each locus. In the current schemes available for diploid *Candida* species, heterozygous genotypes are handled by superimposing the IUPAC one letter code on heterozygous bases (e.g. A or G = R, C or T = Y etc). To reflect this heterozygosity, allelic profiles in diploid species are assigned a diploid sequence type (DST). In *C. albicans*, two independent MLST schemes were initially proposed based on the sequences of six (Bougnoux et al. 2002) or eight (Tavanti et al. 2003) loci. Since then, a consensus scheme has been agreed consisting of seven loci for optimised MLST of *C. albicans*; Table 2.2). The choice of genes for MLST analysis is generally restricted to those with housekeeping functions that are subject to stabilising selection, that is the ratio of non-synonymous to synonymous or silent substitutions (dN/dS ratio) in their nucleotide sequence is less than 1.0 (Odds and Jacobsen 2008). However, the choice of loci must obviously provide sufficient sequence diversity to allow high levels of allelic discrimination.

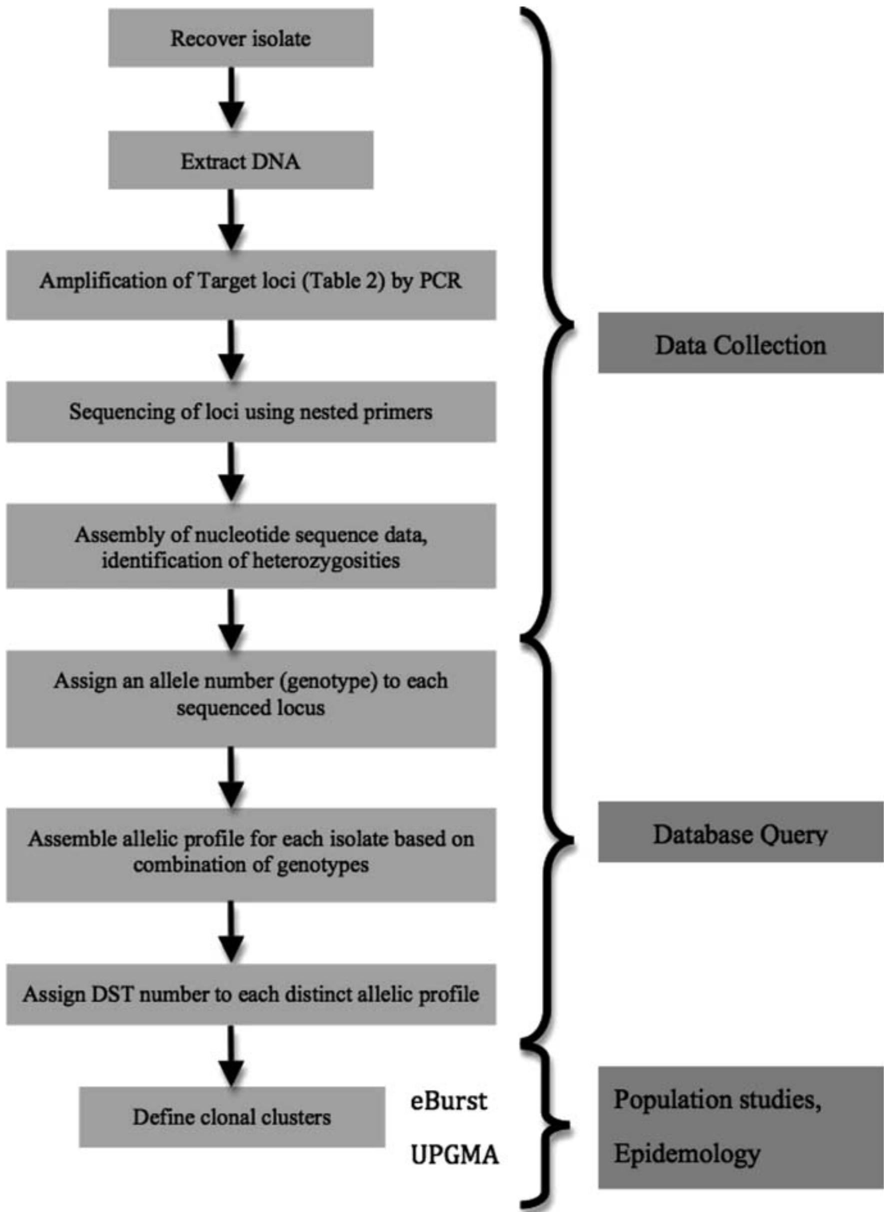

Fig. 2.1 Flow diagram outlining the critical steps in MLST of *Candida* species

Table 2.2 Loci used in the MLST schemes available for analysis of *Candida* species

Species	DSTs/isolates[a]	Gene	Genotypes
C. albicans	1,404/1,771	*AAT1a*	113
http://test1.mlst.net/		*ACC1*	79
		ADP1	93
		PMI1b	85
		SYA1	136
		VPS13	194
		ZWF1b	198
C. dubliniensis[b]	26/50	*AAT1b*	5
		ACC1	4
		ADP1	6
		PMI1b	7
		RPN2	3
		SYA1	5
		exVPS13	4
		exZWF1b	6
C. tropicalis	205/260	*ICL1*	23
http://pubmlst.org/ctropicalis/		*MDR1*	65
		SAPT2	25
		SAPT4	40
		XYR1	74
		ZWF1a	25
C. krusei	99/134	*ADE2*	21
http://pubmlst.org/ckrusei/		*HIS3*	14
		LEU2	17
		LYS2	20
		NMT1	24
		TRP1	24
C. glabrata	70/212	*FKS*	25
http://cglabrata.mlst.net/#		*LEU2*	18
		NMT1	34
		TRP1	23
		UGP1	13
		URA3	20

[a]Data obtained from the Internet MLST database (http://calbicans.mlst.net/) for the relevant species (02/06/09) with the exception of *C. dubliniensis* data, which were obtained from McManus et al. (2008)
[b]Online database not yet available for *C. dubliniensis*

As mentioned above, the great advantage of MLST is that databases of sequences and allelic profiles can be assembled, allowing multiple users to compare data. The consensus *C. albicans* scheme can be queried at http://test1.mlst.net. Here, using a web-based interface, users can assign allele numbers (referred to as ‘genotypes’) to their sequenced loci using a ‘locus query interface’ tool. A second tool, the ‘profile query interface’ can then be used to compare the assembled allelic profile of an isolate to those in the database and to identify isolates with an identical or closely related profile (referred to as the ST in haploid species, or DST in diploids). Users can send details of novel genotypes or sequence types to a database curator for inclusion, permitting rapid expansion of the database.

2.2.2.1 Defining Clonal Clusters by MLST

For epidemiological purposes, different analytical methods can be applied to MLST data for the purpose of defining the relationships between microbes in a population. Traditionally, dendograms based on the unweighted-pair group method with arithmetic mean (UPGMA) technique is widely used for strain typing analysis (Bougnoux et al. 2004). UPGMA analyses MLST data at the level of the individual single nucleotide polymorphisms (SNPs). When applied to *C. albicans* MLST data sets, UPGMA analysis generates dendograms with a clade structure that closely matches that generated by Ca3 fingerprinting (Fig. 2.2) (Tavanti et al. 2005a). Tavanti et al. (2005a) identified four major clades by MLST, referred to as clades 1 to 4, which correspond to clades I, II, III and SA defined by Ca3 fingerprinting. However, isolates from Ca3 clade E were dispersed throughout the MLST clades.

Although useful for visualising population structure, the bifurcating method of lineage splitting implied in dendograms is not a true representation of the way in which microbial lineages emerge and diversify. An algorithm called eBURST was developed, which does not impose a tree-like pattern of descent on population structure (Feil et al. 2004). eBURST compares (D)STs of isolates and gathers isolates that differ at only one of the set of genes sequences (single locus variants, SLVs) into clonal clusters (Fig. 2.3). The model assumes that certain (D)STs will become established in a population and will then diversify by recombination or the accumulation of point mutations resulting in slight variations on the founding genotype. By this model, the microbial population will consist of a series of clonal complexes that can be recognised by the allelic profiles of the strain within the database (Fig. 2.3). Although useful for clustering isolates, BURST analysis of *C. albicans* MLST data highlights large numbers of singletons, that is, isolates that cannot be assigned to a clonal cluster (Odds and Jacobsen 2008). This may be due to the high levels of mitotic recombination in diploid *Candida* species as eBURST analysis is ideally suited to inferring relationships in populations where mutation is the main source of variation.

2.2.2.2 Insights on Population Structure of *C. albicans* from MLST

To date, the largest published MLST study of the population structure of *C. albicans* consisted of 1,391 isolates, most of which (96.7%) could be assigned to one of 17 clades (Odds et al. 2007). Different clades exhibited significant variation in the geographic origins of isolates. However, no association with anatomical source could be identified. As noted in previous studies, reduced susceptibility to azole anti-fungals was associated with homozygosity at the mating type locus (MTL) (Tavanti et al. 2005a). The reason for the association between azole resistance and MTL homozygosity involves the *TAC1* gene, which is located close to the MTL on chromosome 5 (Coste et al. 2006). Mutations in the *TAC1* gene have been identified, that when homozygous, can result in azole resistance. Loss of heterozygosity at the *TAC1* locus is often associated with MTL homozygosity due

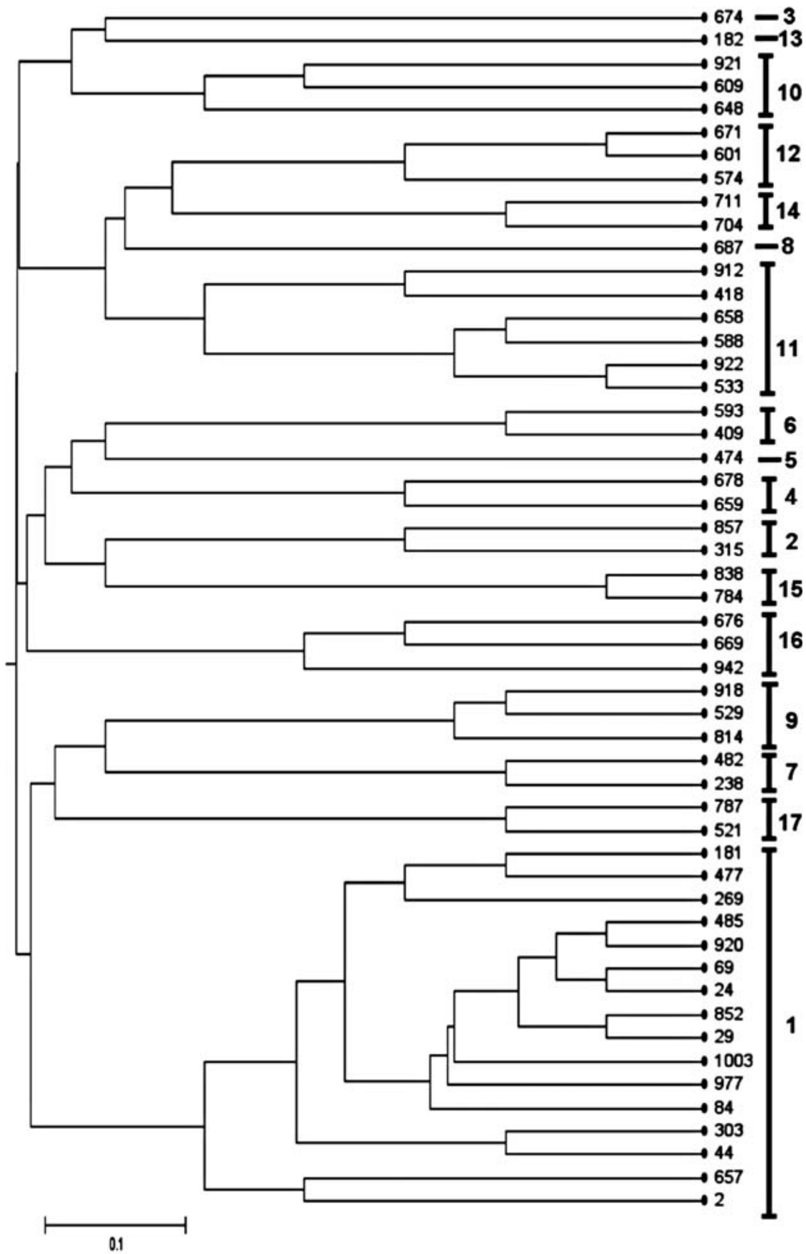

Fig. 2.2 UPGMA dendrogram based on 52 *C. albicans* MLST allelic profiles and their resulting DST numbers. Each of 17 previously defined MLST clades (Odds et al. 2007) are represented and display the genetic relatedness between DSTs in different MLST clades. MLST clades numbers are displayed in bold typeface adjacent to corresponding DST numbers. This dendrogram was generated using START2 software (http://www.ncbi.nlm.nih.gov/pubmed/11751234?dopt=Abstract)

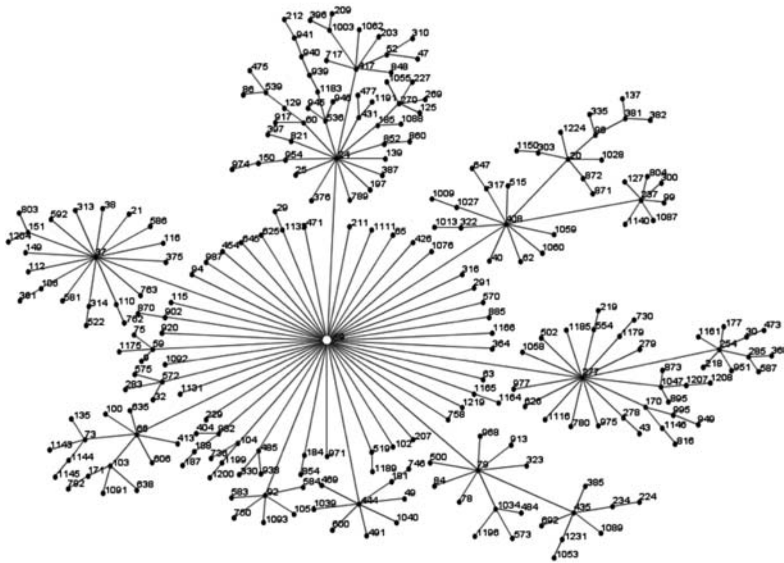

Fig. 2.3 Example of a *C. albicans* clonal cluster generated with eBURST software version 3.0. The primary founder of the clonal cluster (DST 69) is displayed at the centre in white, and DSTs that differ by one of the seven MLST loci (i.e. SLVs) are linked to the primary founder. The lengths of the linkages are not significant. Subgroup founders are also SLVs of DST 69, and are further linked to double locus variants (DLVs) of the primary founder DST

to their close proximity. One of the most striking findings of MLST analysis of *C. albicans* is the tendency of isolates from similar geographic locations to cluster within the same clade (Odds et al. 2007). Clades enriched with isolates from the UK, continental Europe and Asia can be discriminated. However, geographic delineations were not absolute, as would be expected due to movement and migration of human and animal populations. The most common, globally distributed *C. albicans* strain types are those of MLST clade 1. More interesting associations between clade structure and isolate source could be inferred when European isolates were analysed in isolation, thus removing geographical bias from the analysis (Odds et al. 2007). This analysis found that the majority of European clade 1 isolates were commonly associated with commensalism and with superficial infection rather than systemic disease. The ubiquity of these isolates in the human population suggests that they may have evolved characteristics that make them highly efficient colonisers of human mucosal surfaces, perhaps analogous to the 'general purpose genotype' proposed by Schmid et al. (1999). In contrast, clade 4 isolates were significantly enriched with isolates recovered from blood culture (Odds et al. 2007).

C. albicans cells of opposite mating types (i.e. homozygous at the mating locus) have been demonstrated to undergo a process similar to mating in *Saccharomyces cerevisiae* (Hull et al. 2000; Magee and Magee 2000). However, although tetraploid progeny have been generated in vitro and during in vivo experiments, meiosis or

reductive cell division has not been described. The debate on whether natural populations of *C. albicans* undergo mating continues, and MLST has provided significant evidence that mating is, at the very least, extremely rare. MLST can provide data useful for investigating the mating structure of a population. The DST generated by MLST allows one to generate a sequence type for each individual diploid allele, termed the haplotype (Tavanti et al. 2004). Haplotypes allow investigation of allele frequencies in a population and may provide evidence for sexual reproduction. For example, in a sexually reproducing population with random mating, the frequencies of these haplotypes should be in Hardy–Weinberg (H–W) equilibrium due to the random assortment of pairs of haplotypes in diploid cells (Tavanti et al. 2004). Initial analysis of haplotypes generated from *C. albicans* MLST data suggested that the *C. albicans* population may undergo sexual or parasexual reproduction (Odds et al. 2007; Tavanti et al. 2004). Odds et al. (2007) found that some combinations of haplotypes were in H–W equilibrium, providing evidence of chromosomal segregation or intrachromosomal recombination and concluded that although largely clonal, *C. albicans* populations may rarely undergo sexual reproduction (Odds et al. 2007). More recently, Bougnoux et al. (2008) analysed the haplotypes of a larger group of *C. albicans* isolates. This larger group of isolates allowed them to test the hypothesis that mating may only occur in closely related isolates, i.e. between isolates of the same clade. In contrast to other studies, polymorphic nucleotide sites were found to be in H–W disequilibrium with an excess of heterozygotes. The authors concluded that mating within clades in *C. albicans* must be extremely rare. Previous studies have analysed allele frequencies in disparate isolates (i.e. isolates from multiple MLST clades) from different clonal lineages and this may have given the appearance of high levels of recombination, and therefore, mating in the *C. albicans* population. The study of haplotypes also revealed that loss of heterozygosity was a common phenomenon in *C. albicans*, however, selective pressure maintained an excess of heterozygosity. The authors suggest that the excess of heterozygosity is globally maintained as it may mask deleterious alleles and that the maintenance of alternative alleles may confer a selective advantage (Bougnoux et al. 2008).

2.2.2.3 Epidemiological Investigations with MLST

MLST has confirmed much of the existing data regarding strain carriage in *C. albicans*, confirming that strain maintenance, strain replacement and microevolution can occur within an individual (Bougnoux et al. 2006; Odds et al. 2006). Recently, MLST revealed a high incidence of multiple strains of *C. albicans* in samples from healthy individuals (Jacobsen et al. 2008b). Studies have also provided evidence for microevolution through frequent loss of heterozygosity by either chromosome loss or mitotic recombination (Odds et al. 2006).

MLST has recently been applied to analyse nosocomial transmission of *C. albicans* in an intensive care unit in a large UK teaching hospital (Cliff et al. 2008). This study provided evidence for an endemic strain corresponding to DST69,

which was recovered from patients and from health-care workers. However, DST69 is the most common *C. albicans* DST in the MLST database, making it difficult to determine whether transmission of this strain between individuals has occurred in the study, or whether the individuals were coincidentally colonised by the dominant *C. albicans* DST (Cliff et al. 2008). Perhaps a combination of MLST and fingerprinting with the highly discriminatory C1 probe (a fragment of Ca3) could be applied for greater discriminatory power in characterising nosocomial outbreaks.

Recently, Wrobel et al. (2008) applied MLST to examine the genetic relationships between *C. albicans* isolates recovered from humans and non-migratory wildlife in the same geographic locale (central Illinois, U.S.A.). This study found that the clade distribution of human and wildlife isolates was significantly different, indicating limited strain transfer between the two populations (Wrobel et al. 2008). A similar conclusion was reached by Jacobsen et al. (2008a), who found that strains of *C. albicans* from animal hosts were genetically distinct from those recovered from humans (Jacobsen et al. 2008a).

2.3 MLST of Non-*C. albicans Candida* Species

MLST schemes have now been published for all of the major pathogenic *Candida* species, including *C. dubliniensis* (McManus et al. 2008), *C. tropicalis* (Tavanti et al. 2005b), *C. parapsilosis* (Odds et al. 2007), *C. krusei* (Jacobsen et al. 2007b) and *C. glabrata* (Dodgson et al. 2003) (Table 2.2). As *C. dubliniensis* and *C. albicans* are so closely related, the same loci used in the *C. albicans* scheme can be applied to *C. dubliniensis*. However, the level of sequence polymorphism at these loci was found to be significantly less in *C. dubliniensis*, suggesting that the *C. dubliniensis* population is significantly less divergent (McManus et al. 2008). Fewer than 1% of the bases sequenced in *C. dubliniensis* to date exhibit SNPs, compared to 6% of *C. albicans* bases. A scheme consisting of 8 loci, including two loci of extended length (prefixed ‘ex’), have been recommended for maximum discrimination of *C. dubliniensis* isolates (Table 2.2). In an initial study, 50 *C. dubliniensis* isolates were examined and the population structure revealed by MLST confirmed previous findings with the Cd25 fingerprinting probe (Fig. 2.4). However, unlike DNA fingerprint analysis, MLST typing allows one to quantify the level of divergence between *C. albicans* and *C. dubliniensis* populations. By comparing the concatenated sequences for the 8 loci (*AAT1a, ACC1, ADP1, MPIb, SYA1, VPS13, ZWF1b* and *RPN2*) in the recommended *C. dubliniensis* MLST typing scheme, McManus et al. (2008) showed that the two species are separated by 257 bp differences (Fig. 2.4).

Analysis of *C. tropicalis* has revealed a similar clonal population structure to *C. albicans* (Tavanti et al. 2005b). A study that analysed a group of 52 *C. tropicalis* isolates from hospitals in Taiwan identified a clonal cluster consisting of 20 isolates with a high prevalence of reduced fluconazole susceptibility (70%)

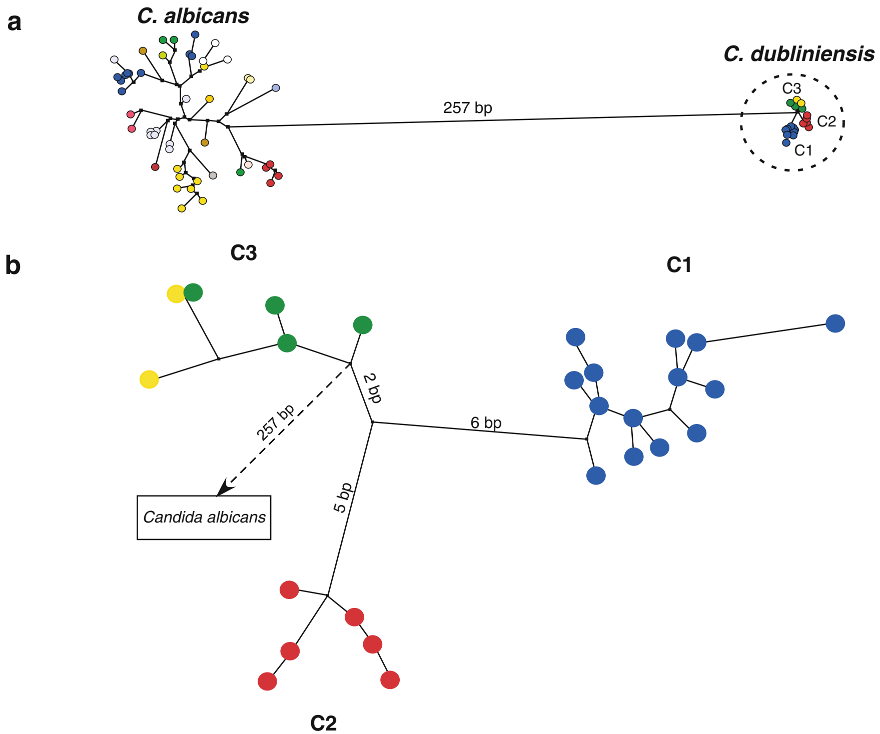

Fig. 2.4 Maximum parsimony tree showing the comparative divergence between 50 isolates each of *C. albicans* and *C. dubliniensis* based on concatenated sequences for the 8 loci (*AAT1a, ACC1, ADP1, MPIb, SYA1, VPS13, ZWF1b* and *RPN2*) in the recommended *C. dubliniensis* MLST typing scheme described by McManus et al. 2008. *C. dubliniensis* isolates were selected from a diverse range of geographic locations and from all four ITS genotypes. *C. albicans* isolates were selected as representatives of the MLST clades described by Odds et al. 2007. Panel (*A*) Comparative divergence between the *C. albicans* and *C. dubliniensis* isolates tested showing that the two species are separated by 257 bp differences. The *C. dubliniensis* isolates formed three closely related groups of isolates or clades (C1–C3). Panel (*B*) shows an enlarged view of the three *C. dubliniensis* major clades encircled in panel (*A*). Clade C1 consists exclusively of ITS genotype 1 isolates, clade C2 consists exclusively of ITS genotype 2 isolates, and clade C3 consists of ITS genotype 3 isolates (*dark grey*) and ITS genotype 4 isolates (*light grey*). Figure adapted from McManus et al. 2008

(Chou et al. 2007). Attempts to generate an MLST typing scheme for *C. parapsilosis* revealed almost a complete absence of DNA polymorphims within isolates of this species, perhaps indicating a very recent evolutionary divergence for this species (Odds et al. 2007). A scheme for the haploid *C. glabrata* has been developed consisting of 6 loci (Dodgson et al. 2003). Analysis of 109 isolates with this scheme revealed a clonal population structure with several clades exhibiting different geographic specificities. In contrast, analysis of six loci in

122 *C. krusei* isolates revealed no evidence for geographical associations with particular subtypes (Jacobsen et al. 2007b).

Analysis of isolates designated as *C. stellatoidea* has revealed interesting findings regarding their position in the current *C. albicans* clade structure (Jacobsen et al. 2007a). *C. stellatoidea* isolates were traditionally identified on the basis of their inability to assimilate sucrose. Type II *C. stellatoidea* are merely sucrose assimilation-negative variants of *C. albicans*. However, the relationship of type I *C. stellatoidea* isolates to *C. albicans* is less clear. Application of the *C. albicans* MLST scheme to four isolates identified as *C. stellatoidea* type I revealed that they clustered with two sucrose negative isolates designated *C. africana* in a group of strains highly distinct from the majority of *C. albicans* strains. These data suggest that *C. stellatoidea* type I may represent a genetically distinct subgroup of *C. albicans* strains (Jacobsen et al. 2007a).

2.4 Future Directions for Typing of *Candida* Species

The *C. albicans* MLST database currently contains data from over 1,500 strains of *C. albicans*. Odds et al. (2007) recently commented that the addition of further *C. albicans* strains to this database is unlikely to reveal anything novel about *C. albicans* population structure. However, the respective databases for the non-*C. albicans Candida* species contain comparatively few strains and continued typing of isolates could reveal new information about the population structures of these species.

At present, MLST is unlikely to be applied to routine screening of clinical isolates as the process is time-consuming and is unlikely to provide data useful to a diagnostic laboratory. Implementation of routine typing for *C. albicans* will depend not only upon the development of cost-effective high-throughput platfoms for SNP analysis but also on the identification of SNPs associated with clinically relevant phenotypic traits (e.g. drug resistance). Microarray technology has the potential for development as a platform for high-through SNP analysis. Lott and Scarborough (2008) recently described an MLST-based SNP microarray for *C. albicans*. The array consisted of oligonucleotide probes specific for 79 SNPs present in 19 discrete loci. One advantage of an array-based platform is the ability to include large numbers of loci without an increase in workload or significant increase in overall cost. The array contains sequences from 12 loci in addition to those in the consensus MLST scheme and includes loci from all 8 *C. albicans* chromosomes. As four of the loci in the consensus MLST scheme are linked (*ADP1* and *ZWF1* on Chr1 and *AAT1* and *PMI1* on Chr2), this leads to a bias in the detection of polymorphisms on these chromosomes. To date, a pilot study analysing 5 isolates has been published and further studies are required in order to determine if this system can offer any new insights into the population structure of *C. albicans* (Lott and Scarborough, 2008). In the future, microarray technology could potentially be useful in the clinical diagnostic laboratory for rapid identification of fungi

from clinical specimens. At present, arrays have been developed for rapid identification of yeasts in clinical specimens using species-specific probes. In order for microarrays to become a routine tool for rapid discrimination of strains in a clinical laboratory environment, they will have to yield clinically relevant data. Arrays have been extensively used to detect anti-microbial resistance genes in prokaryotic organisms and to characterise SNPs associated with anti-malarial drug resistance in *Plasmodium falciparum* (Crameri et al. 2007; Frye et al. 2006.) At present, apart from predicting an isolates susceptibility to 5FC, routine MLST of clinical isolates of *C. albicans* is of little value to a diagnostic laboratory. However, as our knowledge of anti-fungal resistance mechanisms improves in *C. albicans*, SNP arrays may have potential as diagnostic tools in the mycology laboratory. Certain mutations have been identified in *C. albicans* that are associated with azole resistance, including mutations in the transcriptional activators *TAC1* and *MRR1* and in the gene encoding the target of azole anti-fungal drugs, *ERG11*. An array format could potentially be used to screen for SNPs in genes associated with azole resistance. The predictive value of such an array is currently unknown as the full range of potential mutations that can result in azole resistance in *C. albicans* are probably not yet known.

As high-throughput, post-Sanger sequencing technologies improve and become more readily available, the possibility of comparing whole genomes of different strains is becoming feasible. Within a matter of years, epidemiological analysis of *C. albicans* populations will involve whole genome comparisons between strains. At present, next-generation sequencing technologies such as Illumina's Solexa system and the 454 Life Sciences GS FLX system have made the goal of 'a genome in a day' achievable (Medini et al. 2008). However, some technical challenges remain before genome sequencing becomes routine. At present, assembly of genome sequences from such short reads (200-400 bp for the GS FLX) is technically challenging and genomes may have large regions that may be difficult to sequence using these technologies (Medini et al. 2008). In addition, genome comparison software tools are at present unable to efficiently compare a large number of genome sequences simultaneously. Once these technical barriers have been overcome, next generation technologies will allow researchers to compare the genomes of isolates from different clades and to identify specific polymorphisms associated with particular phenotypes, such as drug resistance. Association of polymorphisms with meaningful phenotypes such as virulence or drug resistance may make the task of routine detection of polymorphisms by microarray analysis worthwhile.

2.5 Conclusions

Both DNA fingerprint analysis and MLST have provided useful information regarding the epidemiology and population structure of *C. albicans*. Our knowledge of the epidemiology of the non-*C. albicans Candida* species has also improved. At present, MLST of *C. albicans* has probably yielded as much practical information

as 'low density' genome sequence analysis will allow. Within the next 10 years, our understanding of *Candida* epidemiology will be revolutionised by high-throughput DNA sequencing technologies. Current data suggests that *C. albicans* isolates belonging to different MLST clades may possess different biological properties, with MLST clade 1 isolates more often associated with mucosal colonisation and superficial infection and clade 4 isolates mostly associated with systemic infection. Generation of whole genome sequence data for multiple isolates in different clades may help to explain some of the biological differences between *C. albicans* isolates that MLST analysis can only suggest.

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Chapter 3
Candida Infections and Modelling Disease

Donna M. MacCallum

Abstract *Candida* species are commonly considered harmless commensals, and are isolated from the vagina, mouth and gastrointestinal tracts. When the host-fungus interaction becomes unbalanced, usually due to a change in the host, the fungus is able to initiate infection and cause disease. In the majority of the cases these are superficial mucosal lesions, but in severely ill patients the fungus can enter the bloodstream and cause a disseminated infection. Disseminated *Candida* infections have high mortality rates, usually due to difficulties in diagnosing the infection which leads to delay in the initiation of effective therapy. In the majority of cases, *Candida albicans* is the causative organism, but there is an increased prevalence of non-*albicans Candida* species in some of the patients.

Experimental models play an important role in our attempt to fully understand the development of *Candida* infections and in the development of better antifungal agents and of more effective diagnostics for infection. In this chapter, *Candida* carriage infection and associated species will be discussed. Experimental models of *Candida* infection and their uses will also be discussed.

3.1 *Candida* Species as Commensals

Candida species are commonly found as commensal organisms of the gastrointestinal tract, oral cavity and genital area. In healthy individuals, the asymptomatic oral carriage rate is approximately 40% (Table 3.1), with considerable variation between different studies. A slight increase in carriage rate occurs in both babies and in the elderly. In babies, use of a pacifier was associated with increased oral carriage (Darwazeh and al-Bashir 1995) and in the elderly increased carriage was

D.M. MacCallum
Aberdeen Fungal Group, School of Medical Sciences, University of Aberdeen, Institute of Medical Sciences, Foresterhill, Aberdeen AB25 2ZD, UK
e-mail: d.m.maccallum@abdn.ac.uk

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Table 3.1 Oral carriage of *Candida* species in the general population

Age Group	% Carriage		References
	Mean	Range	
Infants	44.3	8–77	Darwazeh and al-Bashir (1995), Kleinegger et al. (1996), Qi et al. (2005)
Children	37.9	21–70	Kleinegger et al. (1996), Qi et al. (2005), Rozkiewicz et al. (2006), Sanchez-Vargas et al. (2005a), Sanchez-Vargas et al. (2005b)
Adults	40.3	5–69	Belazi et al. (2005), Ben-Aryeh et al. (1995), Bougnoux et al. (2006), Campisi et al. (2002), Carlstedt et al. (1996), Deng et al. (2007), Kleinegger et al. (1996), Liu et al. (2006), Negroni et al. (2002), Qi et al. (2005), Sanchez-Vargas et al. (2005a), Sanchez-Vargas et al. (2005b), Thaweboon et al. (2008), Wang et al. (2006)
Elderly adults	63.8	59–69	Carlstedt et al. (1996), Kleinegger et al. (1996), Wang et al. (2006)

Carriage rates represent the mean values calculated from percentage carriage rates in asymptomatic individuals quoted in recent studies

Table 3.2 Higher *Candida* oral carriage rates are associated with some conditions

Patient Group	% Carriage		References
	Mean	Range	
Dental caries (children)	67	62–71	Rozkiewicz et al. (2006)
Denture prosthetics	71	68–76	Carlstedt et al. (1996), Pires-Goncalves et al. (2007)
Cancer	80	66–100	Davies et al. (2008), Thaweboon et al. (2008)
Diabetes	64	–	Belazi et al. (2005), Pires-Goncalves et al. (2007)
HIV positive	64	29–83	Campisi et al. (2002), Deng et al. (2007), Liu et al. (2006)

Means are calculated from values calculated in recent reports

associated with dental prostheses and xerostomia (dry mouth) (Shimizu et al. 2008). The species most commonly isolated from the oral cavity of healthy individuals is *C. albicans* (~78%) (Belazi et al. 2005; Ben-Aryeh et al. 1995; Campisi et al. 2002; Thaweboon et al. 2008; Wang et al. 2006). The majority of other isolates have been identified as *C. glabrata* and *C. parapsilosis* (Ben-Aryeh et al. 1995; Negroni et al. 2002). Oral *Candida* carriage rates increase in a number of clinical conditions, including diabetes, cancer and HIV-positive status (Table 3.2). Increased oral carriage is also seen in individuals with dental caries (tooth decay) and dental prostheses (Table 3.2).

Estimates of gastrointestinal carriage (from faecal samples) in healthy individuals range from 8 to 77% (Bougnoux et al. 2006; Fong 1994; Knoke 1999; Scanlan and Marchesi 2008). However, gastrointestinal carriage rates may actually be as high as 100%, with the highest *Candida* levels in the duodenum (Kusne et al. 1994). *C. albicans* was isolated in 40–70% of samples, with other isolates identified as *C. parapsilosis*, *C. glabrata, C. tropicalis* and *C. krusei* (Bougnoux et al. 2006; Kusne et al. 1994; Scanlan and Marchesi 2008).

Asymptomatic vaginal carriage of *Candida* species is estimated to occur in approximately 22% of women (Dan et al. 2006; Fong 1994; Pirotta and Garland 2006; Rylander et al. 2004). The majority (~80%) of yeasts isolated are identified as *C. albicans* (de Leon et al. 2002; Grigoriou et al. 2006; Paulitsch et al. 2006; Pirotta and Garland 2006). The other species commonly found in healthy vaginal carriage is *C. glabrata* (Beltrame et al. 2006; Dan et al. 2006). Increased vaginal carriage rates are seen in diabetics and in bed-ridden patients (Dan et al. 2006; de Leon et al. 2002).

3.2 *Candida* Species Associated with Disease

Candida species are capable of causing superficial mucosal lesions in both the oral and vaginal cavity when the balance between host and fungus shifts in favour of the fungus. Predisposing factors for oral and vaginal candidiasis are shown in Tables 3.3 and 3.4.

3.2.1 Oral Candidiasis

Oral candidiasis, commonly known as oral thrush, can be classified into a number of different forms, the most common being acute pseudomembranous and chronic atrophic candidiasis (Richardson and Warnock 1997). Acute pseudomembranous candidiasis occurs in the very young and the very old. The infection appears as white lesions on the cheeks, gums and tongue, which are usually painless. In some cases infection can involve the throat, making swallowing painful (Richardson and

Table 3.3 Risk factors associated with oropharyngeal candidiasis

Risk factor	References
Denture use & xerostomia	Carlstedt et al. (1996), Davies et al. (2008), Pires-Goncalves et al. (2007), Shimizu et al. (2008), Wang et al. (2006)
Dental caries	Rozkiewicz et al. (2006), Wang et al. (2006)
HIV/AIDS	Blignaut (2007), Campisi et al. (2002), Deng et al. (2007), Fong et al. (1997)
Reduced $CD4^+$ T cell counts	Fong et al. (1997), Liu et al. (2006), Vargas and Joly (2002)
Diabetes	Belazi et al. (2005), Guggenheimer et al. (2000), Pires-Goncalves et al. (2007)
Systemic corticosteroid	Davies et al. (2008)
Cancer	Davies et al. (2008)
Age	Kleinegger et al. (1996), Lyon et al. (2006), Qi et al. (2005), Shimizu et al. (2008)
Gender	Lyon et al. (2006)

Table 3.4 Risk factors associated with development of vaginal candidiasis

Risk factor	References
Age under 20	Grigoriou et al. (2006), Paulitsch et al. (2006)
Contraceptive use	Beigi et al. (2004), Cotch et al. (1998), Grigoriou et al. (2006)
Antibiotic treatment	Banerjee et al. (2004), de Leon et al. (2002), Grigoriou et al. (2006), Pirotta and Garland (2006)
Diabetes	de Leon et al. (2002), Grigoriou et al. (2006), Parveen et al. (2008)
Oro-genital sex	de Leon et al. (2002), Reed et al. (2003), Rylander et al. (2004)

Warnock 1997). There is an increased risk of this infection in untreated HIV infection and in cancer patients (Table 3.3).

The most common oral *Candida* infections are chronic atrophic candidiasis cases, more commonly known as denture stomatitis. This infection is often asymptomatic, but redness and swelling under the denture is common. Inflammation in the corners of the mouth is often found associated with this condition (Richardson and Warnock 1997). As suggested by its name, this condition is very common in denture wearers, with approximately 60% suffering from this condition (Daniluk et al. 2006; Figueiral et al. 2007).

In patients with oral candidiasis, a shift in species isolated from the oral cavity can be seen. *C. albicans* remains the most frequently identified species in the mouths of denture wearers with oral candidiasis, but is now reduced to 58% of isolates (Lyon et al. 2006; Pires-Goncalves et al. 2007). Other species that are identified from this patient group include *C. parapsilosis*, *C. tropicalis*, *C. glabrata* and *C. krusei* (Lyon et al. 2006; Pires-Goncalves et al. 2007). *C. albicans* also remains the most frequent species that is identified in HIV-positive and AIDS patients (65%), but is again reduced in frequency when compared to healthy carriers. Other species, namely *C. glabrata*, *C. dubliniensis* and *C. krusei* (Blignaut 2007; Deng et al. 2007) are found in higher frequencies in this patient group. An exception to this species shift associated with oral candidiasis was found in patients undergoing radiation therapies for cancer. In this case, *C. albicans* isolates represented 86% of samples (Thaweboon et al. 2008).

3.2.2 Vaginal Candidiasis

Vaginal candidiasis, or vaginal thrush, occurs in approximately 30% of women (Corsello et al. 2003; Machalski et al. 2006), and represents approximately 12% of all vaginitis cases (Grigoriou et al. 2006). It has been estimated that 75% of all women will experience at least one episode of vaginal candidiasis during their lifetime (Fidel and Sobel 1999). Recurrent vulvovaginal candidiasis (RVVC), where multiple episodes occur within twelve months, is estimated to affect ~8% of women (Corsello et al. 2003; Grigoriou et al. 2006; Paulitsch et al. 2006). Symptoms of vaginal candidiasis include itching (pruritis), vaginal discharge,

soreness and pain during sexual intercourse (Grigoriou et al. 2006). The majority of yeasts isolated from vaginitis samples are identified as *C. albicans* (82%) (Corsello et al. 2003; Paulitsch et al. 2006), with *C. glabrata* and *C. krusei* also found. However, in some population groups there is a shift towards the other species, such as found in bed-ridden elderly patients and Type II diabetics, where the frequency of *C. glabrata* increases to 52% and 54% respectively (Dan et al. 2006; de Leon et al. 2002). Some evidence has been found to link gastrointestinal carriage of *Candida* with likelihood of vaginitis, where higher levels of rectal carriage of *Candida* were found during episodes of vaginitis; however, these levels returned to normal between episodes (Knoke 1999).

3.2.3 Disseminated Candidiasis

Candida species are a common cause of bloodstream infection, occurring in approximately 3 people per 100, 000 population (Table 3.5), and causing approximately 11% of all bloodstream infections (Markogiannakis et al. 2008; Orsi et al. 2006; Sarvikivi et al. 2008). *Candida* bloodstream infections are listed as the third or fourth most common cause of bloodstream infection (Markogiannakis et al. 2008; Orsi et al. 2006; Sarvikivi et al. 2008).

Candida disseminated infections, where fungal cells are found within the internal organs and/or in the bloodstream, are associated with patients who are severely ill. The major risk factors associated with development of a disseminated infection are admission to ICU, presence of an in-dwelling catheter, antibiotic therapy and surgery (Table 3.6). In recent reports, the mortality rate associated with *Candida* bloodstream infections has been estimated to be between 5 and 82%, with an average mortality rate of 44% (Acar et al. 2008; Colombo et al. 2007; Dimopoulos et al. 2008; Falagas et al. 2006; Garey et al. 2006; Kibbler et al. 2003; Pasqualotto et al. 2006; St-Germain et al. 2008). The most common symptom of disseminated candidiasis is fever, with diagnosis made by detection of fungi in the blood or from sterile sites.

The majority of isolates from disseminated *Candida* infections are identified as *C. albicans* (~50% of isolates) (Acar et al. 2008; Badran et al. 2008; Costa-de-Oliveira et al. 2008; Franca et al. 2008; Hinrichsen et al. 2008; Holley et al. 2009; Odds et al. 2007; Sandven et al. 2006; St-Germain et al. 2008; Swinne et al. 2009). However, in a number of studies from India and Singapore, the most common

Table 3.5 Incidence of disseminated *Candida* infection

Incidence	References
1.8 per 1,000 hospital admissions	Anunnatsiri et al. (2009), Celebi et al. (2008), Colombo et al. (2007), Costa-de-Oliveira et al. (2008), Franca et al. (2008), Hinrichsen et al. (2008), Presterl et al. (2007), Schelenz and Gransden (2003)
1.3 per 1,000 patient days	Laupland et al. (2005), Odds et al. (2007), Sandven et al. (2006)
3 cases per 100, 000 population	Acar et al. (2008), Fridkin et al. (2006), Sarvikivi et al. (2008)

Table 3.6 Risk factors predisposing individuals to disseminated candidiasis

Risk factor	References
Intravascular (IV) catheters	Bassetti et al. (2006), Chang et al. (2008); Cheng et al. (2005b), Costa-de-Oliveira et al. (2008), Dimopoulos et al. (2008), Franca et al. (2008), Garey et al. (2006), Hachem et al. (2008), Holley et al. (2009), Kibbler et al. (2003), Odds et al. (2007), Pasqualotto et al. (2007), Sarvikivi et al. (2008), Schelenz and Gransden (2003), Shivaprakasha et al. (2007), St-Germain et al. (2008), Swinne et al. (2009)
Admission to intensive care unit (ICU)	Celebi et al. (2008), Cheng et al. (2005b), Franca et al. (2008), Odds et al. (2007), Sarvikivi et al. (2008), Schelenz and Gransden (2003), Shivaprakasha et al. (2007), St-Germain et al. (2008), Swinne et al. (2009)
Surgery (including organ transplantation)	Bassetti et al. (2006), Chow et al. (2008), Colombo et al. (2007), Costa-de-Oliveira et al. (2008), Jorda-Marcos et al. (2007), Laupland et al. (2005), Playford et al. (2008), Sarvikivi et al. (2008), Schelenz and Gransden (2003), Shivaprakasha et al. (2007), St-Germain et al. (2008), Swinne et al. (2009)
Antibiotic therapy	Bassetti et al. (2006), Celebi et al. (2008), Chang et al. (2008), Franca et al. (2008), Morrell et al. (2005), Odds et al. (2007), Schelenz and Gransden (2003), Shivaprakasha et al. (2007), St-Germain et al. (2008), Swinne et al. (2009)
Parenteral nutrition	Celebi et al. (2008), Chow et al. (2008), Costa-de-Oliveira et al. (2008), Franca et al. (2008), Hartung de Capriles et al. (2005), Jorda-Marcos et al. (2007), Schelenz and Gransden (2003), St-Germain et al. (2008)
Immunosuppression (including neutropenia)	Anunnatsiri et al. (2009), Cheng et al. (2005b), Hachem et al. (2008), Pasqualotto et al. (2006), Schelenz and Gransden (2003)
Cancer	Bassetti et al. (2006), Garey et al. (2006), Laupland et al. (2005), Schelenz and Gransden (2003), Shivaprakasha et al. (2007), Swinne et al. (2009)
Corticosteroid therapy	Cheng et al. (2005b), Dimopoulos et al. (2008), Pasqualotto et al. (2006), Schelenz and Gransden (2003)
Haemodialysis	Chow et al. (2008), Jorda-Marcos et al. (2007), Laupland et al. (2005)
Abdominal condition	Chow et al. (2008), Odds et al. (2007), Playford et al. (2008), Shivaprakasha et al. (2007)
Mechanical ventilation	Acar et al. (2008), Anunnatsiri et al. (2009), Celebi et al. (2008), Shivaprakasha et al. (2007)
Diabetes	Acar et al. (2008), Garey et al. (2006)
Low birth weight	Fridkin et al. (2006)
Prior *Candida* colonisation	Jorda-Marcos et al. (2007)

species was *C. tropicalis* (Chai et al. 2007; Shivaprakasha et al. 2007; Xess et al. 2007). A shift in *Candida* species is also evident in certain patient groups. Patients with haematological malignancies are more likely to have bloodstream infections caused by non-*albicans Candida* species, particularly *C. tropicalis*, *C. parapsilosis* and *C. krusei* (Hachem et al. 2008; Pasqualotto et al. 2006; Presterl et al. 2007; Vigouroux et al. 2006). This shift in species prevalence also occurs in children, where the most commonly identified species are *C. tropicalis* and *C. parapsilosis*,

and where *C. albicans* isolates are associated with only around one fifth of infections (Pasqualotto et al. 2007; Saha et al. 2008). In babies, *C. albicans* remains the most frequent cause of disseminated infection, with fewer *C. glabrata* infections and increased numbers of *C. parapsilosis* and *C. tropicalis* infections (Badran et al. 2008; Fridkin et al. 2006; Hartung de Capriles et al. 2005). This shift in species has been commented on previously, where *C. glabrata* prevalence was linked with age and *C. tropicalis* was linked with youth (Weinberger et al. 2005).

3.3 Modelling *Candida* Carriage and Infection

In order to widen our understanding of these fungal infections and to allow the development of better diagnostic tools and therapies, experimental models of infection are required. These allow us to investigate fungus-host interactions, disease development and efficacy of antifungal therapy. Researchers usually either take a reductionist approach to study *Candida* infection, using single cell types or tissues, or employ a whole animal system. Since *C. albicans* remains the species that is most commonly identified in carriage and disease, the majority of infection models centre on this species.

3.3.1 Experimental Models of Candida Infection: The Reductionist Approach

The reductionist approaches to study *Candida* infection are performed *in vitro* and involve fungal cells interacting with a single cell type, reconstituted human epithelial (RHE), or tissue explants. These models have generated a huge volume of literature on *Candida*-host interactions. These simple systems have allowed the receptor-ligand interactions to be elucidated and have allowed the responses of both fungal and host cells during their interaction to be characterized. Some of the systems used and the information found using these models are described below.

3.3.1.1 Cell-Based Models

The vast majority of research has focussed on immune cell interactions with *C. albicans*, although epithelial and endothelial interactions have also been investigated.

Monocyte–*Candida* Interactions

Monocytes function to move to the sites of infection, and then differentiate into macrophages or dendritic cells to elicit an immune response. Monocytes are

phagocytic cells, with fungicidal activity (Netea et al. 2004). However, while yeasts and hyphae are equally and efficiently killed by monocytes, cytokine production in response to the two different morphologies differs (Chiani et al. 2000; Liu et al. 2001; Torosantucci et al. 2000), with hyphal cells stimulating lower levels of several chemokines (Torosantucci et al. 2000) and IL-12 (Chiani et al. 2000; Liu et al. 2001).

Expression of proinflammatory cytokines and chemokines is induced in monocytes within the first 6 hours of interaction with *C. albicans* (Kim et al. 2005). Stimulation of cytokine production has been used as a measure of the virulence potential of *C. albicans* strains and mutants (e.g. Netea et al. 2006).

Examination of cytokine production induced by specific *C. albicans* cell wall mutants has also allowed the host-fungal cell recognition mechanisms to be defined. β-glucan is hugely important in monocyte function, believed to be the main inducer of chemokine production (Torosantucci et al. 2000) and induces monocytes to differentiate into dendritic cells (Nisini et al. 2007). Production of cytokines by monocytes depends upon the recognition of fungal β-glucan by dectin-1, in combination with TLR2 and TLR4 (Ferwerda et al. 2008; Gow et al. 2007; Torosantucci et al. 2000). Monocytes also affect the ability of *C. albicans* cells to produce biofilms, enhancing their formation (Chandra et al. 2007).

Further insights into the consequences of monocyte-fungal cell interaction have been obtained by examining gene expression changes occurring during their interaction, both for the fungus (Rubin-Bejerano et al. 2003) and for monocytes (Barker et al. 2005; Fradin et al. 2006; Kim et al. 2005).

Macrophage–*Candida* Interactions

Macrophages are another phagocytic cell type with fungicidal activity (Netea et al. 2004) which have a vital role in the clearance of infection (Molero et al. 2005). Proteomic and genomic approaches have been used to analyse responses of the fungus and of the macrophage during interaction (Barelle et al. 2006; Fernandez-Arenas et al. 2007; Lorenz et al. 2004; Y. K. Shin et al. 2005; Yamamoto et al. 1997). *C. albicans* cells have been shown to be starved within the macrophage, with downregulation of carbon metabolism and upregulation of the glyoxylate pathway (Barelle et al. 2006; Fernandez-Arenas et al. 2007; Lorenz et al. 2004). The macrophage responds to the interaction by inducing expression of cytokines and chemokines (Yamamoto et al. 1997).

Macrophage killing assays have been used to estimate virulence of *C. albicans* strains (Corbucci et al. 2007; Tavanti et al. 2006). Anti-*Candida* activity of macrophages occurs via TLR2 (Blasi et al. 2005) and galectin-3 (Kohatsu et al. 2006). Both these molecules, as well as the macrophage-inducible C-type lectin, mincle, dectin-1, SIGNR1 and the mannose receptor are involved in the recognition and phagocytosis of *C. albicans* by macrophages (Bugarcic et al. 2008; Heinsbroek et al. 2005; Jouault et al. 2006; Taylor et al. 2004). Dectin-1 has been shown to be involved only in the recognition of yeasts, and not hyphal cells, by macrophages (Heinsbroek et al. 2005).

Recognition of *C. albicans* by macrophages and other immune cells has recently been reviewed (Netea et al. 2006; Netea et al. 2008). Again, *C. albicans* mutant strains have assisted in determining which receptor interactions produced specific outcomes (Netea et al. 2006). Cytokine production by macrophages depends upon signalling via TLR2, TLR4 and dectin-1 (Blasi et al. 2005; Dennehy and Brown 2007; Ferwerda et al. 2008; Gow et al. 2007; Murciano et al. 2007). Signalling via the TLR pathway leads to tissue macrophage production of KC and MIP-2 which are neutrophil chemoattractants (De Filippo et al. 2008).

Neutrophil–*Candida* Interactions

Neutrophils are involved in phagocytosis and extracellular killing of *C. albicans* cells, playing an essential role within the first three days of infection (Molero et al. 2005). Phagocytosis and killing assays with neutrophils are used to assay the virulence of *C. albicans* strains (Corbucci et al. 2007; Fu et al. 2008). Neutrophils differ from other phagocytic cells as they can form neutrophil extracellular traps (NETs), which allow them to kill both yeast and hyphal cells (Urban et al. 2006). Transcript profiling during the interaction of neutrophils and *C. albicans* demonstrated that no active transcription programme was required by neutrophils to mount an attack on the fungus (Fradin et al. 2006). However, neutrophils determine the main transcriptional response of whole blood (Fradin et al. 2005), reflecting the results of a whole blood phagocytosis assay which gives similar results to assays using fractionated neutrophils (Pattanapanyasat et al. 2007). *C. albicans* cells engulfed by neutrophils induce gene expression of pathways suggestive of starvation (Barelle et al. 2006; Rubin-Bejerano et al. 2003) and also of oxidative stress (Enjalbert et al. 2007). Neutrophil functions are mediated by β-glucan (Lavigne et al. 2006), particularly β-1,6-glucan (Rubin-Bejerano et al. 2007), which is recognized by dectin-1 (Kennedy et al. 2007).

Dendritic Cell–*Candida* Interactions

Dendritic cells are involved in phagocytosis, killing, processing and antigen presentation, although they are less successful *C. albicans* phagocytes when compared to macrophages and neutrophils (Netea et al. 2004; Newman and Holly 2001). Immune responses during dendritic cell–*Candida* interactions have been recently reviewed (Filler 2006; Pepe et al. 2006).

DC-SIGN is a receptor involved in *C. albicans* uptake by dendritic cells (Cambi et al. 2008). The mannose receptor and dectin-1 are also involved in *Candida* uptake (Cambi et al. 2008; Donini et al. 2007; Romani et al. 2004). Binding of *N*-mannan (Cambi et al. 2008) to the mannose receptor led to type I responses and repression of oxidative mechanisms (Donini et al. 2007; Romani et al. 2004), but uptake via dectin-1 led to induction of oxidative activity (Donini et al. 2007). Uptake via FCγ receptors has been shown to suppress type I responses, leading to

induction of type II responses and associated pathology (Romani et al. 2004). TLR2 is involved in induction of type II responses (Re and Strominger 2001), with signalling via TLR4 producing conditions leading to type I responses and activation of the dendritic cells (Re and Strominger 2001) due to signalling via mannoprotein (Pietrella et al. 2006).

Epithelial Cell– and Endothelial Cell–*Candida* Interactions

Epithelial and endothelial cells have been used extensively in attempts to understand *Candida* virulence and disease development (reviewed in Filler and Sheppard (2006)). Their interactions have been shown to be key steps during pathogenesis (Grubb et al. 2008), with oral (Steele et al. 2000) and vaginal (Steele et al. 1999) epithelial cells shown to have anti-*Candida* activity. Monolayers of oral and vaginal epithelial cells have been utilised to examine cytokine production following interaction with, and invasion by, *C. albicans* cells (Lilly et al. 2006; Steele and Fidel 2002; Villar et al. 2005), and have been used extensively to investigate the virulence potential of different *C. albicans* strains and mutants (e.g. Badrane et al. 2005; Bensen et al. 2002; Fu et al. 2008; Martinez-Lopez et al. 2006; Nobile et al. 2008; Phan et al. 2007; Zhao et al. 2007a; Zhao et al. 2007b). Increased adherence of *C. albicans* to oral epithelial cells from AIDS patients (Schwab et al. 1997) has also been seen, which may help to explain the increased incidence of oral candidiasis in this patient group.

Endothelial cells have been used for similar purposes, having been characterized for their production of cytokines in response to interaction with *C. albicans* cells (Villar et al. 2005), and to examine the ability of *C. albicans* mutant strains to adhere to and invade endothelial cells (e.g. Bensen et al. 2002; Martinez-Lopez et al. 2006; Zhao et al. 2007a; Zhao et al. 2007b). Mutants found to be deficient in their ability to damage endothelial cells *in vitro* are generally less virulent *in vivo* (Sanchez et al. 2004). This system has also been utilised to examine gene expression of *C. albicans* (Sandovsky-Losica et al. 2006) and of the endothelial cells (Barker et al. 2008; Filler et al. 1996; Muller et al. 2007) during their interaction. The use of single cell monolayers has also allowed individual molecules involved in fungal cell endocytosis to be defined; involving *N*-cadherin on the endothelial cell (Phan et al. 2005) and (agglutinin-like sequence) Als3p on the fungal cell (Phan et al. 2007).

3.3.1.2 Reconstituted Human Epithelial Models (RHE) and Tissue Explants

In attempts to allow the investigation of interactions occurring in multi-layer tissues, such as mucosa, SkinEthic Laboratories (http://www.skinethic.com/) have developed several Reconstituted Human Epithelial (RHE) models. The most commonly used RHE models for *Candida* infections are those of oral and vaginal mucosa (Schaller et al. 2006). The main uses of these systems have been to examine mucosal gene expression in response to interaction with *Candida* cells (Schaller

et al. 2005), measurement of *C. albicans SAP* (encoding secreted aspartyl proteases) and *ALS* (encoding agglutinin-like sequence proteins) gene expression levels during interaction/invasion of RHE (Cheng et al. 2005a; Green et al. 2004; Naglik et al. 2008b; Schaller et al. 2003) and comparison of *C. albicans* strains ability to invade RHE (Li et al. 2002; Schaller et al. 2003). However, the system has also been used to identify genes required for invasion of epithelial layers (Zakikhany et al. 2007). The system also has the advantage that the additional cells can be added, such as neutrophils, to examine their influence on *Candida*-mucosa interactions (Weindl et al. 2007).

An example of a tissue explant model, that has been used to examine the effect of gene knockouts in *C. albicans* is the murine colon explant model. This system uses sections of mouse colon *in vitro* to examine interaction of fungus with colon mucosa (Bareiss et al. 2008).

3.3.2 Whole Animal Approaches

Whole animal approaches to investigate *Candida* carriage and infection have the advantage that events in multiple organs can be studied and that movement of molecules and cells within the body is possible. This allows the entire immune respond during disease development which is to be studied.

To be of real use in studying *Candida* infections, models should be reproducible, produce similar symptoms and disease progression to the human infection of interest and be easy and cost-effective to perform.

3.3.2.1 Invertebrate Models

A number of invertebrate models have been utilised to test virulence of *Candida* strains, including *Drosophila melanogaster* (Alarco et al. 2004; Chamilos et al. 2006), *Galleria melonella* (Brennan et al. 2002; Dunphy et al. 2003) and *Caenorhabditis elegans* (Breger et al. 2007; Peleg et al. 2008). These models are cheap and simple to perform, and work as a screen to identify *C. albicans* mutants with defective interactions with the innate immune system. However, one drawback of these models is that the innate immune system differs in invertebrates and mammals. These models also do not reflect the patient situation since there is no requirement for any host intervention prior to infection. These models are, hence, not of direct relevance to the clinical situation and do not allow us to investigate infection development as it occurs in mammalian hosts.

3.3.2.2 Vertebrate Models

A number of reviews describing various experimental models of *Candida* infection have been published recently (Capilla et al. 2007; de Repentigny 2004; Naglik et al.

2008a; Samaranayake and Samaranayake 2001). The majority of animal models, like the situation in human hosts, require at least one predisposing factor to be present to allow colonisation and infection to initiate and progress. In setting up animal models of *Candida* infections of interest, we want to take into account the predisposing factors in the human host and see if we can use these to help us establish a similar infection in our experimental model.

Mucosal Models

Mucosal models of carriage and disease cover infections of the oral cavity, the gastrointestinal tract and the vagina (recently reviewed by Naglik et al. 2008a).

Models of Oral and Gastrointestinal Carriage and Infection

A huge research effort has concentrated on the development of experimental models of oral commensalism and disease. An extensive review of models available has been published (Samaranayake and Samaranayake 2001), with the majority of models developed in rodents; mostly rats and mice.

In oral infection protocols, *C. albicans* is administered either in food (Samonis et al. 1990), in drinking water (Narayanan et al. 1991; Sofaer et al. 1982; Taylor et al. 2005), by intra-oesophageal injection (Ishibashi et al. 2007) or applied directly to the surfaces of the oral cavity (Takakura et al. 2003), often in gnotobiotic animals (Schofield et al. 2003). Administration of differing inoculum levels leads either to the clearance of infection, colonisation or dissemination of infection (see below for disseminated infections from gastrointestinal infection) (Samonis et al. 1990). Addition of immunocompromising agents, such as cyclophosphamide or corticosteroids, increased infection, as measured by increased fungal burdens (Cole et al. 1989; Hu et al. 2007; Kamai et al. 2001). Antibiotic therapy and anti-cancer treatments also increased fungal burdens (Clemons et al. 2006; Samonis et al. 2008; Sandovsky-Losica et al. 1992). This is similar to the situation in the human host (Table 3.3). Immunocompromising drugs, antibiotics and anti-cancer drugs could lead to dissemination depending upon the drug concentration used, with *C. albicans* found in the liver, kidneys and spleen (Clemons et al. 2006; Cole et al. 1989; Sandovsky-Losica et al. 1992). However, incidence of disseminated infection was very variable, as were fungal burdens in the organs (Clemons et al. 2006; Sandovsky-Losica et al. 1992). Other models of oral candidiasis include an HIV-transgenic mouse model (de Repentigny et al. 2004) and a rat hyposalivatory model (Meitner et al. 1990).

Models of oral *Candida* infection have determined that $CD4^+$ and $CD8^+$T cells, both have roles in the immune response to infection (Farah et al. 2001; Marquis et al. 2006). Defects in natural killer (NK) cells, T cells and B cells also predispose to increased oral infection (Narayanan et al. 1991; Taylor et al. 2005). There is, however, no evidence for humoral protection in oral infection (Farah and Ashman 2005) and complement deficiencies have no effect (Ashman et al. 2003).

A comparison of immunocompetent and immunosuppressed mice also demonstrated that *C. albicans* gene expression remains unchanged in these two situations, suggesting that the host factors determine susceptibility of infection (Schofield et al. 2003). Villar and Dongari-Bagtzoglou (2008) have recently reviewed immune defense mechanisms involved in oral candidiasis.

C. albicans mutant strains and clinical isolates have been assayed for virulence in experimental models of oral infection (Badrane et al. 2005; Holbrook et al. 1983; Nobile et al. 2008) demonstrating the requirement for yeast-hyphal switching in initiation of infection (Nobile et al. 2008). *C. albicans* gene expression has also been examined during infection development, showing similarities to expression in clinical samples (Green et al. 2006; Stehr et al. 2004).

Models of Vaginal Carriage and Infection

Models of vaginal candidiasis are mainly performed in mice and rats. Both species require oestrogen administration to maintain the animals in pseudo-oestrus, allowing infection by *C. albicans* (Chen and Kong 2007; Fidel and Sobel 1999; Sobel et al. 1985). In addition, rats tend to require surgical intervention to remove the ovaries prior to infection (Sobel et al. 1985), although a newer model involving immunosuppression with cyclophosphamide allowed vaginal infection to establish and be maintained for two weeks without this intervention (Foldvari et al. 2000; Fulurija et al. 1996). Use of immunosuppressants in rats to obtain greater infection agrees with results in immunocompromised mice, which tends to have higher vaginal counts than their immunocompetent counterparts (Fulurija et al. 1996). The requirement for oestrogen in order to maintain carriage and infection agrees with women of childbearing age being more susceptible to vaginal candidiasis.

A new model has been developed recently where both oral and vaginal infection can be studied in the same host (Rahman et al. 2007). This requires administration of oestrogen via two routes, with *C. albicans* administered orally and vaginally. This reduces the number of animals required to study both vaginal and oral infection.

Experimental models have been utilised in attempts to define host factors that predispose to development of infection. Although no difference in carriage rate was found when an anti-neutrophil antibody was used, this treatment led to reduced inflammation (Black et al. 1998). This was confirmed in a human intra-vaginal infection model, where symptomatic infection correlated with influx of neutrophils into the infected mucosa (Fidel et al. 2004). This has led to the hypothesis that an inflammatory reaction in the vaginal is deleterious.

Mouse and rat models have also demonstrated the importance of dendritic cells in immunoregulation during vaginal infection (De Bernardis et al. 2006; LeBlanc et al. 2006). A review summarizing the current knowledge of host defenses in vaginal candidiasis was recently published (Fidel 2007).

Rodent vaginal models have been used to test the virulence of *C. albicans* mutants and clinical isolates (Bader et al. 2006; Fu et al. 2008; Taylor et al. 2000), and also to evaluate antifungal therapies (De Bernardis et al. 2007; Hamad

et al. 2006; Valentin et al. 1993). The model has also been used to measure *C. albicans* gene expression during infection development (Cheng et al. 2005a; Taylor et al. 2005), demonstrating that gene expression in models are similar to that of clinical samples (Cheng et al. 2005a) and showing that, changes in gene expression occur during development of infection (Taylor et al. 2005).

Models of Systemic Infection

Disseminated infections carry the greatest risk of mortality of all *Candida* infections due to difficulties in diagnosis and, hence, delays in initiation of antifungal therapy. Delays in treatment initiation are known to decrease survival (Morrell et al. 2005). It is, therefore, essential that there are models of systemic *Candida* infection that reflect the situation in the human host and which allow us to investigate disease development, test antifungal agents and evaluate new diagnostic tools.

Gastrointestinal Dissemination

Disseminated *Candida* infections are generally believed to be initiated by strains carried by the host. It would, therefore, be advantageous to model disseminated infection using dissemination from the gastrointestinal tract. As mentioned above, immunocompromising drugs, antibiotics and anti-cancer drugs promote dissemination from the gut, but with variable results (Clemons et al. 2006; Cole et al. 1989; Sandovsky-Losica et al. 1992; Tansho et al. 2002). In general models of dissemination from the gastrointestinal tract require use of gnotobiotic animals, usually mice (Cole et al. 1989; Guentzel and Herrera 1982; Herrera and Guentzel 1982; Koh et al. 2008; Ponnuvel et al. 1993), or use of special diets (Samonis et al. 1990; Takahashi et al. 2003). These infections lead to dissemination, with highest fungal burdens usually found for the liver, spleen and kidneys. More reproducible dissemination rates require both damage to the gut and immunosuppression (Koh et al. 2008).

Although these disseminated infection models are highly variable they have been used to evaluate diagnostic tools (Nichterlein et al. 2003) and antifungal therapies (Dromer et al. 2002; Guentzel and Herrera 1982; Herrera and Guentzel 1982).

Intravenous Challenge

Models of disseminated *Candida* infection initiated by an intravenous challenge, are well-characterized and reproducible (Louria et al. 1963; MacCallum and Odds 2005; Papadimitriou and Ashman 1986). The intravenous challenge model leads to systemic infection, with highest burdens found in the kidneys (MacCallum and Odds 2005). In the mouse model, mice die of a severe sepsis, similar to that is seen

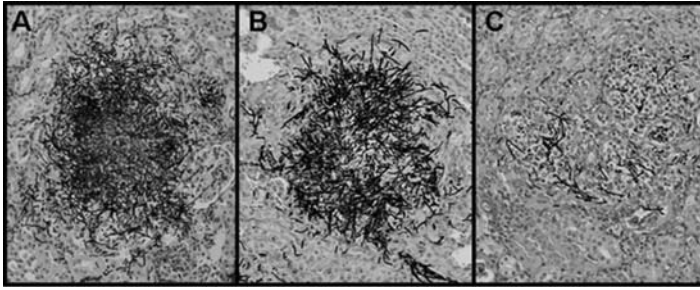

Fig. 3.1 *C. albicans* in kidneys from experimental infections. Wax sections of formalin-fixed kidneys from a mouse (**a**), rabbit (**b**) and guinea pig (**c**) were stained by methenamine silver stain

in severe clinical cases (Spellberg et al. 2005). However, in this model, there is no requirement of the fungus to pass from the gut into the bloodstream.

In general, *Candida* intravenous challenge is carried out in mice, guinea pigs and rabbits, where filamentous forms are seen in the kidney (Fig. 3.1). The choice of species used usually depends upon the pharmacokinetics of the antifungal drug being used (MacCallum and Odds 2002) or the amount of sample required for subsequent procedures (Walker et al. 2009). The mouse model, in particular, has been extensively used in the evaluation of antifungal therapies (examples of studies include Andes et al. 2008; MacCallum and Odds 2004), in virulence testing of mutant strains and clinical isolates (for a list of many of the mutants tested in the mouse IV challenge model see Brand et al. 2004; MacCallum et al. 2009) and in investigation of host defense mechanisms (examples of studies include Ashman et al. 2003; Basu et al. 2008; Murciano et al. 2006; Netea et al. 2006; Villamon et al. 2004). A detailed review of the immune responses occurring in *C. albicans* experimental infections is available (Ashman 2008).

It is clear that there are a number of experimental models available to allow the *Candida* researcher to explore host-fungal interaction, but there still remains a need for the development of a reliable, reproducible gastrointestinal dissemination model. Only when this model becomes available, will it be possible to fully understand the initiation of *Candida* infection and disease from an endogenous source.

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Chapter 4
Candida albicans Cell Wall Mediated Virulence

Carol Munro

Abstract Fungal cells are covered in a polysaccharide-rich coat that protects them from the external environment, acts as a barrier and filter, and resists internal turgor pressure. The tensile strength provided by the mature cell wall is co-ordinated with zones of new polarised growth (germination, septal formation, hyphal tips and branches) where the wall must retain its integrity, as new cell wall material is inserted and assembled. Therefore, there is a careful balance between wall rigidification and wall re-moulding to enable morphogenesis and growth. In this chapter we will discover that the overall cell wall structure can adapt and respond to external and internal factors, and by remodelling the wall in response to such stimuli fungal cells minimise any loss of cellular integrity. In fungal pathogens the cell wall provides the interface with the host and so a number of cell wall associated components have been identified that play important roles in fungal-host interactions. Here we will describe cell wall components that play roles in virulence, either directly or by modulating the host's immune responses using *Candida albicans* as a model fungal pathogen of humans. The main focus will be in recent advances of our understanding of the regulation and dissection of cell wall components using molecular approaches. Readers are also directed to other excellent *C. albicans* cell wall reviews and book chapters (Klis et al. 2001; Chauhan et al. 2002; Ruiz-Herrera et al. 2006; Sohn et al. 2006; Chaffin 2008).

4.1 *C. albicans* Cell Wall Architecture

In the human pathogen *C. albicans* the three main components of the wall are chitin (linear polymer of β(1,4)-*N*-acetyl glucosamine), glucan (β(1,3)- and β(1,6)- chains of glucose) and mannan (highly glycosylated mannoproteins) (De Groot et al. 2007).

C. Munro
Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, UK
e-mail: c.a.munro@abdn.ac.uk

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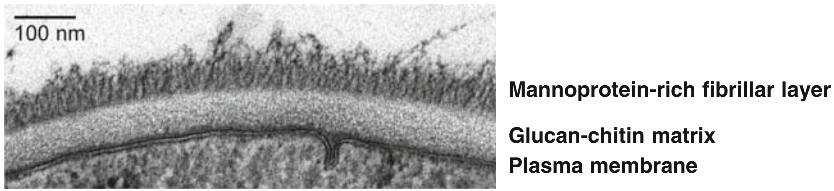

Fig. 4.1 High pressure freeze substitution transmission electron micrograph of *C. albicans* yeast cell wall

Minor components include phospholipomannan (Trinel et al. 1999), sialic acid (Soares et al. 2000), dityrosine (Melo et al. 2008) and possibly chitosan (the deacetylated form of chitin). The core, covalently-linked polysaccharides chitin and β(1,3)-glucan provide the wall with its strength and rigidity. The outer cell wall is enriched with mannoproteins that provide the wall with its fibrillar structure, which is evident by electron microscopy (Fig. 4.1). The cell wall is in close contact with the underlying plasma membrane and several of the important cell wall biosynthetic enzymes, such as the chitin synthase family and β(1,3)-glucan synthase subunits are integral membrane proteins. The major class of covalently attached cell wall proteins, the glycosylphosphatidyl inositol (GPI)-anchored proteins, also transit to the wall via the membrane and some GPI-proteins remain permanently localised in the membrane (see below). In addition, a number of membrane-bound proteins (Mid2, Wsc family and signalling mucins such as Mtl1) are sensors of cell wall damage and transmit their signals to downstream signalling pathways (Levin 2005).

4.1.1 Cell Wall Proteins

GPI-proteins contain both an N-terminal signal peptide and a hydrophobic C-terminal GPI-anchor attachment site. They follow the normal secretory pathway through the ER, where they are processed at the C-terminus and a pre-synthesised GPI-anchor added (Herscovics and Orlean 1993). Assembly of the GPI-anchor is an essential function in *Saccharomyces cerevisiae* and in *C. albicans* (Orlean and Menon 2007; Fujita and Jigami 2008) as evidenced by gene deletion and regulated expression studies. After further processing in the ER and Golgi with the addition of *O*- and *N*-glycans, the GPI-anchored proteins are transported to the plasma membrane. Some GPI-proteins, such as Ecm331, remain tethered to the plasma membrane, (Mao et al. 2008) whereas others are translocated out to the cell wall. Cell wall localisation is dependent upon further modification of the GPI-anchor, which is cleaved, leaving a remnant that becomes covalently attached to β(1,6)-glucan. The enzymes involved in these last processing steps are not known. Likely candidates are other membrane and wall localised proteins, including

other GPI-proteins such as the synthetically lethal Dfg5 and Dcw1 that bear some resemblance to bacterial mannosidases. The basis behind the selection of the final residence of GPI-proteins, i.e. membrane or wall, is not fully understood, but can be predicted by the sequences that are upstream of the GPI-anchor attachment site (Vossen et al. 1997; Frieman and Cormack 2003, 2004; Mao et al. 2008).

The second minor class of covalently attached proteins, which are directly linked to β(1,3)-glucan, comprises only Pir1 in *C. albicans*. Pir1 is orthologous to a family of proteins in *S. cerevisiae*. Characteristic of its class, Pir1 has internal repeats and can be released from the cell wall with mild alkali treatment. It has been suggested in *S. cerevisiae* that glutamine residues within the internal repeats may form ester linkages with glucose sugars of β(1,3)-glucan (Ecker et al. 2006). A number of other cell wall associated proteins (CaScw1, ScScw4, ScScw10 and ScTos1) have been shown to be attached to the wall via a mild alkali-sensitive linkage (Yin et al. 2005). Some of these proteins attached via a mild alkali-sensitive linkage can also be released by treatment with reducing agents such as dithiothreitol (DTT) and β-mercaptoethanol (Cappellaro et al. 1998). Treatment with reducing agents also releases a number of carbohydrate-lytic, secreted proteins from the wall including glucanases ScExg1, ScBgl2 and chitinase ScCts1 (Cappellaro et al. 1998). There are several examples of cell wall proteins that contain cysteine residues, often positioned towards the N-terminal half of the proteins. One example is a family of GPI-proteins that contain eight cysteines at conserved positions constituting putative CFEM (Conserved in several Fungi Extracellular Membrane) domains (Weissman and Kornitzer 2004b; Perez et al. 2006). Therefore, disulphide bridges may be important in tethering non-covalently attached proteins to covalently attached proteins in the wall.

Non-conventional, supposedly intracellular and cytoplasmic proteins that lack a signal peptide have also been detected in cell wall fractions when proteins have been extracted using reducing agents such as β-mercaptoethanol and DTT (Vediyappan et al. 2000; Pitarch et al. 2002; Chaffin 2008). Such conditions have been shown to result in leakage of cytosolic contents by partially solubilising the membrane (Klis et al. 2007). These non-conventional cell wall associated proteins include known immunogens. Antibodies against cytoplasmic proteins have been identified in patient sera suggesting that they become bound to the surface during an infection by an unknown mechanism or as a consequence of their release from lysed cells. Examples are phosphoglycerate kinase Pgk1, enolase Eno1 and heat shock proteins (Angiolella et al. 1996; Pitarch et al. 2002, 2006). Some non-conventional cell wall associated proteins contribute to host-fungus interactions by, for example, binding to plasminogen and other host proteins (Jong et al. 2003; Crowe et al. 2003). Tsa1, the *C. albicans* peroxiredoxin protein, contributes to the oxidative stress response. Although it is expressed equally in yeast and hyphal cells, it remains in the cytosol and nucleus of yeast cells and is only localised to the walls of hyphal cells (Urban et al. 2003, 2005). The localisation of Tsa1 correlated with morphology and was shown to be dependent upon Efg1 (Urban et al. 2005). Therefore, morphology-related changes in cell wall composition can be extended to include non-conventional cell wall associated proteins.

4.2 Cell Wall Remodelling During Morphogenesis and Variation in pH

As *C. albicans* switches between different morphologies in response to environmental stimuli significant remodelling of the cell wall occurs (Fig. 4.2). There are changes in the structural polysaccharides with three- to five-fold higher chitin levels in hyphae compared to yeast (Chiew et al. 1982; Sullivan et al. 1983; Munro et al. 1998). Structural differences also exist between yeast and hyphal β(1,3)-glucan (Lowman et al. 2003). The mannan structure is also altered with more β-1,2-linked mannose in the acid labile yeast mannan than the hyphal form (Shibata et al. 2007) and there are a number of morphotype-specific cell wall proteins. Expression analyses have identified genes encoding cell surface proteins that are differentially regulated in response to morphogenesis (Table 4.1). Hyphal induced or hyphal-associated genes include *ALS3* (Hoyer et al. 1998), *CSA1/WAP1 (*Braun et al. 2000*),*

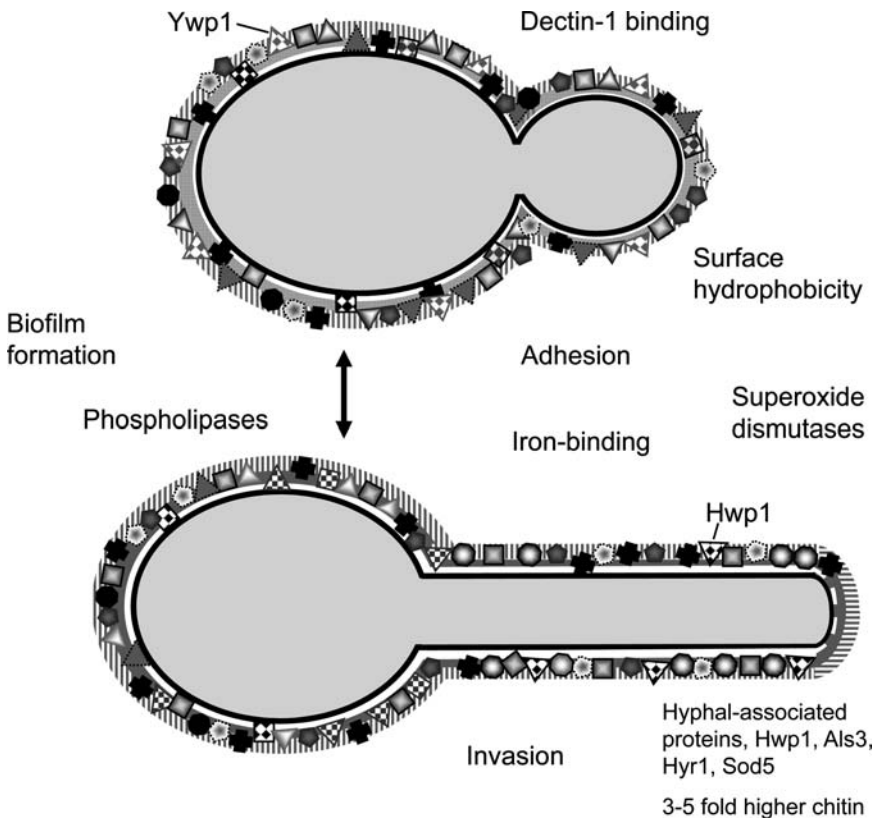

Fig. 4.2 Virulence attributes associated with *C. albicans* yeast and hyphal cell wall. Black line represents plasma membrane, chitin is white, glucan grey and outer fibrillar layer is shown. Specific mannoproteins are represented as different coloured and patterned shapes and may be attached to the plasma membrane or the cell wall

HWP1 (Staab et al. 1996), *HYR1* (Bailey et al. 1996), *IHD1*/*PGA36* (Nantel et al. 2002) and *RBT1* (Braun et al. 2000). In contrast, *YWP1* is yeast-form specific (Granger et al. 2005). In addition, members of the Gas-like Phr family of transglycosidases respond to changes in pH, a parameter that also contributes to morphogenesis. Phr1 is expressed in alkaline pH conditions related to hyphal induction (Saporito-Irwin et al. 1995), whereas Phr2 is expressed in acidic conditions (Muhlschlegel and Fonzi 1997). *In vivo* models have indicated that the *phr1*Δ mutant is defective in causing systemic infection, but had normal ability to cause disease in a rat vaginitis model, whereas the *phr2*Δ mutant had the inverse phenotype (Ghannoum et al. 1995; De Bernardis et al. 1998). Therefore, the mutants display niche-specific phenotypes commensurate with their differential expression under these conditions. Another cell surface pH-regulated antigen, Pra1, acts as a ligand for leukocyte integrin alphaMbeta2 and is immunogenic in a mouse model of candidiasis (Soloviev et al. 2007). Transcript profiling has identified pH responsive genes in *C. albicans* (Lotz et al. 2004; Bensen et al. 2004). As well as the aforementioned *PHR1* and *PHR2*, the differentially regulated genes included a number of other cell wall related genes such as the one encoding the GPI-anchored protein Rbr1/Pga20, which was shown to be repressed by Rim101 and activated by Nrg1 (Lotz et al. 2004). A number of the hyphal-induced genes were also activated in response to alkaline pH; *RBT1* (repressed by Tup1), *CSA1*, *ECE1*, *HYR1*, *HWP1* (Bensen et al. 2004), and *RIM101*. The latter encodes a zinc finger transcription factor orthologous to PacC (Ramon and Fonzi 2003), which participates in the regulation of the alkaline pH response and plays a role in virulence as the *rim101*Δ mutant is defective in endothelial damage assays and in a murine model of systemic candidiasis (Davis et al. 2000). The attenuated virulence of the *rim101*Δ mutant in an oropharyngeal model of candidiasis has been attributed to its altered cell wall as over-expression of Als3, Cht2, Pga7/Rbt6 and Skn1 resulted in remediation of the *rim101*Δ avirulent phenotype (Nobile et al. 2008b).

In addition to gene expression studies, proteomics analyses have identified proteins that are differentially expressed in yeast and hyphal cell walls and membrane fractions (Pitarch et al. 2002; Ebanks et al. 2006; Alvarez and Konopka 2006; Castillo et al. 2008) (Table 4.1). *C. albicans* is found as yeast, pseudohyphae and hyphae as it grows in host tissues and so all combinations of different surface components may be present in a single infection lesion. So the host's immune response may be activated by yeast and hyphae-associated components at the same time. By switching morphologies and hence altering cell surface properties, *C. albicans* elicits different immune responses (Torosantucci et al. 1990, 2000), and this could also act as an immune evasion mechanism (Torosantucci et al. 2004). Altering morphology has also been shown to affect *C. albicans* recognition via the pathogen recognition receptor Dectin-1 (Heinsbroek et al. 2005). Dectin-1 elicits uptake of *C. albicans* by macrophages by binding to β(1,3)-glucan. However, β(1,3)-glucan is normally shielded from the host's immune cells by an outer layer of mannoproteins except when it is exposed on the surface of bud scars of mother yeast cells. Dectin-1 recognition triggering engulfment by macrophages is specific to yeast cells, *C. albicans* hyphae lacking bud scars were not recognized by Dectin-1

Table 4.1 Morphotype-associated GPI-anchored[a] proteins

Protein	Expression profile	Approach/cellular fraction	Function	Reference
Ywp1/Pga24	Yeast	Promoter-GFP-reporter fusion Cell wall proteomics	May aid dispersal of yeast cells and mask underlying cell wall components	Granger et al. (2005), Castillo et al. (2008)
Als3	Hypha	Northern analysis/mRNACell wall proteomics	Adhesion, invasion, iron-sequestration, biofilm formation	Hoyer et al. (1998), Zhao et al. (2004), Oh et al. (2005), Zhao et al. (2006), Phan et al. (2007), Almeida et al. (2008), Ebanks et al. (2006), Castillo et al. (2008)
Cht2	Yeast	Cell wall proteomics	Chitinase	Iranzo et al. (2002), Ebanks et al. (2006), Castillo et al. (2008)
Hyr1	Hypha	Northern analysis/mRNA	Unknown	Bailey et al. (1996)
Hwp1	Hypha	Immunofluorescence assay with monospecific Hwp1 anti-sera	Adhesion, substrate for mammalian transglutaminases	Staab et al. (1996, 1999)
Pga10/Rbt51/Rbt8	Hypha-induced	mRNA/transcript profiling	Plasma membrane protein involved in heme-iron utilization	Bensen et al. (2004)
Pga36/Ihd1	Hypha-induced	mRNA/transcript profiling	Unknown	Nantel et al. (2002), Kadosh and Johnson (2001), Bensen et al. (2004)
Rbt1	Hypha	Cell wall proteomics mRNA/transcript profiling	Unknown	Kadosh and Johnson (2001); Sohn et al. (2003); Bensen et al. (2004); Castillo et al. (2008)
Sod5	Hypha-induced	Northern analysis/mRNA	Cu/Zn-containing superoxide dismutase	Martchenko et al. (2004)

[a]Proven or predicted

and not taken up by macrophages (Gantner et al. 2005). Recent evidence has revealed that, as infection progresses deeper, glucan-rich layers of the *C. albicans* cell wall become unmasked and so different pathogen associated molecular patterns (and possibly antigens) may become exposed to host cell receptors in the different stages of infection (Wheeler et al. 2008). Furthermore treatment with caspofungin aided the unmasking of glucan and was biased towards hyphal cells.

4.3 Phenotypic Switching

Phenotypic switching is a fascinating phenomenon demonstrated by *C. albicans*. The ability to switch between an opaque (mating competent) and a white (mating incompetent) form has been carefully dissected at the molecular level (Slutsky et al. 1987; Sonneborn et al. 1999; Miller and Johnson 2002; Soll et al. 2003; Zordan et al. 2006; Srikantha et al. 2006; Vinces and Kumamoto 2007; Yi et al. 2008; Huang et al. 2009). Accompanying the switch is altered morphology and cell surface differences, with opaque cells having a more elongated, ellipsoidal cell shape and a cell surface covered in prominent pimples (Anderson et al. 1990). Not only does the switch phenotype alter mating competency but also affects interactions with host's phagocytes. Murine-derived macrophage cell line Raw264.7 as well as *Drosophila* hemocyte-derived S2 cells preferentially engulfed white cells over opaque cells. This led the authors to propose that the ability to switch between white and opaque form was an immune evasion strategy (Lohse and Johnson 2008). The differentially expressed cell wall components that elicit altered phagocytosis have not been defined.

4.4 Comparative Cell Wall-Omics

Cell wall related genes are identifiable in fungal genomes by sequence comparison and other *in silico* analyses. *C. albicans* possesses families of chitin synthases (Chs.1, 2, 3 and 8) and β(1,3)-glucan synthase subunits (Gsc1/Fks1, Gsl1/Fks3, Gsl2/Fks2). The prediction of proteins that are GPI-anchored has identified between 104 and 180 proteins depending on the algorithm used (De Groot et al. 2003; Lee et al. 2003; Alberti-Segui et al. 2004; Eisenhaber et al. 2004). Cell wall proteins appear not to have the evolutionary constraints of other proteins, perhaps because they are surface localised or because they do not have many interacting partners (Coronado et al. 2007). Inter-species genome-wide prediction of GPI-anchored proteins has demonstrated that each species in the so-called CTG clade species (Fitzpatrick et al. 2006) related to *C. albicans* contains a sub-set of species-specific GPI-proteins (Butler et al. 2009). Within the CTG clade GPI-proteins that are conserved and found in other species may play important roles in cell wall biosynthesis, assembly or re-modelling. These include transglycosidases such as the Gas/Phr proteins that modify β(1,3)-glucan (Mouyna et al. 2000; Carotti et al. 2004; Vinces and Kumamoto 2007;

Hurtado-Guerrero et al. 2009) and the Crh family that links chitin to β(1,6)-glucan (Cabib et al. 2007, 2008). Orthologous proteins can be divided into those that are singletons, i.e. a unique sequence in each species and those that are members of one of the 22 families (Butler et al. 2009; Richard and Plaine 2007). Inter-species comparison has discerned expansion and contraction of these families and pinpointed two examples of *C. albicans* and *C. dubliniensis*–specific families (Munro et al. unpublished). Whether these families as well as other *C. albicans* and *C. dubliniensis*–specific singleton proteins contribute to virulence remains to be established.

4.5 Glycosylphosphatidylinositol Anchored Cell Wall Proteins

Only around 30% of predicted GPI-proteins have been characterised so far in terms of their function (Richard and Plaine 2007; Plaine et al. 2008a). Those that have been characterised play roles in cell wall assembly and maintenance (Phr/Gas, Crh and yapsin families) and act as adhesins, chitinases, phospolipases and superoxide dismutases. GPI-anchored proteins also contribute to *C. albicans* virulence by acting as adhesins and lytic enzymes, by sequestering iron, participating in biofilm formation and in the case of Als3 performing as an invasin. Approaches to characterize novel predicted GPI-anchored proteins have included systematic gene disruption (Plaine et al. 2008a) and over-expression (Fu et al. 2008). Disruption of a number of predicted GPI-anchored proteins altered *C. albicans* sensitivity to caspofungin (Plaine et al. 2008a). Therefore, there is much evidence to suggest that GPI-anchored proteins have an important role in cell wall robustness and viability. This is also reflected in mutants that are defective in GPI-anchor biosynthesis. Disruption of *GPI7* that encodes a GPI-anchor modifying enzyme affected cell wall attachment of GPI-anchored proteins and resulted in an enhanced pro-inflammatory response in mice (Richard et al. 2002; Plaine et al. 2008b). Grimme et al. (2004) demonstrated that *C. albicans* Smp3 added the fourth mannose to a trimannose core in the biosynthesis of the GPI-anchor and was essential for viability. Switching off the *MAL2* promoter-regulated expression of *SMP3* caused yeast cells to aggregate, the cells had abnormal morphology and became enlarged and were hypersensitive to Calcofluor White (CFW) (Grimme et al. 2004).

4.6 Cell Wall Associated Virulence Attributes

4.6.1 Adhesion

Cell wall components contribute to different stages of infection. Initially during colonisation, *C. albicans* must adhere to host cells to avoid being washed away in

the fluids that are constantly bathing host mucosal surfaces. The ability to adhere to a wide range of substrates enables *C. albicans* to stick on to and grow in many different niches in the body (Calderone and Braun 1991; Calderone 1993; Sundstrom 2002; Calderone and Gow 2002; Hoyer et al. 2008). In addition, adhesion to inanimate substrates such as indwelling catheters allows *C. albicans* to initiate biofilm formation that renders *C. albicans* more resistant to antifungal agents (Ramage et al. 2001; Kumamoto 2002; Douglas 2003; d'Enfert 2006; Nett and Andes 2006).

Progress has been made in deciphering the adhesive attributes of a number of cell surface proteins. These are likely to be important for superficial as well as systemic infections. Yet, we know very little about *C. albicans*' ability to adhere to the surface of the gut epithelium during commensalism and how it adheres to and interacts with the resident gut microbiota. One family of proteins that plays a key role in adhesion is the Als family of GPI-anchored cell wall proteins.

4.6.1.1 Als family

C. albicans has an eight membered family of agglutinin-like sequences (Als) proteins (Hoyer 2001; Hoyer et al. 2008) comprising Als1 to Als7 and Als9. Each protein consists of a unique N-terminal functional domain that elicits cell-to-cell adhesion and adhesion to host proteins and tissues, a central domain that contains characteristic mid-repeats and a C-terminus that houses the GPI-anchor attachment site. Expression of the different family members in *S. cerevisiae* and domain-swapping experiments has shown that different family members have specificity for selected host proteins (Sheppard et al. 2004). The fibronectin-binding and cell-to-cell adhesion properties of Als5 have been shown to be mediated by the N-terminal IgG-like domain with participation from the threonine-rich tandem repeats (Rauceo et al. 2006). Als5 also has the potential to form amyloid-like fibers and the sequences involved are conserved in other family members (Otoo et al. 2007). *In vitro* - prepared Als5 amyloids had the capacity to bind Congo Red and shared other properties associated with amyloids. Als amyloid-like structures may contribute to cell-to-cell adhesion, with Als proteins on one cell interacting with Als proteins on an adjacent cell, and adhesion to other substrates (Rauceo et al. 2006; Klotz et al. 2007). In addition to Als-to-Als interactions aiding *C. albicans* cell-to-cell adhesion, interactions between Hwp1 and Als proteins enhance the attachment of cells to each other in biofilm formation (Nobile et al. 2008a). In *S. cerevisiae* cooperation in cell-to-cell adhesion termed flocculation is mediated via the "green beard" gene *FLO1* (Smukalla et al. 2008). *FLO1* encodes the major cell surface protein responsible for flocculation and has high inter-strain variability in terms of expression and sequence, the latter brought about by recombination between intragenic tandem repeats (Verstrepen et al. 2005).

4.6.1.2 Eap1

A successful approach to identify *C. albicans* adhesins is to express *C. albicans* genes in *S. cerevisiae* and to monitor for consequential increases in adhesion. This approach identified *ALA1*, now re-annotated as *ALS5* (Gaur and Klotz 1997; Gaur et al. 1999) and *EPA1* (Li and Palecek 2003; Li et al. 2007). Heterologous expression of Epa1 increased adhesion of a *S. cerevisiae flo8*Δ mutant and a *C. albicans efg1*Δ mutant to polystyrene and to a kidney epithelial cell line (Li and Palecek 2003). An *epa1*Δ mutant also had reduced adhesion to polystyrene and epithelial cells (Li et al. 2007). Expression of *EPA1* increased during biofilm formation and *EPA1* expression was required for the generation of biofilms *in vitro* and *in vivo* (Li et al. 2007). Epa1 has a modular domain structure that resembles many other cell wall proteins with an N-terminal signal peptide, followed by a serine/threonine-rich domain containing tandem repeats and a C-terminal GPI-anchor attachment site. The N-terminal domain is required for cell-to-cell adhesion, whereas the serine/threonine tandem repeat region is necessary for exposure of the N-terminal domain on the cell surface and attachment to polystyrene (Li and Palecek 2008).

4.6.1.3 Hwp1

Another key player in adhesion is the hypha-specific Hwp1. Isolated as a proline-rich and glutamine-rich protein with integral repeats specific to the hyphal cell surface (Staab et al. 1996), Hwp1 has been shown to act as a substrate for mammalian transglutaminases (Staab et al. 1999). This property is resident in the disulphide-bridged, coiled N-terminal domain and enables cross-links to form between *C. albicans* hyphal cells and host mucosal proteins (Staab et al. 2004). The C-terminal modified GPI-anchor was also essential for this property. Deletion of *HWP1* resulted in cells that could no longer attach stably to host mucosa and that were avirulent in a mouse systemic model (Staab et al. 1999).

Dissection of the *HWP1* promoter has pinpointed a region of 368 bp that mediates hyphal specific expression and this was recognised by transcription factors Nhp6 and Gcf1p (Kim et al. 2007). In the formation of conjugation tubes during mating *HWP1* expression was found to be **a**/**a** cell specific and Hwp1 localised to the region of the **a**/**a** conjugation tube that the first daughter cell emerged from (Daniels et al. 2003).

4.6.1.4 Ywp1

In contrast to *HWP1*, *YWP1* (*PGA24*) is most highly expressed in the yeast form and has reduced expression in stationary phase and in hyphae. The *ywp1*Δ mutant had enhanced biofilm formation and cell-to-cell aggregation suggesting that in yeast

cells Ywp1 acts to keep the cells separated perhaps by masking other cell wall components (Granger et al. 2005).

4.6.1.5 Int1

Int1 was isolated as an integrin-like protein that localised to the yeast cell surface (Gale et al. 1996). Subsequently, Int1 has been implicated in adhesion to host epithelia, filamentation, intestinal colonisation and virulence (Gale et al. 1998; Bendel et al. 1999; Kinneberg et al. 1999). Int1 interacts with septins, the cortical cues that mark the axis for septum formation at the mother-bud neck and its localisation pattern with the septin Cdc3 differentiated yeast and pseudohyphae from hyphae (Gale et al. 2001). A peptide derived from the N-terminus of Int1 has super-antigen like properties, including the ability to activate T-lymphocytes independent of antigen processing and presentation and triggering the release of the interferon-γ cytokine (Devore-Carter et al. 2008).

4.6.2 Cell Surface Hydrophobicity

The physical properties of the wall including surface hydrophobicity are known to contribute to virulence. Hydrophobic strains are more adherent and more resistant to killing by phagocytes (Hazen et al. 1991, 2000). *C. albicans* isolates exhibit different fibrillar architectures on their outer surfaces. Those with hydrophilic surfaces have dispersed long fibrils, whereas more hydrophobic isolates have shorter, more compact, aggregated fibrils (Hazen and Hazen 1992). Outer chain *N*-glycans and specifically acid-labile β(1,2)-oligomannoside side chains (also called phosphomannan) contribute to surface hydrophobicity with hydrophobic cells having longer β(1,2)-oligomannoside side chains (Masuoka and Hazen 1997, 1999). *C. albicans* can be classified into serotype A and B isolates that have different cell surface properties due to different mannan structures (Suzuki 2002). Serotype A isolates have additional β(1,2)-oligomannoside side chains added to the acid stable *N*-glycan as well as in the acid-labile phosphomannan portion. Different levels of hydrophobicity correlate with the length of the acid-labile β(1,2)-oligomannoside side chains whereas differences in the acid-stable β(1,2)-mannose oligomers of serotype A isolates do not relate to hydrophobicity changes (Masuoka and Hazen 2004).

The protein Csh1 that resembles members of the aldo-keto reductase family has been identified in a screen for cell surface hydrophobic proteins (Singleton et al. 2001). Deletion of *CSH1* initially reduced surface hydrophobicity by 75% but the null mutant was able to regain hydrophobicity upon frozen storage (Singleton et al. 2005a).

The outer layer of the *C. albicans* wall is negatively charged due to the presence of the phosphate group of the phosphomannan moiety of *N*-mannan. This is

reflected in the ability of *C. albicans* cells to bind the cationic dye alcian blue and can provide a quick and easy screen for altered cell wall charge. For example, serotype A and serotype B *mnn4*Δ mutants that have defects in phosphomannan production have significantly decreased alcian blue binding (Hobson et al. 2004; Singleton et al. 2005b). The change in surface charge does not affect virulence as the *mnn4*Δ mutant in the serotype A background is as virulent as wild type cells in a murine model of systemic candidiasis (Hobson et al. 2004).

4.6.3 Superoxide Dismutases (SODs)

A number of GPI-anchored proteins bear similarity to catalytic enzymes. Among these are the Sod4, Sod5 and Sod6 proteins that resemble Cu, Zn superoxide dismutases (De Groot et al. 2004). Superoxide dismutases represent one of a number of antioxidant strategies used by microbes to combat the damaging free oxygen radicals generated by immune cells in the defence of the host from invading microbes (Hamilton and Holdom 1999). SODs convert O^{2-} to H_2O_2, which can then be broken down by catalases. *SOD5* was identified as a hyphal-induced gene whose expression was also activated in response to oxidative stress (Martchenko et al. 2004). The null *sod5*Δ mutant was attenuated in virulence in a murine systemic model but was as efficiently killed by macrophages as its parental control strain (Martchenko et al. 2004). Fradin et al. (2005) went on to show that *SOD5* expression was significantly activated when *C. albicans* yeast cells were exposed to neutrophils (Fradin et al. 2005). When independently-constructed *sod5*Δ and double *sod4*Δ*sod5*Δ mutants were challenged with bone marrow derived macrophages and myeloid dendritic cells more reactive oxygen species (ROS) accumulated with the mutants than with control strains (Frohner et al. 2009). In addition, both the *sod5*Δ and *sod4*Δ*sod5*Δ mutants had significantly reduced viability when challenged *in vitro* with macrophages. Therefore, surface-associated SODs do seem to play a role in the ability of *C. albicans* to protect itself from host assault.

4.6.4 Phospholipases

Among the GPI-anchored, covalently attached cell wall proteins are proteins that resemble phospholipases Plb3 to Plb5 (De Groot et al. 2003). *C. albicans* has two further phospholipase B proteins, Plb1 and Plb2, that are secreted (Chap. 9). Phospholipases breakdown phospholipids and lysophospholipids, and function in cellular processes such as signal transduction as well as virulence (Ghannoum 2000). Disruption of *PLB5* results in reduced *in vitro* phospholipase A_2 activity and decreasesorgan burdens in a murine systemic model (Theiss et al. 2006), indicating surface-attached phospholipases do contribute to *C. albicans* virulence. Redundancy

may occur between different family members and so multiple mutations may be required to see a phospholipase B minus phenotype.

4.6.5 Iron Binding Proteins

The ability to scavenge essential iron from the host is a known virulence attribute of microbial pathogens. Als3 as well as a number of other GPI-anchored proteins have iron-binding capabilities. *RBT51/PGA10/RBT8* has the ability to confer the utilisation of iron hemoglobin to *S. cerevisiae* and expression of a closely related gene, *RBT5*, was induced upon iron starvation (Weissman and Kornitzer 2004a). Disruption of the *RBT5* gene alone diminished *C. albicans*' ability to use heme and hemoglobin as sources of iron (Weissman and Kornitzer 2004a). An *S. cerevisiae*-based screen identified mutants that were defective in iron hemoglobin utilisation and pointed to an endocytosis-mediated mechanism (Weissman et al. 2008). One of the major classes of mutants identified were in the ESCRT (Endosomal Sorting Complex Required for Transport), proteins involved in the internalisation of mono-ubiquitinated proteins that are targeted to the vacuole (Hurley and Emr 2006). The generation of equivalent mutants in *C. albicans* proved this was the case. Mutants of the ESCRT were defective in heme and hemoglobin uptake (Weissman et al. 2008). Rbt5 and Rbt51 are members of a family that includes predicted GPI-anchored proteins Csa1/Wap1 and Pga7/orf19.5635 as well as Csa2 that is not predicted to be GPI-anchored (Weissman and Kornitzer 2004a). Each of these proteins has cysteines at conserved positions in the N-terminal half of the amino acid sequences similar to other proteins with CFEM domains. Members of this family also contribute to biofilm formation (Perez et al. 2006).

4.6.6 Als3 a Multi-Functional Adhesin, Invasin and Ferritin-Binding Protein

The GPI-anchored, hyphal specific protein Als3 not only acts as an adhesin but also plays a second important role in *C. albicans* virulence by acting as an invasin. Phan et al. (2007) elegantly showed that the Als3 protein structure mimics E- and N-cadherins of epithelial and endothelial cells and mediates endocytosis of *C. albicans*. In addition, Als3 mediates iron acquisition by binding ferritin (Almeida et al. 2008). Therefore, Als3 is a multifunctional protein with a major role in *C. albicans* virulence. Interestingly, the closely related but less pathogenic species *C. dubliniensis* lacks an Als3 orthologue (Jackson et al. 2009). *C. dubliniensis* has a reduced ability to cause systemic infection compared to *C. albicans,* but is a successful pathogen in the oral cavity, the extent to which the absence of Als3 impacts on this remains to be experimentally tested.

4.6.7 Biofilm Formation

C. albicans has the ability to form biofilms – communities of cells surrounded by extracellular matrix (ECM) – that contribute to pathogenicity (Douglas 2003; Nett and Andes 2006). In the clinic, *C. albicans* biofilm development on inanimate material such as indwelling central venous catheters, complicates antifungal therapies as cells within biofilms are more resistant to azole drugs and amphotericin B (d'Enfert 2006). Biofilms are discussed in detail in Chapter 6; therefore, here, we will mention only the contribution of cell wall components to biofilms. It is easy to speculate that biofilm ECM is a modified and extended version of the cell wall and yet, no firm evidence suggests that any of the cell wall biosynthetic enzymes contribute to biofilm formation. However, several lines of evidence including chemical composition analysis (Al Fattani and Douglas 2006) and the ability of β(1,3)-glucanase to degrade ECM suggests that *C. albicans* biofilm ECM is rich in β(1,3)-glucan (Nett et al. 2007b). Nett et al. (2007a) showed that medical device-associated infection led to increased secreted β(1,3)-glucan and led them to propose that detecting elevated levels of secreted β(1,3)-glucan may be an indicator of, and therefore a potential diagnostic for, abiofilm formation associated with medical devices. In addition, the echinocandin class of antifungal drugs seems to be effective in inhibiting growth of sessile cells within biofilms

Screening Tn-mutagenised libraries has highlighted a number of genes that are involved in biofilm formation (Nobile and Mitchell 2005; Richard et al. 2005) that encode signalling proteins and cell wall proteins. Included is the transcription factor Bcr1, which regulates expression of Als1, Als3 and Hwp1 that are required for biofilm generation (Nobile and Mitchell 2005; Nobile et al. 2006a,b). Analysis of the function of the Tor1 kinase of *C. albicans*, a kinase that regulates the transcriptional responses to nutrients, has revealed a novel role for Tor1 signalling in negatively regulating cell-to-cell adhesion (Bastidas et al. 2009). A subset of genes encoding cell wall proteins, hyphae-associated proteins and hyphal growth regulators have elevated expression in response to rapamycin, the Tor1-specific inhibitor. Inhibition of Tor1 by rapamycin inhibited filamentation, increased the expression of *ALS1*, *ALS3*, *HWP1* and *ECE1* and caused cells to aggregate. The hyphal repressors Tup1 and Nrg1 were identified as downstream targets of Tor1 signalling as were the transcription factors Efg1 and Bcr1 with the latter participating in the induction of the adhesin genes in response to rapamycin (Bastidas et al. 2009). Both filamentation and adhesin expression are vital for biofilm formation and so Tor1 signalling may play an important role in regulating biofilm formation in response to environmental and nutritional cues.

Microarray analysis comparing the transcriptomes of planktonic and sessile cells (Garcia-Sanchez et al. 2004) has also provided some leads to other factors involved in hyphal development and biofilm production (Goyard et al. 2008). As described above, a number of predicted GPI-proteins have also been shown to contribute to biofilm formation. Cell surface GPI-proteins are in general heavily glycosylated. Cells treated with tunicamycin, the *N*-glycosylation inhibitor, are defective in

biofilm development but tunicamycin has little impact on mature biofilms (Pierce et al. 2009).

4.6.7.1 Interactions with Host Cells

The molecular basis of host immune recognition of *C. albicans* continues to be an active area of research. As already mentioned, specific receptors on host cells, so-called Pattern Recognition Receptors (PRRs), recognise microbial components called PAMPs (Pathogen Associated Molecular Patterns) often associated with the cell surface. Binding of PAMPs by PRRs activates and modulates cytokine production and induces phagocytosis and fungal killing. One of the best studied is the recognition of β(1,3)-glucan by the C-type lectin Dectin-1 (Gow et al. 2007; Tsoni and Brown 2008; Reid et al. 2009). However, the ability of the innate immune system to recognise fungal pathogens is highly complex and involves the detection of several fungal PAMPs (Netea et al. 2008). Surface-exposed cell wall epitopes include the mannose-rich glycans that are added post-translationally to proteins destined for the cell wall and membrane. Characterisation of mutants that are defective in the addition of *O*-glycans (Timpel et al. 1998; Munro et al. 2005; Rouabhia et al. 2005; Prill et al. 2005; Peltroche-Llacsahuanga et al. 2006) and in the synthesis of acid-stable (Bates et al. 2005) and acid-labile *N*-glycan (Hobson et al. 2004) or glycosylation in general (Bates et al. 2006) have been insightful in determining PAMP-PRR interactions. *O*-glycans are recognized by TLR4, *N*-glycans by the mannose receptor (Netea et al. 2006) and TLR2 has been implicated in the recognition of glucan and phospholipomannan (Jouault et al. 2003). There is also much cross-talk between the different PRRs with activation of different PRR combinations resulting in synergy. For example, Dectin-1 synergises with TLR2 and TLR4 to induce TNFα production by monocytes and macrophages (Ferwerda et al. 2008). Indeed, an association between Galectin-3 recognition and signalling via TLR2 has been implicated in *C. albicans* specific recognition by macrophages (Jouault et al. 2006).

Phospholipomannans (PLMs) are glycolipid structures, members of the mannoseinositolphosphoceramide family, that contain linear chains of β(1,2)-linked oligomannoside (Trinel et al. 1999, 2002). β(1,2)-linked oligomannosides are not found in *S. cerevisiae*. Phospholipomannans contribute to adhesion, induce cytokine production, elicit the production of protective antibodies and, as discussed earlier, are the differentiating factor between serotype A and serotype B *C. albicans* isolates (Trinel et al. 2005). A family of β(1,2)-mannosyltransferase enzymes, Bmt1-9, have been identified (Mille et al. 2008). Mutants lacking *BMT1-BMT4* were generated and demonstrated that Bmt1 and Bmt2 are responsible for addition of the first β(1,2)-linked mannose residue to acid stable and acid-labile α(1,2)-linked glycan chains whereas Bmt3 and Bmt4 catalyzed elongation of β(1,2)-linked oligomannosides. The synthesis of PLM β(1,2)-linked oligomannosides were not affected in these mutants. Fractionation of cell walls by releasing proteins with different treatments revealed that β(1,2)-linked oligomannosides are added to a range of

proteins that cover the different classes of cell wall proteins discussed previously. Included were proteins linked to the wall via disulphide bridges, via β(1,3)-glucan and to β(1,6)-glucan via GPI-anchor remnants (Fradin et al. 2008).

To date evidence of chitin as a fungal PAMP has been sparse, prompted by the model that chitin is positioned in the deeper layers of the cell wall and may only be exposed to any extent in bud scars. But recent evidence has been provided that chitin fragments do activate an innate immune response (Lee et al. 2008; Da Silva et al. 2008, 2009). It is not only the carbohydrates attached to cell surface proteins but the proteins themselves that interact with innate cells. Mp65/Scw1 is a cell wall glucanase that has been developed as a potential vaccine (see below). When deprived of its glycosylation Mp65 can still be taken up by macrophages and dendritic cells, induce cytokine production and activate a T-cell response (Pietrella et al. 2006, 2008; Corbucci et al. 2007).

4.6.7.2 Wall as Potential Source of Novel Therapies

A number of potential fungal vaccines are currently under development with a particular emphasis on cell wall and cell envelope molecules (Cutler et al. 2007; Cassone 2008). A number of different approaches have been used to identify *C. albicans* cell wall proteins that are important targets for both humoral and cell mediated immune responses (Pitarch et al. 1999, 2001, 2006; Lopez-Ribot et al. 2004; Pitarch et al. 2006; Chaffin 2008). Examples include the putative glucanases Bgl2 (Pitarch et al. 2006), Camp65/Scw1 (De Bernardis et al. 2007) and the adhesins Als1 and Als3 (Ibrahim et al. 2006). Vaccines using the latter three proteins have been protective in animal models of candidiasis. A fungicidal monoclonal antibody, C7, that gives protection in a mouse candidiasis model was also shown to react with Als3 (Sevilla et al. 2006; Brena et al. 2007), once again highlighting that Als3 is truly a potent virulence factor and worthy target of immunotherapies.

4.7 Future Perspectives

The fungal cell wall is a multi-faceted, highly dynamic organelle that maintains cell integrity and is the point of contact between the fungus and its environment. Continuous remodelling of the cell wall architecture occurs in response to internal cues during different phases of growth and development and external signals indicating changes in the environment. Cell wall remodelling is a necessary adaptation to different stresses signalled via a variety of pathways and must be overcome to render cell wall-targeted therapies effective.

In fungal pathogens the cell wall is the interface between fungus and host. Tools and technologies to look at cell wall remodelling during an infection are being developed. Recent advances using *ex vivo* immunofluorescence have revealed that β-glucan is masked early in infection but becomes unmasked in later stages and

treatment with the echinocandin drug caspofungin preferentially unmasks β-glucan on hyphal cells (Wheeler et al. 2008). Therefore the cell wall structures and surface-exposed molecules *in vivo* may be very different to those of cells grown in laboratory culture conditions.

The introduction of the echinocandin class of antifungals that targets cell wall biosynthesis is a realisation of the potential of the cell wall as a target of antifungal drugs that has been proposed for many years. The echinocandins are not only welcome in the clinic but have provided researchers with important tools to improve our understanding of how the cell wall is assembled and re-modelled (Walker et al. 2008). Advances in atomic force microscopy that enables physical properties such as elasticity to be accurately measured are likely to be informative approaches in the analysis of fungal cell wall mutants. Current phenotypic analyses rely mainly on biochemical measurements and sensitivities to cell wall perturbing agents.

In the post-genomics era comparisons of cell wall related genes from pathogenic and non-pathogenic species have informed that conserved genes are likely to be important for synthesis and maintenance of a robust wall. Divergent genes such as those encoding predicted GPI-anchored proteins that appear to be evolving rapidly may have specific roles in the fungus-environment interface. An example is the *C. albicans* Als family (Hoyer et al. 2008). Investigation of the *C. albicans* cell wall with emphasis on elucidating the function of all cell wall related genes, the pathways that regulate them and the identification of the surface components that elicit and modulate the host's immune response are key to understanding *C. albicans* virulence.

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Chapter 5
Secreted *Candida* Proteins: Pathogenicity and Host Immunity

Julian R. Naglik and Bernhard Hube

Abstract Protein secretion is a vital process in all microbes. In pathogenic fungi, such as *Candida* species, secreted proteins are not only necessary for growth and proliferation but can also play important roles in fungal pathogenicity and host immunity. The majority of those that have been specifically targeted for study are the hydrolytic enzymes of *C. albicans*, including the secreted proteinases, phospholipases and lipases. However, recent and powerful advances in technologies, such as proteomics, have permitted unprecedented means of identifying and predicting novel cell wall and secreted proteins from these important fungal pathogens. Although the functions of the majority of recently identified proteins are currently unknown, it is only a matter of time before we add a considerable number of new secreted proteins to the arsenal of *Candida* species, which will significantly advance our understanding of why these fungi are such a successful human pathogens.

5.1 Introduction

The aetiology of microbial infections may be regarded as an encounter between the virulence of a microorganism and the ability of the host to resist microbial colonisation, invasion, and damage. All pathogenic microorganisms have developed mechanisms that allow successful colonisation or infection of the host (Finlay

J.R. Naglik (✉)
Department of Oral Immunology, King's College London Dental Institute, King's College London, Floor 28, Guy's Tower, St Thomas Street London SE1 9RT, United Kingdom
e-mail: julian.naglik@kcl.ac.uk

B. Hube
Department of Microbial Pathogenicity Mechanisms, Leibniz Friedrich Schiller University Jena and Institute for Natural Product Research and Infection Biology – Hans Knoell Institute Jena (HKI), Beutenbergstraße 11a 07745, Jena, Germany
e-mail: bernhard.hube@hki-jena.de

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and Falkow 1989). However, unlike pathogenic bacteria, which often develop unambiguous ways of causing host infections, the eukaryotic fungal pathogen *Candida albicans* has more advanced resources in order to cause disease and overcome host defences. *C. albicans* is highly adapted to humans as a commensal organism and, accordingly, has developed an effective battery of commensal factors to colonise host tissues and with the potential to cause disease. Therefore, *C. albicans* possesses attributes distinct from those of the closely related but non-commensal and non-pathogenic yeast *Saccharomyces cerevisiae* (Hube and Naglik 2001).

The virulence factors expressed or required by *Candida* spp., and in particular *C. albicans*, to cause infections will vary depending on the infection site (e.g. mucosal or systemic), the stage of infection, and the nature of the host response (Naglik et al. 2003a). Transition from harmless commensal to disease-causing pathogen is finely balanced and can be attributable to the delicate interplay of an extensive repertoire of virulence determinants selectively expressed under suitable predisposing conditions (Hube and Naglik 2001). It seems apparent that not only single factors but also a panel of virulence attributes are involved in the infective process. Although many factors have been suggested to be virulence attributes for *C. albicans*, hypha formation, the production of cell wall-associated surface adhesion/recognition molecules, invasion, phenotypic switching, and secreted protein production are still thought to be the most significant fungal processes (Calderone 2002; Nather and Munro 2008; Hruskova-Heidingsfeldova 2008). In this chapter, we shall specifically address the secreted proteins of *Candida* spp., their role in fungal virulence, and how the host responds to their presence.

5.2 The Secretory Pathway in Fungi

Fungal proteins destined for secretion are packaged along the secretory pathway to the cell surface where they ultimately become covalently or non-covalently linked to the cell membrane or cell wall or secreted from the cell. Newly synthesised mRNA is transferred from the nucleus to the cytoplasm and translated into a pre-protein, while transported into the endoplasmic reticulum (ER). Within the ER lumen the protein is folded and modified. Quality control of proteins at this stage determines whether the protein continues its path to the surface or, if misfolded or incorrectly modified, delivered to proteasomes (Hampton 2002) or vacuoles (Coughlan et al. 2004) for degradation. Proteins are then transported to the Golgi apparatus for further modification, where their ultimate destinations are determined (e.g. secreted and vacuolar). The Golgi apparatus in filamentous growing fungi are often found at apical regions of hyphae (Cole et al. 2000; Kuratsu et al. 2007) and as protein secretion is believed to mainly occur at hyphal apices, the data are consistent with a dominant distribution of ER/Golgi at hyphal tips that promote protein secretion. Vacuolar proteins are sorted at the Golgi apparatus and delivered to vacuoles via endosomes (Shoji et al. 2008). The vacuole has many functions, which

Table 5.1 Generic classes of *Candida* proteins destined for the cell wall and extracellular secretion

Class description	Cell wall moiety link	Linkage type	Ultimate destination
GPI protein	β-1,6-Glucan	Covalent	Cell wall
PIR protein	β-1,3-Glucan	Covalent	Cell wall
Mannosylated and non-mannosylated proteins	Not linked	Non-covalent; alkali-labile	Cell wall
Mannosylated and non-mannosylated proteins	Not linked	N/A	Secreted from cells

include storage, protein degradation, maintenance of cytosolic homeostasis, regulation of cell size and determination of branching frequency (Klionsky et al. 1990; Veses et al. 2009). In the final stage of the process the secreted and plasma membrane proteins are delivered by exocytosis from the Golgi apparatus and become incorporated into the plasma membrane, cell wall or are secreted from the cell.

5.3 The Secretory Proteins of *Candida* spp.

There are different classes of proteins in *Candida* spp. that are destined for the cell wall or secretion from the cell, which are transported via the secretory pathway (Table 5.1) (Chaffin 2008). The most abundant class are the glycophosphatidylinositol (GPI) proteins, which become either incorporated into the cell membrane (GPI-anchored proteins) or covalently linked to β-1,6-glucan structures in the cell wall (GPI-proteins). The second class are Pir proteins (proteins with internal repeats) and these become covalently linked to β-1,3-glucan structures in the cell wall. For more detailed information regarding the cell wall and proteins covalently linked to cell wall structures the reader is directed to Chap. 4. A third class of protein does not become covalently linked to any cell wall structure (non-covalent linkages), but given that their substrates are present in the cell wall remain wall-associated. The fourth and final class of proteins are directly secreted from the cell into the extracellular environment. In this Chapter, we shall concentrate on the latter two classes of secreted proteins.

5.4 Non-Covalently Linked Cell Wall Associated Secreted Proteins

The cell wall is a highly dynamic structure that is in constant flux as a result of polarised cell growth and in response to changes in local environmental conditions. Cell wall integrity is continually of fundamental importance and many of the major proteins that control alterations in cell wall structure and remodelling are the GPI-anchored or GPI- proteins and non-covalently wall-associated secreted proteins.

These include chitinases, glucanosyltransferases, transglucosidases and glucanases. Many of these are GPI-linked (e.g. Phr1, Phr2, Cht1, Cht2, Crh11, Crh12, Exg2, Pga4, Spr1, Utr2) and the reader is guided to an excellent recent review by Chaffin (2008) for more information regarding these proteins. Several non-covalently wall-associated proteins have also been identified and these include Kre9, Bgl2, Mp65, Xog1, Eng1, Sun41, Cht3, Cht4, and Atc1 (Chaffin 2008). All are proposed to belong to the glycoside hydrolase (GH) family of enzymes but only Atc1, which is an acid trehalose, does not play a role in cell wall remodelling/integrity. GH enzymes hydrolyse the glycosidic bond between carbohydrates or between carbohydrates and a non-carbohydrate moiety. Some of the functional roles of these non-covalently wall-associated enzymes have been deduced from mutant analysis and these are summarised in Table 5.2 and briefly below.

Kre9 is a member of the GH16 family and is required for β-1,6-glucan synthesis; β-1,6-glucan is essential for linking GPI proteins to the cell wall. Some fungal GPI-linked proteins are likely to have important roles in binding host receptors or other moieties; hence, defects in non-covalent wall-associated enzymes involved in β-1,6-glucan synthesis will probably have serious downstream effects with regard to host-pathogen interactions.

Bgl2 (like Mp65) belongs to the GH17 family and is a β-1,3-glucosyltransferase that catalyses β-1,3-glucan digestion (removing a disaccharide), thereby permitting β-1,3-glucan side chain elongation and β-1,6-glucan side chain linkages. Bgl2 probably contributes to general cell wall structure and remodelling.

Xog1 is an exo-β-1,3-glucanase and a member of the GH5 family, which has specificity for β-1,3-glucoside linkages (Stubbs et al. 1999). Xog1 is regulated by a protein phosphatase encoded by *SIT4* (Lee et al. 2004) and is upregulated (mRNA) and controlled during filamentation, but is not directly required for morphogenesis (Lee et al. 2004; Lotz et al. 2004; Murad et al. 2001).

Eng1 is an endo-β-1,3-glucanase and a member of the GH81 family, which catalyses the removal of glucose from the non-reducing end of β-1,3-glucan (Esteban et al. 2005). Eng1 probably plays a role in cell separation and is regulated with the cell cycle (Mulhern et al. 2006; Dunkler and Wendland 2007).

Mp65 is a likely β-glucanase (GH17 family) which cleaves β-glucans, usually β-1,3-glucans. Mp65 is required for hypha formation and can act as an adhesin (Sandini et al. 2007). *MP65* gene expression is responsive to a number of external stimuli and cell wall specific antifungals (Bennett and Johnson 2006; Castillo et al. 2006; Liu et al. 2005; Bensen et al. 2004) and it is thus thought to contribute to general cell wall structure, remodelling and metabolism. Sun41 is also a probable β-glucanase and appears to be regulated at the gene level by numerous external stimuli and during morphogenesis (Castillo et al. 2006; Liu et al. 2005; Setiadi et al. 2006; Lotz et al. 2004). Sun41 is involved in general cell wall integrity, structure and cell separation.

C. albicans possesses four chitinases (Cht1-4), which are members of the GH18 family. Cht1 and Cht2 are GPI-anchored, Cht3 is secreted but cell wall associated, and Cht4 is probably a cytoplasmic protein (Chaffin 2008). Cht3 is the major chitinase of *C. albicans* (Dunkler and Wendland 2007; Selvaggini et al. 2004)

Table 5.2 Phenotypes and functional roles of *C. albicans* non-covalent cell wall associated secreted proteins

Protein	Phenotype of null mutant	Pathogenicity studies	References
Kre9	• Poor growth on many carbon sources • Unviable when grown on glucose • Reduced β-1,6-glucan on galactose • No hypha production in serum	• Null mutant attenuated in systemic model	Lussier et al. (1998), Norice et al. (2007)
Bgl2	• Accounts for 50% of β-1,3-glucosyltransferases activity in cell wall • No change in β-1,3-glucan or 1,6-glucan content. Possibly increased chitin • Sensitive to Nikkomycin (chitin synthesis inhibitor) • Contributes to adhesion?	• Not tested	Sarthy et al. (1997)
Xog1	• Accounts for the majority of exo-β-1,3-glucanase in the cell wall • Yeast and hyphal growth unaffected • Minor increase in sensitivity to chitin and glucan synthesis inhibitors	• No attenuation in systemic model	Gonzalez et al. (1997)
Eng1	• Yeast and hyphal growth unaffected • Separation defect after cytokinesis	• Not tested	Esteban et al. (2005)
Mp65	• Yeast growth unaffected • Severely impaired morphogenesis • Reduced adherence to polystyrene	• Null mutant attenuated in systemic model • Immunisation partially protects against infection in vivo	Sandini et al. (2007), De Bernardis et al. (1997)
Sun41	• Defective morphogenesis, cell separation • Loss of viability in stationary phase • Sensitive to caspofungin and Congo Red • Unaffected by Nikkomycin or calcofluor white	• Null mutant attenuated in systemic and oral models • Defective biofilm formation • Reduction in adherence to Caco-2, but not to FaDu or HUVEC cells	Firon et al. (2007), Hiller et al. (2007), Norice et al. (2007)
Cht3	• 60% reduction in yeast chitinase activity • 80% reduction in hyphal chitinase activity • Possible increase in chitin content • Sensitive to calcofluor white • Separation defect	• Not tested	Selvaggini et al. (2004)
Atc1	• Does not grown on trehalose • Resistant to heat, osmotic and oxidative stress • Reduced germ tube formation	• Null mutant attenuated in systemic model	Pedreno et al. (2007)

and present in both yeast and hyphal forms, but has predominant activity in hyphae. Cht3 plays a role in cytokinesis, which is daughter cell driven.

Atc1 is a member of the GH65 family and is one of two *C. albicans* enzymes (the other being neutral trehalase (Ntc1) that is cytosolic) that hydrolyses trehalose, which is a non-reducing disaccharide (Pedreno et al. 2004, 2007). Atc1 is the only secreted cell wall associated protein identified so far that does not play a direct role in cell wall structure or integrity. Instead, Atc1 appears to contribute to resistance of *C. albicans* to oxidative stress.

Although many phenotypic studies have been undertaken on mutants lacking the above enzymes, few pathogenicity studies exist. However, when performed, virulence was always assessed in murine models of disseminated infection, except for one study with the *sun41* null mutant that assessed virulence in a mucosal model also (Table 5.2). Therefore, the role of these cell wall associated enzymes in mucosal infections is still largely unknown and no data exists regarding the precise mechanism by which these mutants are attenuated or how the host responds to these proteins.

5.5 *Candida* Proteins Secreted from the Cell

Most of the known extracellular proteins produced by *Candida* spp. are hydrolytic enzymes. Hydrolytic enzymes contribute to the pathogenicity of not just pathogenic yeasts and fungi (Ogrydziak 1993; Naglik et al. 2003a), but also bacteria (Finlay and Falkow 1989) and protozoa (McKerrow et al. 1993). In *C. albicans* hydrolytic enzyme production can be classified into three main families: secreted aspartyl proteinases, phospholipases, and lipases. These proteins are thought to have a range of biological functions ranging from basic nutritional needs, subtle alterations of the host cell for the benefit of fungal adhesion and invasion, and direct host cell toxicity.

5.5.1 Candida Secreted Aspartyl Proteinases

All proteinases hydrolyse peptide bonds (CO–NH) in proteins and are characterised on the catalytic mechanism of their active site. Based on the catalytic mechanism they are classified into serine, cysteine, aspartyl (aspartic), and metalloproteinases (Barrett and Rawlings 1991). Extracellular proteinases of saprophytic fungi such as *Aspergillus niger* or *Neurospora crassa* are primarily secreted to provide nutrients for the cells. However, pathogenic fungi have adapted this biochemical property to fulfil more specialised functions during the infective process in addition to nutrient acquisition (Naglik et al. 2003a). *Candida* spp. only possess extracellular proteinases of the aspartyl family. These are more commonly referred to as secreted aspartyl proteinases, or Saps, and have been extensively investigated in the past two

decades. *C. albicans* boasts the greatest number of proteinases (ten), but it is not the only species that produces Saps. *C. dubliniensis* possesses eight *SAP* genes, but lacks *SAP5* and *SAP6*, leaving it with only one member (*SAP4*) of the *SAP4–6* sub-family that is present in *C. albicans* (Moran et al. 2004). *C. tropicalis* is thought to possess four *SAP* genes (Zaugg et al. 2001), whereas *C. parapsilosis* possesses at least three *SAP* genes (de Viragh et al. 1993; Trofa et al. 2008), two of which remain largely uncharacterised (Merkerova et al. 2006). However, annotation of the *C. tropicalis and C. parapsilosis* genomes is ongoing and a more definitive number of *SAP* genes will soon become available. *C. glabrata* possesses multiple orthologs of the *S. cerevisiae* GPI-linked aspartyl proteinases (the yapsins), but little extra-cellular enzyme activity has been detected (Kaur et al. 2007). Limited information is available regarding the presence of *SAP* genes or extracellular enzyme activity in other *Candida* spp.

5.5.1.1 *Candida* Secreted Aspartyl Proteinases: Processing, Activation and Structure

In *C. albicans* the transcription of the *SAP* genes (specifically *SAP2*) is initiated by the transcription factor Stp1 (Martinez and Ljungdahl 2005). Other transcription factors that specifically target the *SAP* gene family in a morphologically-independent manner are likely to be involved. For example, *SAP4–6* have been shown to be regulated by Efg1 and it has been speculated that the attenuated virulence potential of an *efg1* mutant is at least partially due to reduced *SAP4–6* expression (Felk et al. 2002). The transcription factors that activate *SAP* gene expression in other *Candida* spp. are currently unknown, although Stp1 homologs are likely to exist. Assuming that a similar process occurs in all *Candida* spp., each *SAP* gene encodes a preproenzyme approximately 60 amino acids longer than the mature enzyme. The N-terminal secretion signal is cleaved by the signal peptidase in the ER (von Heijne 1985). The resulting proenzyme is then further processed after Lys-Arg sequences by the Kex2 proteinase (Togni et al. 1996), alternative proteinases or by autocatalysis (Togni et al. 1996; Newport and Agabian 1997; Koelsch et al. 2000; Beggah et al. 2000). The mature enzyme (ranging from 340 to 544 amino acids in length) contains sequence motifs typical for all aspartic proteinases, which includes two conserved aspartic acid residues in the active site and conserved cysteine residues implicated in the maintenance of the three-dimensional structure. Most Saps contain few putative *N*-glycosylation sites and debate remains with regard to whether all Saps become glycosylated (Hube and Naglik 2002; Naglik et al. 2003a). However, it is clear that *C. albicans* Sap9 and Sap10 contain several *N*-glycosylation sites and are in fact heavily glycosylated (Albrecht et al. 2006). Once activated, the enzymes are packaged into secretory vesicles, transported to the surface, and are either incorporated into the membrane via a GPI-anchor or linked to glucan as GPI-proteins (*C. albicans* Sap9 and Sap10), or secreted from the cell (*C. albicans* Sap1–8). Sap1–3, Sap4–6 and Sap9–10 constitute three major sub-families within the *C. albicans SAP* gene family (Fig. 5.1).

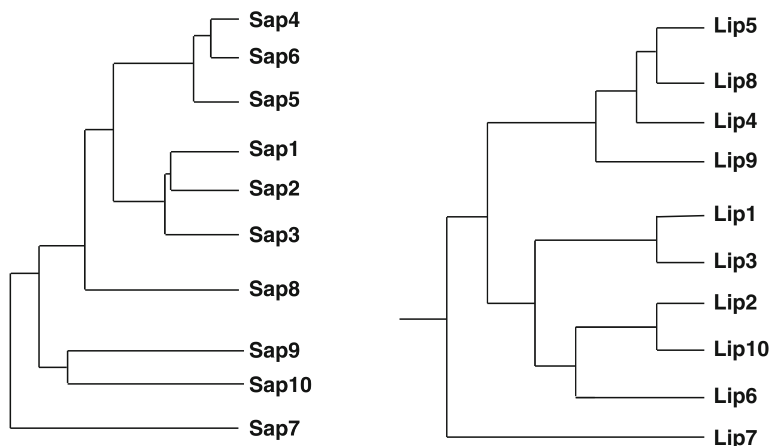

Fig. 5.1 Dendrogram of the *C. albicans* Sap and Lipase families. The Sap family can be clustered into three distinct groups. Sap1–3 are up to 67% identical, Sap4–6 up to 89% identical, while Sap7 is only 20–27% identical to other Saps. Sap9 and Sap10 both have C-terminal consensus sequences typical for GPI-linked proteins. Similar families, but with fewer numbers, exist in *C. dubliniensis* and *C. tropicalis*. All *LIP* genes encode lipases with high similarities to each other and with the same overall structure. Amino acid sequence identity ranges from 33 (between Lip2 and Lip7) to 80% (between Lip5 and Lip8). When clustered, the Lip isoenzyme family can be divided into two subgroups. Lip4, Lip5, Lip8 and Lip9, which were more than 73% identical to each other, and Lip1, Lip2, Lip3, Lip6 and Lip10, which were at least 54% identical to each other. Lip7 was the most divergent lipase in this isoenzyme family

Initial structural studies of the *C. albicans* proteinase family concentrated on Sap2 (Cutfield et al. 1995; Abad-Zapatero et al. 1996), which is the most abundant secreted protein in vitro when grown in the presence of protein as the sole source of nitrogen (Hube et al. 1994; White and Agabian 1995). More recently, the structures of Sap1, Sap3 and Sap5 have also been determined (Borelli et al. 2007; Borelli et al. 2008). The secondary structures of Sap1–3 and Sap5 are highly conserved, but Sap5 differs from Sap1–3 in the entrance to the active site cleft and in its overall electrostatic charge. With the structures of nearly half the *C. albicans* Sap proteins resolved, and potentially structures of Saps in other species to be completed, it should finally become feasible to design specific inhibitors for different Sap sub-families to more fully explore the contribution of these important extracellular enzyme families in fungal pathogenesis.

5.5.1.2 *Candida* Secreted Aspartyl Proteinases: Role in Pathogenicity

The restriction of proteinase families to only the most pathogenic *Candida* spp. is indicative of the potential roles and functions of these enzymes in fungal pathogenicity. Most of the pathogenicity-related data over the past two decades has been deduced from in vitro and in vivo experiments using predominantly *C. albicans*. One of the distinct features of the *C. albicans* Saps is the range of pH activity

(2.0 and 7.0) within the family. Although not directly proven, this versatile property is thought to be necessary for the success of *C. albicans* as an opportunistic pathogen by allowing the fungus to adapt to, colonise and infect a variety of different mucosal surfaces and internal organs.

An obvious function for the proteinases is nutrient acquisition, as a result of degradation of complex proteins to peptides for cell uptake and utilisation. In support of this is the broad substrate specificity of the *Candida* Saps (notably Sap2), which are able to digest a whole host of human proteins that are found on mucosal surfaces (e.g. mucin, extracellular matrix proteins) (Naglik et al. 2003a). Sap activity probably also contributes to the evasion of host defences by degrading these and other immune-related molecules such as secretory IgA, salivary lactoferrin, lactoperoxidase, cathepsin D (an intracellular lysosomal enzyme of leukocytes), α_2-macroglobulin (a natural proteinase inhibitor in human plasma), cystatin A (a cysteine proteinase inhibitor found in human epidermal tissues and fluids), and complement (Naglik et al. 2003a). Furthermore, Sap2 can activate the proinflammatory cytokine IL-1 β from its precursor suggesting roles for the *Candida* proteinases in the activation and maintenance of the inflammatory response at epithelial surfaces. Such wide-ranging proteolytic activity is likely to assist or promote *C. albicans* colonisation and infection.

Numerous strategies have been employed to assess the role of the *Candida* Saps in pathogenicity, but two of the most common and effective strategies include the analysis of *SAP* gene expression and functional studies assessing the modulation of virulence using *SAP*-disrupted mutants or aspartyl proteinase inhibitors (Table 5.3) (Naglik et al. 2003a, 2004; Schaller et al. 2005a). For *C. albicans*, the conclusions drawn from the functional studies indicate that the Sap1–3 sub-family contribute predominantly to mucosal infections and the Sap4–6 family to systemic infections (Naglik et al. 2003a). Expression studies, however, do not always conform to such neat segregation of function and the data can often be difficult to interpret. This is likely due to the fact that the *SAP* gene family is co-regulated with other virulence attributes including adhesion (Sap1–3) (Schaller et al. 1999, 2003a; Korting et al. 1999; Borg-von Zepelin et al. 1998, 1999; Bektic et al. 2001; Ghannoum and Abu Elteen 1986), phenotypic switching (Sap1 and Sap3) (Kvaal et al. 1999; Morrow et al. 1992, 1994; White and Agabian 1995; Hube et al. 1994) and the yeast-hypha transition (Sap4–6) (White and Agabian 1995; Hube et al. 1994; Schweizer et al. 2000; Schroppel et al. 2000; Stoldt et al. 1997; Felk et al. 2002; Staib et al. 2002). Therefore, it is unsurprising that diverse *SAP* gene expression profiles are detected when different models are employed and several experimental conditions assessed. Recently, two studies have questioned the role of Sap1–3 in mucosal infections as no differences in *SAP1–3* expression (Naglik et al. 2008) or activation (Lermann and Morschhauser 2008) were evident during infection or invasion of organotypic oral and vaginal models over a time course of 24 h. Likewise, in both studies, no differences in cell damage were observed when *sap1–3* null mutants were compared with the wild type. Interestingly, the only *SAP* gene that does appear to be consistently upregulated or activated in both mucosal and systemic infections is *SAP5* (Naglik et al. 2003a, 2008; Lermann and Morschhauser 2008; Staib et al. 2000).

Table 5.3 Functional roles of *C. albicans* extracellular secreted proteins

Gene[a]	Functional role[b]	Host response	References
SAP1	• Adhesion to buccal epithelial cells • Promoting tissue damage in an organotypic oral epithelial model • Promoting virulence in mucosal (rat) and disseminated infections	• IgA induction • Cytokine induction?	Hube et al. (1997), Borg-von Zepelin et al. (1999), Watts et al. (1998), Schaller et al. (1999), De Bernardis et al. (1999a), Sanglard et al. (1997), Millon et al. (2001), Drobacheff et al. (2001)
SAP2	• Adhesion to buccal epithelial cells • Promoting tissue damage in an organotypic oral epithelial model and in endothelial cells • Promoting virulence in mucosal (rat) and disseminated infections	• IgA induction • Cytokine induction? • Immunisation partially protects against infection or colonisation in vivo	Hube et al. (1997), Borg-von Zepelin et al. (1999), Watts et al. (1998), Schaller et al. (1999), De Bernardis et al. (1999a), Sanglard et al. (1997), Cassone et al. (1999), Ibrahim et al. (1998), Korting et al. (1999), Millon et al. (2001), Drobacheff et al. (2001), Schaller et al. (2005b), Rahman et al. (2007), De Bernardis et al. (1997)
SAP3	• Adhesion to buccal epithelial cells? • Promoting tissue damage in an organotypic oral epithelial model? • Promoting virulence in mucosal (rat) and disseminated infections?	No data available	Hube et al. (1997), Borg-von Zepelin et al. (1999), Watts et al. (1998), Schaller et al. (1999), De Bernardis et al. (1999a), Sanglard et al. (1997)
SAP4	• Inhibition of adhesion to buccal epithelial cells? • Promoting virulence in murine peritoneal and disseminated infections	• Evasion of phagocytic killing by macrophages	Hube et al. (1997), Sanglard et al. (1997), Kretschmar et al. (1999), Borg-von Zepelin et al. (1999), Staib et al. (2002), Borg-von Zepelin et al. (1998)
SAP5	• Inhibition of adhesion to buccal epithelial cells? • Promoting virulence in murine peritoneal and disseminated infections • Degradation of epithelial cell junction protein E-Cadherin	• Evasion of phagocytic killing by macrophages	Hube et al. (1997), Sanglard et al. (1997), Kretschmar et al. (1999), Borg-von Zepelin et al. (1999), Staib et al. (2002), Borg-von Zepelin et al. (1998), Villar et al. (2007)
SAP6	• Inhibition of adhesion to buccal epithelial cells? • Promoting virulence in murine peritoneal and disseminated infections	• Evasion of phagocytic killing by macrophages • IgA induction	Hube et al. (1997), Sanglard et al. (1997), Kretschmar et al. (1999), Borg-von Zepelin et al. (1999), Staib et al. (2002), Borg-von Zepelin et al. (1998), Felk et al. (2002), Millon et al. (2001), Drobacheff et al. (2001)

SAP7	• Potentially contributes to disseminated infection but not vaginal infections	No data available	Taylor et al. (2005)
SAP8	• No data available	No data available	N/A
PLB1	• Potentially promotes translocation across the murine gut wall	No data available	Mukherjee et al. (2001)
PLB2	• No data available	No data available	N/A
LIP1-7, 9-10	• No data available	No data available	N/A
LIP8	• Contributes to disseminated infection	No data available	Gacser et al. (2007b)
Cp*LIP1-2*	• Contributes to biofilm formation • Promote tissue damage in oral epithelial model • Protect against macrophage killing • Enhance virulence in murine intraperitoneal model	No data available	Gacser et al. (2007c)

N/A Not applicable

[a]Genes are from *C. albicans* unless stated (Cp: *C. parapsilosis*)

[b]Data observations from experiments using null mutants and proteinase inhibitors. Data were often obtained using double or triple mutants, thus the listed function is probably due to only a single member (or potentially two) of the sub-family

Together with the recent finding that *C. albicans* Sap5 is able to degrade E-cadherin on epithelial cells (Villar et al. 2007; Frank and Hostetter 2007) the data suggest that Sap5 might be an important proteinase in promoting *Candida* pathogenicity at mucosal surfaces.

Studies using HIV proteinase inhibitors (ritonavir, indinavir, saquinavir) and pepstatin A, which inhibit general aspartyl proteinase activity, also indicate a role for the *C. albicans* Saps in adherence to buccal epithelial cells and causing mucosal tissue damage (Table 5.3) (Borg-von Zepelin et al. 1999; Korting et al. 1999; Bektic et al. 2001), but no role in interfering with phagocytic killing (Bektic et al. 2001). In vivo experiments also indicated beneficial effects of pepstatin A and the HIV aspartyl inhibitors on mucosal *C. albicans* infections (Cassone et al. 1999; De Bernardis et al. 1997, De Bernardis et al. 1999b) but not systemic infections (Edison and Manning-Zweerink 1988; Fallon et al. 1997) (Table 5.3). With regard to other *Candida* spp. the proteinase inhibitor pepstatin A has been shown to block the penetration of *C. parapsilosis* through mucosal surfaces and can reduce histopathological alterations during experimental cutaneous candidiasis (Gacser et al. 2007a; Schaller et al. 2003b), but little data exists regarding other *Candida* spp.

In summary, it is highly probable that the main role of the *Candida* proteinases is to provide nutrition for the cells. However, these enzymes probably also contribute to fungal penetration, invasion and immune evasion, depending on the species. Given that the precise biochemical and proteolytic properties of many of the *Candida* proteinases are still unknown, further studies are clearly warranted in order to determine the full functional repertoire of the Sap family during *Candida* infections.

5.5.2 Candida Secreted Phospholipases

The term phospholipase (Pl) describes a heterogeneous group of enzymes capable of hydrolysing one or more ester linkages in glycerophospholipids. Pl's have been implicated as putative virulence factors in many bacterial, fungal and protozoan infections as phospholipid molecules are major components of host cell membranes. Depending on the target within the phospholipid molecule and the mode of action, in *C. albicans* these enzymes can be divided into four different subclasses: PlA (Banno et al. 1985), PlB (Barrett-Bee et al. 1985; Hoover et al. 1998; Sugiyama et al. 1999), PlC (Pugh and Cawson 1977; Bennett et al. 1998) and PlD (Hube et al. 2001; Kanoh et al. 1998). Although early studies using the egg yolk based assay showed that only *C. albicans* isolates possessed Pl activity (Lane and Garcia 1991), more recent studies indicate that non-*C. albicans* spp. such as *C. glabrata, C. parapsilosis, C. tropicalis, C. lusitaniae* and *C. krusei* also secrete Pl's (Ghannoum 2000). *C. albicans* contains genes for at least three classes of Pl: *PLB1-5*, *PLC1-3*, *PLD1* (d'Enfert et al. 2005). Unlike PlC1-3 and PlD1, only the PlB's are thought to be secreted extracellularly (Schaller et al. 2005a; Hube and Naglik 2002). Three members of the PlB protein family (PlB3-5) contain putative GPI anchors, so here we will only discuss the secreted PlB1 and PlB2 enzymes (Table 5.3).

PlB's from *C. albicans* have significant homology to PlB's from *S. cerevisiae* and *Schizosaccharomyces pombe* (Ghannoum 2000). *C. albicans PLB1* encodes a protein 605 amino acids in length and contains *N*-glycosylation sites and potential tyrosine phosphorylation sites (Leidich et al. 1998). *C. albicans PLB2* encodes a protein 609 amino acids in length and contains six *N*-glycosylation sites (Sugiyama et al. 1999). Like the Saps, PlB1 and PlB2 follow the same pathway of secretion from the cell. However, unlike the Saps, the structures of the *C. albicans* PlB's have not yet been resolved.

5.5.2.1 *Candida* Phospholipases: Role in Pathogenicity

PlB is produced in both yeast and hyphal cells, particularly at the growing tip of hyphae (Pugh and Cawson 1977; Leidich et al. 1998) and their main substrates are proposed to be host phospholipids and lysophospholipids. PlB production or gene expression has been detected in vivo in murine dissemination (Leidich et al. 1998), gastrointestinal (Mukherjee et al. 2001; Schofield et al. 2003) and oral (Ripeau et al. 2002a) models, in addition to human mucosal infections (Naglik et al. 2003b). Given that PlB1 accounts for the vast majority of the extracellular PlB activity in *C. albicans*, most pathogenicity studies have targeted PlB1. Virulence of *C. albicans* mutants lacking *PLB1* is significantly attenuated in systemic (Leidich et al. 1998) and intragastric (Ghannoum 2000) models, and reintroduction of *PLB1* into *C. albicans* has been shown to restore virulence in a murine disseminated model (Mukherjee et al. 2001). No data has been published evaluating the *plb2* null mutant in animal models. In addition, β-blocker-like compounds, which inhibit Pl activity, are able to prevent fatality in mice that have been inoculated with lethal doses of *C. albicans* (Hanel et al. 1995), seemingly through inhibiting *C. albicans* tissue penetration. However, no beneficial effect was observed after exposure to fungicidal concentrations of the cell wall targeting antifungal caspofungin (Ripeau et al. 2002b).

Together, although the data implicates a role of PlBs in *C. albicans* infections, their precise functions are still not known. Nonetheless, probable roles include disruption of host membranes to aid cell penetration and adhesion to epithelial cells. Finally, very little data is available with respect to the role of Pl's in other *Candida* spp. and it remains to be determined whether they play a significant role in those respective infections (Trofa et al. 2008).

5.5.3 *Candida*-Secreted Lipases

Compared with the proteinases and phospholipases, the secreted lipases of *C. albicans* have been less intensively studied, despite the fact that lipases of non-pathogenic *Candida* spp. are commonly used in biotechnology. Like esterases, lipases are able to catalyse the hydrolysis of ester bonds of mono-, di- and triacylglycerols or even phospholipids. Extracellular lipase activity in *C. albicans* was described over four decades ago (Werner 1966), while secreted esterase

activity (lipolytic activity on soluble lipids) was more recently characterised (Tsuboi et al. 1996). In *C. albicans* ten *LIP* genes are present (Fig. 5.1), but *LIP7* lacks a secretion signal. *LIP1* was identified just over a decade ago (Fu et al. 1997) with the remaining nine genes characterised in 2000 (Hube et al. 2000). Sequences similar to *C. albicans LIP1-10* are also present in other pathogenic *Candida* spp. such as *C. tropicalis*, *C. parapsilosis* and *C. krusei*, but not in *C. glabrata* or *S. cerevisiae* (Fu et al. 1997; Hube et al. 2000). In *C. parapsilosis*, two lipase genes, Cp*LIP1* and Cp*LIP*2, have been identified, although only Cp*LIP2* codes for an active protein (Brunel et al. 2004; Neugnot et al. 2002). Extracellular lipase enzyme activity has also been detected in most other pathogenic *Candida* spp. (Bramono et al. 2006). The *C. albicans* lipases have 80% identity at the protein level and are between 426 and 471 amino acids in length. Each lipase contains four conserved cysteine residues and putative *N*-glycosylation sites. Like the Pl's, no structural information has yet been resolved from the *Candida* lipases.

5.5.3.1 *Candida* Lipases: Role in Pathogenicity

Few studies have assessed the role of lipases in pathogenicity of *Candida* spp., although recently a number of studies dealing with lipases as potential virulence factors of *Candida* spp. have been published. *LIP* gene expression can be detected during the yeast-to-hypha transition (Hube et al. 2000), but debate remains whether expression of the family is associated specifically with the morphological switch, even though *LIP8* disrupted mutants have been shown to produce more hyphal growth (Gacser et al. 2007b). Studies have demonstrated *LIP* gene expression during murine intraperitoneal infections (Hube et al. 2000) and in patient oral samples (Stehr et al. 2004), and others have shown significant reduction in virulence of specific null mutants (*lip8*) indicating a potential role in virulence (Gacser et al. 2007b). Finally, the *C. albicans* lipases appear to directly induce cytotoxicity in mammalian macrophages and hepatocytes and promote the deposition of lipid droplets in the cytoplasm (Paraje et al. 2008). In *C. parapsilosis*, data from functional studies using lipase inhibitors and Cp*LIP1* and *LIP2* disrupted mutants indicated that the *C. parapsilosis* lipases contribute to biofilm formation, resistance to macrophage killing, tissue damage in experimental infection models of reconstituted human oral epithelium, and virulence in a murine intraperitoneal model (Gacser et al. 2007a, 2007c). Therefore, putative roles in pathogenicity may include the digestion of lipids for nutrient acquisition, adhesion to host tissues, lysis of competing microflora and evasion of host inflammatory processes (Trofa et al. 2008).

5.6 Other Secreted Enzymes

A number of other secreted enzymes have been identified in *Candida* spp., predominantly *C. albicans*. These include extracellular phosphatases (Pho100, Pho112, Pho113), glucoamylases (Gca1, Sga1) and β-*N*-acetylhexoaminidase (Hex1)

(Chaffin 2008; Hruskova-Heidingsfeldova 2008). Most of the data available relate to their gene expression phenotypes in culture, although *GCA1* expression has been detected during rat oral infections (Sturtevant et al. 1999) and the *hex1* null mutant is less pathogenic in a mouse model than the wild type parent (Jenkinson and Shepherd 1987). No other pathogenicity or immunologically related functional data are available.

5.7 The *Candida* Proteome and secretome

Using computer-based prediction algorithms, Lee et al (2003) identified 495 ORFs that were predicted to encode proteins with *N*-terminal signal peptides. In the set of 495 deduced proteins with *N*-terminal signal peptides, 350 were predicted to have no transmembrane domains (or a single transmembrane domain at the extreme *N*-terminus) and 300 of these were predicted not to be GPI-anchored. After eliminating proteins with mitochondrial targeting signals the final computationally-predicted *C. albicans* secretome was estimated to consist of up to 283 ORFs. However, few of these proteins have been experimentally shown to be secreted by *C. albicans*.

Monteoliva et al. (2002) used a systematic approach to identify secreted proteins in *C. albicans*. In this genetic screening protocol in-frame fusions with an intracellular allele of the *S. cerevisiae* invertase gene *SUC2* were used to select and identify putatively exported proteins in the heterologous host *S. cerevisiae*. Eighty-three clones were selected that contained sequences conferring protein export. Comparing these sequences with the genome assembly 6, 11 of the sequences were found to correspond to known sequences encoding proteins with predicted *N*-terminal signal sequences (Chaffin 2008). Currently in the Candida Genome Database (http://www.candidagenome.org/) 63 of these sequences have been identified (Chaffin 2008).

Several more proteins have been isolated from culture supernatants using proteomics approaches (summarised in (Chaffin 2008)). Many of these are not considered to be proteins that enter the secretory pathway and some are in fact prototypic cytoplasmic proteins. These include abundant glycolytic enzymes such as Tdh1, Tdh2, and Tdh3 (Klis et al. 2002). Other proteins such as members of the heat shock protein (Hsp) family are also frequently found extracellularly. However, it is not clear whether these proteins originate from lysed cells or, as frequently claimed, are exported by a non-conventional secretory mechanism (Klis et al. 2002). Irrespective of whether these proteins are secreted or not, it is possible that these proteins do play an extracellular role for a given *C. albicans* cell population during interactions with the host.

5.8 The Host Response to *Candida* Secreted Proteins

While there have been a plethora of studies evaluating the virulence properties of *Candida* secreted proteins, particularly the Saps, surprisingly few studies have investigated the host response to these proteins. Global proteomic studies

evaluating the host response to *C. albicans* indicate that this fungus induces strong immune responses (Pitarch et al. 2006; Rupp 2004; Diez-Orejas and Fernandez-Arenas 2008), but only a handful of studies have specifically targeted the analysis of host responses to the *Candida* secreted proteins, and most of these are based on the proteinase family (e.g. Sap2) and Mp65.

In humans, proteinase-specific IgG antibodies have long been detected in sera of patients with disseminated candidiasis (Macdonald and Odds 1980; Ray and Payne 1987). Likewise, in mucosal infections total IgA levels against the *C. albicans* proteinases (Sap1, Sap2 and Sap6) were raised during fungal infection and this was related to their HIV status (Millon et al. 2001; Drobacheff et al. 2001). The latter study also indicated that variations in *C. albicans* colonisation levels in the oral cavity and episodes of oropharyngeal candidiasis correlated with variations in salivary anti-Sap6 IgA antibody levels (Millon et al. 2001)

In experimental models of infection using organotypic vaginal epithelium the addition of the proteinase inhibitor pepstatin A strongly reduced the cytokine response (interleukin-1α IL-1 β, IL-6, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF), tumour necrosis factor (TNF)-α) initiated by *C. albicans*. Furthermore, *sap1* and *sap2* null mutants (but not a *sap4–6* null mutant) induced significantly reduced levels of cytokine expression compared to the wild type strain (Schaller et al. 2005b). This indicates that the epithelium-induced proinflammatory cytokine response correlates with the presence of *C. albicans* Sap1 and Sap2. However, it is unclear whether the host response was the consequence of tissue damage caused by hyphal invasion or the presence of Sap1 and Sap2. Another in vitro study demonstrated that a *sap4–6* null mutant (but not *sap1–3* mutant) was killed approximately 50% more effectively after contact with macrophages than the wild-type strain (Borg-von Zepelin et al. 1998). These data, together with virulence experiments assessing the *sap* null mutants in animal and experimental models (Table 5.3), were the foundation on which the proposal was suggested that Sap1–3 contribute to mucosal infections and Sap4–6 to systemic infections (Naglik et al. 2003a).

In vivo animal model data suggests that immunisation with Sap2 is able to partially protect against *C. albicans* infection in rat model (De Bernardis et al. 1997) or accelerate clearance of *C. albicans* in a murine oral and vaginal colonisation model (Rahman et al. 2007). Furthermore, administration of an anti-Sap2 monoclonal antibody or anti-Sap2 antibody-containing vaginal fluids, partially protected rats against candidal vaginitis (De Bernardis et al. 1997). However, it is unlikely that direct enzyme neutralisation accounts for the mode of protection (Naglik et al. 2005). Furthermore, cross-reactivity of the anti-Sap2 antibodies with other proteinases such as Sap1 and Sap3 (White et al. 1993; White and Agabian 1995; Smolenski et al. 1997) may contribute to the protective nature of these antibodies. Similar protective responses were observed against the β-glucanase Mp65 of *C. albicans* (De Bernardis et al. 1997). Although the mechanism of protection induced by Sap2 and Mp65 is not fully known, it appears to be T-cell dependent (Nisini et al. 2001). Little information is yet available concerning a protective role of Sap antibodies against systemic *Candida* infections.

5.9 Conclusion

Of the *Candida* secreted proteins studied to date, it is evident that they potentially play important roles in fungal pathogenicity. Furthermore, being present in the cell wall or directly secreted from the cell, these secreted proteins are likely to also interact with or modulate host surface moieties. The production of a number of hydrolases is a highly regulated and tightly controlled process and suggests that these enzymes are adapted to and contribute to the complex pathogenicity of *Candida* infections. The proteinases in particular appear to be key virulence attributes of *C. albicans* and are known to assist this species in the colonisation and invasion of host tissues. It is likely, however, that only a small portion of the total secretome of *Candida* spp. has been identified and in the next decade or two more secreted proteins will undoubtedly be identified and functions assigned to them. Only then will we be able to fully appreciate the importance of this class of proteins in *Candida* biology and pathogenicity, which may provide vital information as to why this fungal pathogen is such a successful coloniser of humans. This, in turn, may lead to the development of new prophylactic and therapeutic strategies targeting these secreted enzymes, which would be a valuable addition to the limited repertoire of antifungal agents currently available.

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Chapter 6
Yeast Biofilms

Gordon Ramage, Eilidh Mowat, Craig Williams, and Jose L. Lopez Ribot

Abstract Yeast biofilms are an escalating clinical problem, which affect both the healthy and immunocompromised, and are related to significant rates of mortality within hospitalized patients. *Candida albicans* is the most notorious yeast biofilm former and as a result the most widely studied; however, other *Candida* species and yeasts such as *Cryptococcus neoformans* are also implicated in biofilm-associated infections. Yeast biofilms have distinct developmental phases, including adhesion, colonization, maturation and dispersal, which have been examined utilizing various in vitro and in vivo model systems. Furthermore, the complex molecular events governing biofilm development are slowly being elucidated, including the role of quorum sensing. Clinically, biofilms act as reservoirs for systemic infection, and also induce localized pathology and tissue damage. However, the key virulence factor is their recalcitrance to antifungal therapy. This chapter will discuss our current understanding of the role that yeast biofilms play in the clinical setting.

6.1 Introduction

Infections caused by yeasts represent an escalating problem in health care as advances in modern medicine prolong the lives of severely ill patients, including HIV-infected, cancer, transplant, surgical, and ICU patients, but also newborn

G. Ramage (✉) and E. Mowat
Section of Infection and Immunity, Glasgow Dental School, Faculty of Medicine, University of Glasgow, 378 Sauchiehall Street, Glasgow, G2 3JZ, UK
e-mail: g.ramage@dental.gla.ac.uk

C. Williams
Microbiology Department, Royal Hospital for Sick Children, Yorkhill Division, Glasgow, G3 8SJ, UK

J.L. Lopez Ribot
Department of Biology and South Texas Center for Emerging Infectious Diseases, The University of Texas at San Antonio, San Antonio, TX, USA

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infants. Use of broad spectrum antibiotics, neutropenia, parenteral nutrition, indwelling catheters, immuno-suppression and disruption of mucosal barriers due to surgery, chemotherapy and radiotherapy are among the most important predisposing factors for these infections (Calderone 2002; Odds 1988). As fungi are eukaryotic cells and more complex than bacteria, these infections are often difficult to diagnose and treat, and carry unacceptably high mortality rates. In addition, the ability of yeasts to exist as biofilms further confounds this difficulty in clinical management. *Candida* species are among the most common etiologic agents of yeast-related biofilm infections (Kumamoto and Vinces 2005). However, infections due to other yeasts have been implicated in biofilm-related pathogenesis, such as *Cryptococcus, Blastoschizomyces, Malassezia*, *Trichosporon,* and *Saccharomyces* (Cannizzo et al. 2007; D'Antonio et al. 2004; Di Bonaventura et al. 2006; Reynolds and Fink 2001; Walsh et al. 1986). The clinical impact of biofilm infections and the increasing body of knowledge relating to yeast biofilms will now be addressed.

6.2 Clinical Significance

Clinically, yeast biofilms are an increasingly significant problem. Biofilms provide a safe sanctuary and act as reservoirs for persistent sources of infections, and it is clear that yeast biofilms adversely impact the health of an increasing number of immunocompromised patients, with soaring associated costs. Understanding the role of yeast biofilms during infection should help the clinical management of these recalcitrant infections. These sessile structures can form on a wide variety of implanted medical devices, and the various substrates play key roles in the ability of the yeast to form biofilms (Kojic and Darouiche 2004). Examples of implant-related yeast infections are detailed in Table 6.1. Among the pathogenic yeasts, *C. albicans*, a normal commensal of human mucosal surfaces and opportunistic pathogen in immunocompromised patients, is most frequently associated with biofilm formation (Douglas 2003; Ramage et al. 2006). Indwelling devices can become colonized, either endogenously or exogenously, which develop into adherent biofilm structures from which cells can then detach and cause acute fungemia. Implant-associated infections are inherently difficult to resolve and often result in the implant having to be physically removed from the patient and long-term antifungal therapy administered to control the infection.

Different types of biomaterials often used in the clinics support colonization and biofilm formation by *C. albicans*, and the increase in candidiasis in past decades has virtually paralleled the increase in use of a variety of medical implant devices (Ramage et al. 2006). Of note, *C. albicans* is the third leading cause of intravascular catheter-related infections, with the second highest colonization to infection rate and the overall highest crude mortality (Wisplinghoff et al. 2004). *C. albicans* is the main pathogenic *Candida* species and its ability to form biofilm structures has been extensively studied as a result (Blankenship and Mitchell 2006). Other non-*albicans Candida* species are also associated with biofilm formation, catheter-related bloodstream infections and device-related infections, including *C. glabrata*,

Table 6.1 Case reports of medical implant related yeast infections

Device	Species	References
Cardiac pace maker	*Candida*	Joly et al. (1997), Kurup et al. (2000); Roger et al. (2000)
Prosthetic heart value	*Candida*	Darwazah et al. (1999), Lusini et al. (2008), Lye et al. (2005), Nagaraja et al. (2005)
CNS shunt	*Candida* *Cryptococcus*	Agus et al. (2000), Carter et al. (2008), Duffner et al. (1997), Walsh et al. (1986)
Oral environment	*Candida* *Saccharomyces*	Coco et al. (2008), Infante-Cossio et al. (2007), Jarvensivu et al. (2004), Lamfon et al. (2003), Sen et al. (1997)
Breast implants	*Candida* *Trichosporon*	Penk and Pittrow (1999), Reddy et al. (2002), Saray et al. (2004), Young et al. (1997)
Joint replacements	*Candida*	Bruce et al. (2001), Fabry et al. (2005), Wada et al. (1998)

C. parapsilosis, *C. krusei,* and *C. tropicalis* (Choi et al. 2007; Coco et al. 2008; Hawser and Douglas 1994; Shin et al. 2002; Tumbarello et al. 2007).

Cryptococcus neoformans is an encapsulated opportunistic yeast that causes life-threatening meningoencephalitis in immunocompromised individuals. Colonization and subsequent biofilm formation by *C. neoformans* on ventricular shunts, peritoneal dialysis fistulas, and cardiac valves have also been reported (Bach et al. 1997; Banerjee et al. 1997; Walsh et al. 1986). Contrary to *C. albicans*, which is only found inside its host, *C. neoformans* is ubiquitous in nature, and it is conceivable that biofilm formation can also contribute to its survival in hostile environmental conditions and against predation (Joubert et al. 2006; Martinez and Casadevall 2007).

The opportunistic *Trichosporon* species can cause disseminated life threatening infections and have also been associated with medical implant-related infections, including catheters, breast implants, and cardiac grafts (Krzossok et al. 2004; Pini et al. 2005; Reddy et al. 2002). In addition, *Blastoschizomyces capitatus* has been associated with patients with catheter-related fungemia, *Malassezia pachydermatis* has been isolated from patients undergoing parenteral nutrition and *Saccharomyces* has been detected from dentures of stomatitis patients (Cannizzo et al. 2007; Coco et al. 2008; D'Antonio et al. 2004). The spectrum of yeasts shown to colonize surfaces and form biofilm structures is vast.

6.3 What are Biofilms?

Most microbiology investigations have traditionally focused upon free living (planktonic) cells in pure-culture, resulting in the common perception that microorganisms are unicellular life forms. Nevertheless, Costerton and colleagues were one of the first to link the surface-attached growth state to microbial pathogenesis and human infection (Costerton et al. 1981). Extensive research has now revealed that a wide range of bacteria and fungi alternate between planktonic and surface-attached

multicellular communities, a growth modality that is commonly referred to as a biofilm. Within their natural ecosystems, most microbes have been shown to exist as attached communities of cells within an organized biofilm and not as planktonic organisms. In fact, it is estimated that up to 80% of all bacteria in the environment exist in sessile biofilm communities and over 65% of human microbial infections involve biofilms (Donlan 2002). Biofilms are by definition highly structured communities of microorganisms that are surface-associated, and/or attached to one another, enclosed within a self-produced protective extracellular matrix (Costerton et al. 1995). These can form in the natural environment as well as inside the human host, and can be considered as complex cities of microbes that cooperatively interact in an altruistic manner (Coghlan 1996). The advantages to an organism of forming a biofilm include protection from the environment, resistance to physical and chemical removal of cells, metabolic cooperation, and a community-based regulation of gene expression (Jabra-Rizk et al. 2004). In recent years, there has been an increased appreciation of the role that microbial biofilms play in human medicine, particularly because microbes growing within biofilms (sessile cells) exhibit unique phenotypic characteristics compared to their planktonic counterpart cells, including increased resistance to antimicrobial agents and protection from host defenses (Brown and Gilbert 1993). Therefore, they pose a major problem to clinicians as the dose required to eradicate the biofilm can exceed the highest therapeutically attainable concentrations (Rasmussen and Givskov 2006). Some of the biological characteristics of yeast biofilms will now be addressed.

6.4 Fungal Biofilm Model Systems

A wide range of biofilm model systems have been developed to study bacteria and yeasts in vitro. Many factors affect in vitro yeast biofilm formation, which have been elucidated using model systems. These include strain, species and substrate specificity, and the role of conditioning film and bacterial competitors (Adam et al. 2002; Ramage et al. 2001b; Thein et al. 2006). However, the primary function of many of these models is to investigate biofilm developmental properties and their susceptibility to antimicrobial agents.

An early fungal biofilm model involved adherent populations of *Candida sp.* developing on catheter discs (Hawser and Douglas 1994). Static biofilm growth was quantified using dry weight measurements, tetrazolium salt (MTT) reduction assays and incorporation of [^{3}H] leucine, of which the latter two methods showed excellent correlation to the dry weight of the biofilm. Six different species of *Candida* were investigated for their ability to form biofilms. *C. albicans* showed superior biofilm formation compared to *C. parapsilosis*, *C. tropicalis*, *C. pseudotropicalis,* and *C. glabrata* on catheter material. Latex material produced the best biofilm, followed by PVC and polyurethane. Biofilm formation on silicone was found to be more variable, with the surface topography and hydrophobicity differing between the two types of catheter discs (Hawser and Douglas 1994).

A biofilm model for *C. albicans* has been developed using polymethyl-methacrylate strips (Chandra et al. 2001a). Total biofilm biomass (dry weight) and the metabolic activity of cells (XTT reduction assay) of *C. albicans* cells were determined using this model system. The authors found that inoculum size, adherence time, incubation time, and exposure to carbohydrate (especially glucose) and saliva all influenced biofilm development of *C. albicans* within this model system. In this denture biofilm model, *C. albicans* was found to be significantly more resistant to a range of antifungals compared to planktonic cells (Chandra et al. 2001b).

Traditionally, most models for the formation of microbial biofilms, including those formed by fungal species, are cumbersome, requiring expert handling, longer processing times and the use of specialized equipment not generally available in a regular microbiology laboratory. Moreover, these complex and technically demanding biofilm models are generally not amenable to high throughput screening since relatively few equivalent biofilms can be produced at the same time. Ramage and colleagues were the first to describe a standardized high throughput 96 well microtiter plate model for the formation of *C. albicans* biofilms (Pierce et al. 2008b; Ramage et al. 2001a). This model has now been adopted by a number of other groups to evaluate various experimental parameters of biofilm formation (Ramage et al. 2001a; Thein et al. 2007; Tumbarello et al. 2007). The XTT (2,3-bis (2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide) reduction assay is based on initial candidal adhesion and antifungal drug susceptibility studies (Hawser 1996; Tellier et al. 1992). This methodology was found to be rapid and highly reproducible, and particularly amenable for biofilm susceptibility testing against a range of current antifungal agents (Ramage et al. 2001b, 2002c). This colorimetric assay is non-invasive and non-destructive, requiring minimal post-processing of samples as compared to other alternative methods (such as viable cell counts). Using this technique, multiple microtiter plates can be processed simultaneously without compromising on accuracy, and is important due to its utility for testing of biofilms, which are inherently more resistant to antifungal therapy compared to their free floating planktonic cell counterparts (Pierce et al. 2008b; Ramage and Lopez-Ribot 2005). Martinez and Casadevall also developed a microtiter plate biofilm assay for *C. neoformans* to determine the susceptibility profiles of sessile structures in vitro using this XTT-based reduction assay (Martinez and Casadevall 2006a). Whereas the assay is useful for antifungal testing to evaluate the effects of the drug on a sessile population in comparison to an untreated control, metabolic variability between different isolates make its usefulness in quantifying biofilm development limited, and caution should therefore be taken when interpreting the data obtained from this metabolic assay (Kuhn et al. 2003).

The presence of flowing liquid over the biofilm can increase the amount of matrix formed compared to statically developed sessile populations (Hawser et al. 1998). Therefore, flow systems have been utilized by many researchers to model biofilm development (Garcia-Sanchez et al. 2004; Ramage et al. 2008). A "seed and feed" modified Robbin's device has been described, which permits multiple

biofilms to be formed under constant flow conditions (Ramage et al. 2008). The production of polymeric material was increased under flow conditions, with the architecture of the resultant biofilms altered with respect to water channels, porosity, topography, and thickness compared to biofilms grown statically. Conversely, recent studies have shown that shear flow can reduce the biofilm thickness whilst increasing overall cellular density (Mukherjee et al. 2008). This highlights the intrinsic variability in biofilm flow systems modeling. Other flow systems include cylindrical cellulose filters, constant depth film fermenters, perfusion fermenters and a Robbin's device (Baillie and Douglas 1998a, 1999; Chandra et al. 2001a, b; Lamfon et al. 2003). For flow systems, although perhaps more representative of certain physiological conditions, limitations to this type of apparatus are evident. These include their poor availability and accessibility to many laboratories, difficulty in implementation, and their limited utility to high throughput screening.

The majority of yeast biofilm research to date is carried out in vitro, but it has proved important to validate laboratory-based models with those formed in vivo, which has revealed structures with equivalent architecture (Andes et al. 2004; Schinabeck et al. 2004). With conditions encountered in situ distinctive from those in vitro, it is practically impossible to reproduce all the environmental permutations experienced by the biofilm, particularly the host-pathogen relationship in relation to the host immune response system (Nett and Andes 2006). To date two key specific yeast biofilm models have been developed within animal hosts. The first model, described by Schinabeck and coworkers, was developed on central venous catheters within New Zealand white rabbits for *C. albicans* biofilms to investigate antifungal lock therapy (Schinabeck et al. 2004). Similarly, Andes and coworkers described a central venous catheter biofilm model using rats (Andes et al. 2004). Both groups noted a similar time course of biofilm formation over 24 h and confirmed the presence of a multilayered structure with extracellular matrix using microscopy. The catheter biofilms were also found to exhibit increased resistance to antifungal therapy, with differential expression of two efflux pump genes.

6.5 Biofilm Developmental Characteristics

The colonization of complex, adherent yeast populations on biological and innate surfaces, such as the oral mucosa or denture material substrates is commonplace for clinically relevant yeasts (Holmes et al. 1995). Analogous to bacterial biofilms, yeast biofilms have defined developmental phases. Various groups have endeavored to develop suitable and robust models of yeast biofilm development. Although slight variations exist within individual models, such as the substrate, incubation time and growth media, the overall premise remains universal. These key stages include arrival at an appropriate substratum, adhesion, colonization, polysaccharide production, biofilm maturation, and dispersal (Blankenship and Mitchell 2006). These phases of biofilm formation of *C. albicans* are illustrated in Fig. 6.1. A wide variety of environmental factors contribute to the initial surface attachment of a

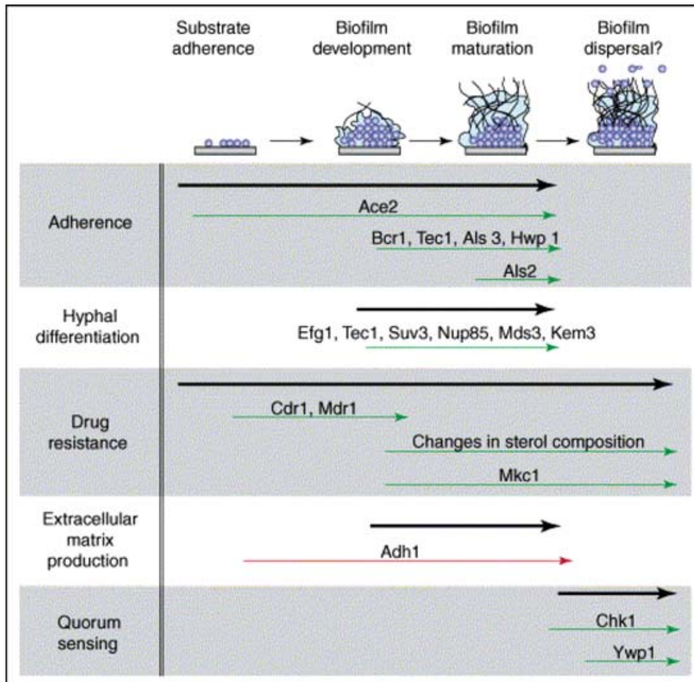

Fig. 6.1 Overview of *C. albicans* biofilm development. A timeline of formation and dispersal is depicted at the top of the figure. The categories listed at the side represent important processes in biofilm development; the adjacent thick black lines represent the phase(s) during which they are important to the biofilm life cycle. The thin arrows within each category represent the phase(s); the listed proteins or events contribute to the respective process. *Green arrows* represent a positive role of the genes or events indicated; the reed arrow indicates a negative role. Permission for the reproduction of this figure was kindly permitted by the authors (Blankenship and Mitchell 2006) and Elsevier

microbe. These include the flow velocity of the surrounding medium (urine, blood, saliva), pH, temperature, presence of antimicrobial agents, and presence of extracellular polymeric substances (Chandra et al. 2001a).

The best defined eukaryotic organism with regard to biofilm formation is the yeast *C. albicans*. Endogenous or exogenous *C. albicans* cells must firstly colonize a suitable substrate and quickly adhere to its surface. This initial attachment phase is mediated by both nonspecific factors, including hydrophobicity of the cell surface and electrostatic forces as well as by specific adhesins on the surface of *C. albicans* that bind to ligands on the conditioning film (fibrinogen and fibronectin) (Dranginis et al. 2007; Verstrepen and Klis 2006). *Candida* species can also directly attach to one another or to bacterial organisms that have already colonized the biomaterial (Coco et al. 2008; El-Azizi et al. 2004).

After the initial attachment phase, growth ensues and microcolonies are formed, *C. albicans* then begins to multiply by budding, a filamentous scaffolding is

produced and the initial deposition of extracellular matrix material occurs and subsequent biofilm development follows (Chandra et al. 2001a). Filamentous growth, although not strictly essential for biofilm formation per se, strengthens the entire structure and provides protection and adhesion sites for the budding yeast cells (Ramage et al. 2005). Microscopical analysis has demonstrated that *C. albicans* biofilm formation could be separated into three distinct developmental phases: Early (0–11 h), intermediate (12–30 h) and maturation (38–72 h) (Chandra et al. 2001a). Following initial adhesion by blastospores, microcolonies of budding yeast are detected in the 3 rd and 4th hour, with pseudo-hyphae and true hyphae being present at 4 h and 8 h, respectively (Ramage et al. 2001b). Microcolonies are later conjoined by hyphal extensions, leading toward a confluent monolayer (intermediate phase). This phase is made distinct by the development of an opaque film covering the fungal microcolonies made of predominantly noncellular material. The cloudy appearance is due to the extracellular material, composed of predominantly cell-wall-like polysaccharides (Al-Fattani and Douglas 2006; Baillie and Douglas 2000). Yeast cells make up the basal layer, while filamentous cells compose the structural framework (Baillie and Douglas 1998a). In the maturation phase, the quantity of this extracellular material increases in a time-dependant manner, until the microbial communities are entirely enclosed to form a mature biofilm (Chandra et al. 2001a). Recent work has shown the exopolymeric substance (EPS) to consist of proteins, chitins, DNA and β-1,3 glucan carbohydrates (Al-Fattani and Douglas 2006). It covers the biofilm and it is thought to act as a protective barrier by preventing penetration of host immune factors, antifungals, and impeding physical disruption of underlying cells.

Confocal laser scanning microscopy has shown mature *C. albicans* biofilms to be complex three dimensional structures that can range from anything between 50 and 350 μm thick, depending on the model (Mukherjee et al. 2008; Nobile et al. 2006a; Ramage et al. 2001b). Images obtained from scanning electron microscopy (SEM) have shown that mature *C. albicans* biofilms consist of yeasts, pseudohyphal forms, and true hyphae. The scanning electron micrographs in Fig. 6.2 illustrate the different architecture of biofilms formed by both *C. albican*s and *C. glabrata*, either alone or mixed. Whereas *C. glabrata* are sparse and consist of clumps, *C. albicans* biofilms are dense and heterogeneous, characterized by different morphological forms. *C. glabrata* appears to use *C. albicans* as a scaffold to maintain biofilm integrity (El-Azizi et al. 2004). *Candida* species tend to vary in their biofilm developmental characteristics (Parahitiyawa et al. 2006).

Like *C. albicans, T. asahii* biofilms were identified to contain yeast cells and hyphal elements (Di Bonaventura et al. 2006). Martinez and Casadevall reported that *C. neoformans* also have the ability to form biofilm structures in vitro (Martinez and Casadevall 2006a). They found that *C. neoformans* displayed the typical sequence of events of biofilm formation and production of polymeric material. Similarly, other yeasts undergo the similar developmental characteristics, but are restricted to their yeast morphology, and rely specifically on adhesion, cell wall glycoproteins, and the production of matrix material (El-Azizi et al. 2004; Hawser and Douglas 1994; Kuhn et al. 2002a; Reynolds and Fink 2001)

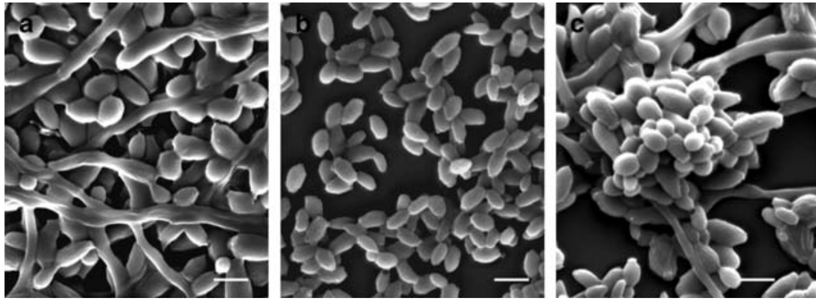

Fig. 6.2 Scanning electron micrographs of (**a**) *Candida albicans*, (**b**) *Candida glabrata* and (**c**) Mixed *Candida albicans* and *Candida glabrata* biofilms formed over 24 h on Thermanox™ coverslips. Note the differing biofilm structures of each population. *C. albicans* produces a dense matrix of yeast, pseudohyphae and hyphal cells, whereas *C. glabrata* biofilms are sparse and consist of clumps of smaller yeast cells. Note the architecture of the mixed species biofilm, with *C. albicans* providing attachment sites and a stable matrix for *C. glabrata* to attach to. The scale bars are 2 μm

Overall, it is proposed that the architecture of biofilms is highly ordered to enable the perfusion of nutrients and expulsion of waste products. Mature biofilms exhibit spatial heterogeneity with microcolonies and water channels being present. These features are common to both bacterial and fungal biofilms (Chandra et al. 2001a; de Beer et al. 1994; Lawrence et al. 1991). This complexity is governed by defined genetic pathways.

6.6 Molecular Mechanisms of Biofilm Development

Historically, numerous biofilm models have helped to deduce the basic biological processes involved in the development of biofilm structures, which are described in these reviews (Kumamoto and Vinces 2005; Mukherjee et al. 2005; Ramage et al. 2005). This work primarily concentrated on the phenotypic characteristics associated with biofilm formation and recalcitrance to antifungal agents. Although a large number of groups have continued in this line of research, a significant number of groups began to examine these complex structures at the molecular level with the aim of understanding the molecular pathogenesis of yeast biofilm infections. An article published in 2003 entitled "Are there biofilm-specific physiological pathways beyond a reasonable doubt?" made it clear that there still remained doubt within the research community regarding the impact of the biofilm lifestyle, and indeed whether this was unique and worthy of attention (Ghigo 2003). However, the extensive effort over the past five years has categorically shown this to be the case, with leading groups addressing the problem with vigor and tenacity.

Early studies showed that morphogenesis play a pivotal role in *C. albicans* biofilm development, in which hyphae are essential elements for providing

structural integrity and for multi-layered architecture (Baillie and Douglas 1999). A gene encoding a major regulator of hyphal development is *EFG1*, which has been shown to be involved in regulation of the morphological transition and associated with ability to form coherent biofilm structures on both polystyrene, polyurethane and glass (Garcia-Sanchez et al. 2004; Lewis et al. 2002; Ramage et al. 2002d). *CPH1* has also been shown in these studies to play an associated role. Given that Efg1p also regulates numerous genes whose products include many cell surface proteins, it is not surprising that this protein is important for biofilm formation (Sohn et al. 2003). For example, Efg1p regulates expression of *EAP1*, which encodes a predicted cell wall, GPI-anchored protein. When expressed in adhesion defective *S. cerevisiae* cells, this resulted in attachment to polystyrene, suggesting that this surface protein can mediate attachment to polystyrene (Li and Palecek 2003). Further studies have shown that the Eap1p mediates *C. albicans* biofilm formation and that this is specific to the *EAP* domain (Li et al. 2007; Li and Palecek 2008). *ACE2* and *NOT4* have also been shown to have defective adhesion phenotypes leading to reduced biofilm formation (Kelly et al. 2004; Krueger et al. 2004)

Cell wall proteins with a demonstrated role in biofilm formation include the *ALS* gene family, which have been shown to be expressed on both innate and biological substrates, and which may facilitate coaggregation with other yeasts and bacteria (Green et al. 2004; Klotz et al. 2007). Another gene encoding the transcriptional regulator Bcr1p activates cell-surface protein and adhesin genes required for biofilm formation (Nobile et al. 2006a). This has also been shown to play a role in *C. parapsilosis* biofilm formation (Ding and Butler 2007). It has also been shown that *HWP1*, when overexpressed in the biofilm deficient *bcr1/bcr1* mutant background regains the ability to form biofilms in vivo, indicating the pivotal role of Hwp1p for biofilm formation (Nobile et al. 2006b). A recent study has shown that there may indeed by a complementary adhesion function in biofilms, where *als1/als1*, *als3/als3,* and *hwp1/hwp1* biofilm-deficient mutants were able to form a biofilm both in vitro and in vivo when mixed (Nobile et al. 2008). These authors proceed to hypothesize that the complementary adhesion be analogous to the roles of sexual agglutinins in mating reactions, which is subject to a recent review by authors who have previously demonstrated that a unique signaling system exists in between opaque and white cells of the *C. albicans* white–opaque switching system to form biofilms (Daniels et al. 2006; Soll 2008).

Sun41p, a glycosidase, is also involved in biofilm formation, cytokinesis, cell wall biogenesis, adhesion and virulence, with its deletion resulting in a cell wall damaged phenotype (Norice et al. 2007). Glycosylated mannoproteins have been shown to be an important factor in adhesion and virulence (Bates et al. 2005, 2006; Munro et al. 2005). In vitro biofilm studies have shown that Mnt1, Mnt2, Och1, and Pmr1 were also important in *C. albicans* biofilms, as mutants were defective in their abilities to form biofilms on polystyrene (Ramage, unpublished observation). Insertion mutant studies have also shown that *SUV3, NUP85*, *MDS3,* and *KEM1* are involved in biofilm formation through their morphogenic defects (Richard et al. 2005). An interesting study has also shown that upon contact, the mitogen-activated protein kinase (MAPK) protein, Mck1p signal transduction factor, is involved in

invasive hyphal growth and biofilm development, and the mutant (*mck1*) is azole-sensitive within its altered biofilm phenotype (Kumamoto 2005). This implies that Mck1p may also be involved in biofilm resistance. With regards to dispersal from the biofilm to initiate colonization at a distal site, studies have indicated a potential role for Ywp1 (Granger et al. 2005).

Although many of these experiments on molecular mechanisms of biofilm formation in yeasts were obtained piece-meal, mostly by using single mutant strains with defined genetic defects and assessing their biofilm-forming ability, the post-genomic era has allowed the implementation of powerful techniques such as DNA microarray and proteomic analyses to analyze global patterns of gene and protein expression during the biofilm lifestyle. Microarrays containing the entire genome of various fungi are now available, enabling transcriptomic analysis under various conditions, including, biofilm development and their response to antifungal agents and quorum sensing molecules (Cao et al. 2005; Lepak et al. 2006). Early studies of planktonic cells and biofilms grown in different conditions (nutrient flow, aerobiosis and glucose concentration) showed transcriptional correlation of culture conditions between the biofilms, indicating that similar and specific transcriptional events occur during biofilm formation, independent of the growth conditions (Garcia-Sanchez et al. 2004). Three specific phases of biofilm development were subsequently examined of biofilms grown on both denture acrylic and catheter substrates, and it was shown that the transcriptional profiles were both phase- and material-specific (Yeater et al. 2007). Murillo and colleagues investigated the early stages of biofilm formation and found that after only 30 min, there was a substantial difference in gene expression between adherent and non-adherent *C. albicans* cells (Murillo et al. 2005). Key regulators of morphogenesis were also shown to be differentially expressed during biofilm growth, such as *NRG1* and *EFG1* (Kadosh and Johnson 2005; Sohn et al. 2003). Moreover, genes encoding for drug efflux pumps and implicated in azole resistance have been reported to be differentially regulated upon exposure to antimicrobial agents, which include *CDR1, CDR2* and *ERG11*, genes which have been previously shown to be implicated within the biofilm developmental phase (Lepak et al. 2006; Mukherjee et al. 2003; Ramage et al. 2002a). These detailed transcriptional studies indicate that biofilm development is a highly regulated and multifactorial event.

A parallel approach has been used to examine biofilm characteristics using proteomics. More recently two different groups have used these approaches (two dimensional gel electrophoresis and mass spectrometry techniques) to compare cell surface-associated proteins from *C. albicans* planktonic and biofilm cultures (Vediyappan and Chaffin 2006). These studies described that the protein profiles associated with the planktonic and biofilm extracts were very similar, although a few proteins were identified that were differentially expressed during the biofilm mode of growth. In addition, Thomas and colleagues reported that proteins in the biofilm exopolymeric material were similar to those found in liquid culture supernatants obtained from planktonic (liquid) cultures. Proteins found in the biofilm matrix included a few predicted to form part of the secretome, but also many non-canonically secreted proteins (Thomas et al. 2006). Another proteomic study

identified alcohol dehydrogenase as a key regulator of biofilm formation in *C. albicans* (Mukherjee et al. 2006). Pierce and colleagues utilized proteomics to assess changes in *N*-glycosylation levels of cell wall mannoproteins after treatment with tunicamycin that inhibited biofilm formation, suggesting a key role for *N*-linked glycosylation during biofilm development in *C. albicans* (Pierce et al. 2008a).

6.7 Pathogenesis and Host Immunity

The high density of cells present within a biofilm represents a challenge to the host through direct and indirect interaction of the sessile cells and associated products, which ultimately lead to inflammation and pathology. The general host pathogen relationship will be dealt with elsewhere in this book. Certain individuals suffer from denture stomatitis because the denture provides a reservoir for the heterogeneous yeast biofilm, which subsequently induces inflammation of the oral mucosa. It has been shown that both *Candida* biofilm diversity and quantity can contribute to high level inflammation (Coco et al. 2008). To date, there are limited in vitro studies that have examined the expression of defined yeast virulence factors within the context of the biofilm phenotype. However, the oral mucosa, and models thereof, represent useful tools to extrapolate the pathogenic role of biofilm infections. Several studies by Naglik have shown that the biofilm-specific protein Hwp1p is an important determinant of mucosal infection, and that secreted aspartyl proteases (Saps) play key roles with proteolytic activity in vivo (Naglik et al. 2003). However, recent publications have re-examined the role of Saps during superficial infections and demonstrated that no single Sap appears to play a predominant role in mucosal invasion (Lermann and Morschhauser 2008; Naglik et al. 2008). Coco has recently demonstrated that members of the *SAP* family are expressed in a biofilm phase-dependant manner using an in vitro biofilm model, and that mixed *C. albicans* and *C. glabrata* biofilms are associated with higher grades of inflammation and expression of *SAPs* than mono-species *C. albicans* biofilms alone (Coco et al. 2008; Coco 2009). Previous studies have also shown the contributory role of lipolytic enzymes, such as the phospholipases (Naglik et al. 2003).

Limited information is available on the role of the host immune response in relation to biofilm formation, as this subject is in its infancy. However, it was recently shown that the epithelial surfaces can be protected from the development of mucosal infections through a TLR4-mediated protective mechanism, which is PMN-dependent (Weindl et al. 2007). One of the few papers to directly investigate biofilm immunology in any detail reported that upon exposure to *C. albicans* biofilms adherent human peripheral blood mononuclear cells secreted significant amounts of IL-1β, IL-10, and MCP-1 compared to planktonic yeast cells (Chandra et al. 2007; Wang et al. 2008). Biofilm matrices are thought to play a key role in protecting microbial biofilms from host immune responses. For example, increased phagocytosis and killing of staphylococcal biofilms has been reported for knock-out

strains missing polysaccharide intercellular adhesion, which is a main constituent of staphylococcal biofilms (Vuong et al. 2004). Antibodies are also thought to fail to penetrate biofilms due to the coating of biofilms by matrix material.

In *C. neoformans* biofilm formation is dependent on the presence of a polysaccharide capsule composed primarily of glucuronoxylomannan (GXM) and specific "protective" antibodies against this component inhibited biofilm formation (Martinez and Casadevall 2005). In the same report, the authors indicated that lactoferrin, a component of the innate immune system, was unable to prevent fungal biofilm formation despite its previously reported efficacy against bacterial biofilms. However, under certain circumstances, specific antibodies can antagonize the action of antifungal drugs and reduce their activity against *C. neoformans* biofilms (Martinez et al. 2006b). Also, *C. neoformans* cells within biofilms were more resistant than their planktonic counterparts to oxidative stress, but remained susceptible to cationic antimicrobial peptides (Martinez and Casadevall 2006b). Interestingly, antibody-mediated agglutination and biofilm formation can also mediate resistance to and escape from phagocytosis inside macrophages (Alvarez et al. 2008). However, antibody-guided alpha radiation has been proven effective to damage fungal biofilms, a technique in which a radioactive molecule is attached to an antibody, which is specific to epitopes localized within the biofilm (Martinez et al. 2006a).

6.8 Antifungal Resistance

The commonly used CLSI broth microdilution techniques for antifungal susceptibility testing are based on the use of planktonic populations and will not enable prediction of the drugs efficacy against fungal biofilms. These increased levels of resistance typically associated with biofilms in comparison to planktonic populations, underscore the importance of developing standardized assays to test biofilm antifungal susceptibilities and to allow systematic studies to determine the effectiveness of different antifungal agents and regimens against fungal biofilms (Pierce et al. 2008b). One of the defining characteristics of biofilms is their increased resistance to antimicrobial agents. Bacteria and fungi have been reported to be up to 1,000-fold less susceptible to antimicrobial therapy than planktonic free floating cells in a wide variety of in vitro and in vivo biofilm models, although this recalcitrance to antimicrobial therapy is not fully understood (Di Bonaventura et al. 2006; Tre-Hardy et al. 2008). Although some antifungal agents have been shown to be efficacious against yeast biofilms, particularly liposomal amphotericin B formulations and the echinocandins (Bachmann et al. 2002, 2003; Kuhn et al. 2002b; Ramage et al. 2002c), the high level resistance exhibited by these complex structures has promoted detailed investigation.

Factors that are potentially responsible for the increased resistance of biofilms to antifungals, amongst others, include the structural complexity and increased cell density of sessile populations, physiological state of the cell, and differential gene

expression of biofilms compared to planktonic cells (Perumal et al. 2007; Ramage et al. 2002a). The architecture of biofilms and the presence of exopolymeric material may reduce the diffusion of antifungal drugs. Studies have shown that polymeric material production is increased with biofilms grown under shaking conditions compared to static culture (Baillie and Douglas 2000). *C. albicans* biofilm developed under shaking conditions were also shown to be 20% more resistant to amphotericin B when compared to resuspended cells (Baillie and Douglas 1998b). In addition, it has been shown that preformed biofilms are not affected by high concentrations of most antifungal agents, and that even newly adherent cells could still grow, proliferate, and form biofilms in the presence of high concentrations of fluconazole (Ramage et al. 2002a). However, the biofilm ultrastructure and presence of matrix material are not entirely responsible for antifungal resistance, as biofilms that have been detached and dispersed were at least eight times more susceptible to fluconazole compared to intact sessile structures (Ramage et al. 2002a). These results indicate that the matrix material does play a role in the resistance of biofilms, however, as it has been shown that antifungal agents can diffuse through this, then its precise function is as yet to be determined (Al-Fattani and Douglas 2004).

The physiological state of cells in sessile populations has also been suggested to influence the susceptibility profiles of biofilms. The effect of growth rate on *C. albicans* biofilm resistance has been investigated using a perfused biofilm fermenter, in which it was shown that under glucose limited conditions, high level resistance for amphotericin B was still observed (Baillie and Douglas 1998a). The development of semi-quantitative metabolic dye assays (for example XTT-based assays) has confirmed that cells within biofilms are metabolically active (Chandra et al. 2001a; Hawser 1996; Kuhn et al. 2003; Ramage et al. 2001a). Therefore, resistance of *C. albicans* biofilms is not solely due to a slow growth rate or nutritional limitations.

In an attempt to further understand the molecular basis of biofilm resistance, the expression of efflux pump genes (*MDR1*, *CDR1,* and *CDR2*) and ergosterol biosynthetic genes (*ERG*), already implicated in azole drug resistance in *C. albicans* planktonic populations, has been investigated in vitro by several groups (Mukherjee et al. 2003). Animal studies have also shown that biofilms formed on catheters express *CDR* pumps (Andes et al. 2004). Initially examination of mRNA transcripts indicated that these genes were upregulated transiently in *C. albicans* biofilms, and that they may play a defined role in resistance. Similar results were recently reported in *C. glabrata* (Won Song et al. 2008). Subsequent analysis using *C. albicans* efflux pump mutants, which are planktonically hypersensitive to fluconazole, showed that high level sessile resistance phenotypes were still observed, thus implying that antifungal resistant in biofilms is multifactorial, with membrane sterol composition and differentially expressed genes playing a combined role in sessile recalcitrance (d'Enfert 2006). It has been demonstrated that there is less ergosterol present in the later stages of biofilm formation, therefore some antifungal drugs have less target molecule (Mukherjee et al. 2005). In addition, elevated levels of β-1,3 glucan in the cell wall of *C. albicans* sessile cells and in the biofilm matrix bind fluconazole and may contribute to the overall resistance of biofilm populations (Nett et al. 2007).

One mechanism of resistance that has gathered some attention recently is the persister cell theory (Khot et al. 2006). The internal metabolic environment of a biofilm has been described as markedly different from planktonic cells, with changes in pH, oxygen level, and sugar availability all altered within the different layers of the biofilm (Stewart and Costerton 2001). A small subset of blastospore cells in a *C. albicans* biofilm have been described as highly resistant to amphotericin B following adhesion, and this is independent to up-regulation of efflux pumps and cell membrane composition (LaFleur et al. 2006). This provides the biofilm community a greater opportunity of survival whilst limiting the advantages to these individual blastospores for growth. This strain-specific behavior has yet to be deduced as either genetic or environmental stimulation, but the dormant persistent phenotype appears to be another defined biofilm resistance mechanism (LaFleur et al. 2006). More recently persisters were found in biofilms of one of two strains of *C. albicans* and in biofilms of *C. krusei* and *C. parapsilosis*, but not in biofilms of *C. glabrata* or *C. tropicalis*, suggesting that persister cells are not solely responsible for drug resistance in *Candida* species biofilms (Al-Dhaheri and Douglas 2008). In addition, in a series of very detailed experiments, the Chaffin group unequivocally demonstrated the contribution of cell density to antifungal drug resistance in *C. albicans* biofilms and concluded that azole drug tolerance at high cell density differs mechanistically from tolerance at low cell density (Perumal et al. 2007). This is particularly interesting as cell density is a key aspect of quorum sensing, which plays a crucial role in biofilm regulation.

6.9 Cell–Cell Communication

Quorum sensing is defined as the ability of microorganisms to communicate and coordinate their behavior via the secretion of signaling molecules in a population dependent manner. Quorum sensing was first described in aquatic *Vibrio fischeri* and is associated with bioluminescence (Nealson et al. 1970). Since then a vast amount of research has been undertaken to gain a better understanding of quorum sensing networks in bacteria (Venturi 2006).

Quorum sensing in eukaryotic organisms was first described in *C. albicans*. Hornby and colleagues were the first to identify the quorum sensing molecule as farnesol *trans*, *trans* 3,7,11-trimethyl-2,6,10-dodecatrien-1-ol (Hornby et al. 2001). In parallel, Oh and colleagues found that another strain of *C. albicans* produced a closely related compound called farnesoic acid (Oh et al. 2001). Farnesol has been shown to inhibit the yeast to hyphal transitional stage of *C. albicans* (Hornby et al. 2001). This morphological switching is important for the pathogenicity of *C. albicans* (Saville et al. 2003). Ramage and colleagues found that farnesol was able to reduce biofilm development in a concentration dependent manner (Ramage et al. 2002b). However, adherent cell populations that had begun to germinate before being exposed to farnesol were not inhibited and hyphal formation resulted in almost typical biofilm structures being formed (Ramage et al. 2002b). The effect

of farnesol also resulted in alterations of gene expression with *HWP1*, a hyphal wall protein displaying decreased gene expression upon exposure to farnesol (Ramage et al. 2002b). Other studies have subsequently confirmed these observations, exploring the effects of this quorum sensing molecule using microarray analysis (Cao et al. 2005; Enjalbert and Whiteway 2005). Cao and colleagues (2005) concluded that exposing *C. albicans* to farnesol resulted in a genome wide response involving hyphal developmental genes (*TUP1* and *CRK1)*, cell surface hydrophobicity genes and genes involved in drug resistance (*FCR1* and *PDR16*) (Cao et al. 2005). Similar findings were reported, with farnesol repressing the expression of hyphal-related genes and inducing the expression of drug resistance genes (Enjalbert and Whiteway 2005). Both studies confirm that the exposing farnesol to *C. albicans* results in multifactorial transcriptional events. A recent study has now demonstrated that quorum sensing events in *C. albicans* is likely driven by the two component regulatory system of Chk1p (Kruppa et al. 2004). The production of farnesol has not been reported for any other fungi to date. However, farnesol has been found to induce production of reactive oxygen species in *S. cerevisiae* and apoptosis in both *Aspergillus nidulans* and *Fusarium graminearum*. Therefore, the efficacy of farnesol is not solely specific to *C. albicans* (Semighini et al. 2006).

A second quorum sensing molecule called tyrosol has been isolated from *C. albicans* (Chen et al. 2004). Tyrosol was found to promote germ tube formation, suggesting *C. albicans* morphology is under a complex regulatory system of positive (tyrosol) and negative (farnesol) control. Further work identified tyrosol production by both *C. albicans* planktonic cells and biofilms (Alem et al. 2006). Biofilms produced significantly more tyrosol compared to planktonic cell cultures. The early stages of biofilm development (2–6 h) were enhanced with exposure to tryosol, and the ability of tryosol to abolish the inhibitory effects of farnesol was dependent on the concentration of farnesol present (Alem et al. 2006).

More recently, Martins and colleagues demonstrated that *C. albicans* and *C. dubliniensis* planktonic and biofilm cells produce a series of chemical signaling molecules, including isoamyl alcohol, 2-phenylethanol, 1-dodecanol, E-nerolidol, and E,E-farnesol which affected morphogenetic transitions, and secretion of these alcohols was species, culture mode, and growth time-specific (Martins et al. 2007). It is expected that further insights into yeast quorum sensing pathways will provide novel methods for controlling clinical yeast infections. For example, Ywp1p has been implicated as a biofilm dispersal agent, as deletion of the gene results in enhanced adherence (Granger et al. 2005). This soluble factor is released into the supernatant, and can subsequently block adhesion of strains that do not express Ywp1.

6.10 Conclusions

Over the past decade, there has been an extensive research effort undertaken to understand the biological processes governing the development and characteristics of yeast biofilms, in particular those of *C. albicans*. Through the development of

robust biofilm models, many of the fundamental phenotypic and genotypic characteristics of biofilms have been elucidated. With technological advances accelerating and the necessary sequence information for other clinically relevant yeasts being made available, the application of powerful molecular biology techniques should unravel their intricate biofilm processes. Whether we can harness the knowledge obtained from biofilm development, drug resistance and quorum sensing to create new therapeutic strategies remains to be seen. Nevertheless, this will be an exciting challenge.

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Chapter 7
Cryptococcus: Spectrum of Disease and Treatment

Tihana Bicanic and Thomas Harrison

Abstract Cryptococcosis is a major fungal opportunistic infection in increasing populations of immunocompromised hosts worldwide. Both *Cryptococcus neoformans*, which favours the immunosuppressed host, and *Cryptococcus gattii,* the cause of a recent outbreak in immunocompetent hosts, produce clinical presentations ranging from asymptomatic pulmonary infection to disseminated disease involving the central nervous system. Cryptococcal antigen testing of serum and cerebrospinal fluid is highly sensitive and specific for rapid diagnosis, though culture remains the gold standard. Initial treatment is with fungicidal amphotericin B-based combinations, followed by consolidation and maintenance with long-term azole therapy. Optimal management includes early recognition and management of raised intracranial pressure and immune reconstitution inflammatory syndrome. Restoration of host immunity is critical for the immunosuppressed host. Future efforts to improve the ongoing high mortality in patients with HIV infection in areas of high cryptococcal incidence must be directed at prevention of severe disease by screening for early sub-clinical infection.

7.1 Introduction

Cryptococci are encapsulated yeasts that are ubiquitous environmental saprophytes. The two species of *Cryptococcus* causing illness in humans, *Cryptococcus neoformans* (var. *grubii* (serotype A) or var. *neoformans* (serotype D) and *Cryptococcus gattii* (previously *C. neoformans* serotypes B and C) (Kwon-Chung and Varma 2006) produce a wide spectrum of disease, from asymptomatic, self-limiting pulmonary infection to life-threatening meningoencephalitis.

T. Bicanic and T. Harrison (✉)
Department of Cellular and Molecular Medicine, St. George's University of London, Cranmer Terrace, London, SW17 ORE, UK
e-mail: tbicanic@sgul.ac.uk; tharriso@sgul.ac.uk

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C. neoformans has emerged as a major opportunistic pathogen as populations of immunocompromised hosts increase worldwide, be it due to HIV (McCarthy et al. 2006; Mirza et al. 2003; Dromer et al. 1996) or immunosuppressive treatment of cancer or organ transplantation (Singh et al. 2008; Pappas et al. 2001). In parts of Africa with highest HIV sero-prevalence, *C. neoformans* has become a more common cause of meningitis than the usual bacterial culprits (Hakim et al. 2000; Gordon et al. 2000; Bekondi et al. 2006), and is a major infectious cause of death, excluding HIV itself, after malaria and gastroenteritis (Park et al. 2009). *C. gattii*, previously thought to be confined to tropical and subtropical areas (Kwon-Chung and Bennett 1984), caused a recent outbreak of cryptococcosis in relatively immunocompetent patients on Vancouver island (Kidd et al. 2004).

In the immunosuppressed host with deficient T-cell mediated immunity, disseminated disease is usual. Infection of the immunocompetent host often produces focal lesions (cryptococcomas) localized by a more effective host granulomatous response to infection.

7.2 Clinical Presentations

Humans are exposed to cryptococci by inhalation of encapsulated yeast cells or basidiospores from the environment (Powell et al. 1972). In the majority of cases, this results in an asymptomatic pulmonary infection that may be associated with a hilar lymph node complex (Baker 1976; Salyer et al. 1974). In the majority of hosts with intact cell-mediated immunity, the infection is cleared or is contained and enters a period of latency or subclinical infection similar to pulmonary tuberculosis (Garcia-Hermoso et al. 1999). In the event of current or subsequent immunosuppression, the organism enters the blood stream to produce disseminated infection in almost any organ in the body, with a predilection for the central nervous system (that might be determined by its production of the enzyme laccase, which catalyses the formation of melanin in the fungal cell wall from catecholamine precursors widespread in the CNS).

7.2.1 Risk Factors: Immunosuppressed v Immunocompetent

Risk factors for cryptococcosis include all conditions associated with defective cell-mediated immunity: most commonly HIV infection, prolonged corticosteroid treatment or immunosuppressive drugs used for prevention of organ transplant rejection (e.g. calcineurin inhibitors cyclosporin and tacrolimus) (Singh et al. 2007; Singh et al. 2008; Vilchez et al. 2002) or for treatment of chronic inflammatory conditions such as Crohn's disease (e.g. TNF-α inhibitor infliximab (Hage et al. 2003)). Less-common risk factors include diabetes mellitus, haematological malignancy, cirrhosis, connective tissue diseases and sarcoidosis (Pappas et al. 2001). Up to 25% of

patients have no apparent risk factor (Dromer et al. 2007; Pappas et al. 2001). Predictably, these immunocompetent patients tend to present with disease characterized by a greater host inflammatory response leading to more localised disease and a smaller yeast burden at the site of infection (Lee et al. 1996). In HIV-infected patients, cryptococcal meningoencephalitis is associated with profound immunosuppression, usually occurring at CD4 counts <100 cells/μL. Compared to HIV-negative patients, the presentation tends to be more acute, and is associated with higher fungal burdens (higher rates of India Ink positivity, higher cryptococcal antigen titres and more frequent positive blood cultures) and a poorer CSF inflammatory response (<20 WBC/μL).

7.2.2 *C. neoformans* v *C. gattii*

For unknown reasons, *C. neoformans* has a predilection for the immunosuppressed host, causing over 90% of cryptococcal infections in AIDS patients (McCarthy et al. 2006; Speed and Dunt 1995), whilst *C. gattii* favors the immunocompetent host. Although the clinical presentation and spectrum of disease is generally indistinguishable, *C. gattii* causes cryptococcomas in lung and brain more commonly than *C. neoformans* (Chen et al. 2000). Patients with cryptococcomas tend to have more chronic clinical courses, slower response to antifungal treatment with occasional requirement for surgical excision, but better survival than immunosuppressed patients with *C. neoformans* infection (Speed and Dunt 1995). The geographic distribution of disease is likely to be a product of both intensity of exposure to the organism as well as the prevalence of risk factors in the population. In Africa and most developing countries, the vast majority of cryptococcal disease is currently HIV-related and caused by *C. neoformans*.

7.2.3 Central Nervous System (CNS)

Meningoencephalitis is the most frequent manifestation of cryptococcosis. Infection of the meninges and subarachnoid space is accompanied, and probably preceded (Charlier et al. 2005), by involvement of the brain parenchyma (Lee et al. 1996). Cryptococcal meningoencephalitis should always be included in the differential diagnosis of meningoencephalitis, particularly in patients with known immunocompromise. Patients usually present with fever and headache, progressing to confusion or drowsiness over 2 to 4 weeks, though presentation in HIV infection may be more acute. Signs are often absent but may include meningism, papilloedema, cranial nerve palsies, depressed conscious level and raised intracranial pressure. In *C. gattii* infection, and, less commonly, *C. neoformans* infection, cerebral cryptococcomas may present with symptoms of a space-occupying lesion: seizures, focal neurological signs or obstructive hydrocephalus.

Diagnosis is by lumbar puncture with measurement of CSF opening pressure and removal of a minimum of 5 ml of CSF for laboratory diagnostic tests (see below). In immunosuppressed patients, and in the presence of focal neurology or abnormal mental status (confusion, decreased conscious level, seizures) a CT head scan should be done before lumbar puncture, if resources allow. Scans may show gyral enhancement, single or multiple enhancing or non-enhancing nodules (cryptococcomas), or hydrocephalus (Cornell and Jacoby 1982). In HIV infection CT scans are often normal, even in the presence of raised intracranial pressure (Graybill et al. 2000). MRI scans are more sensitive for detection of small nodules and dilated perivascular (Virchow-Robin) spaces (Charlier et al. 2008).

7.2.4 Lung

The presentation ranges from asymptomatic infection to severe pneumonia with acute respiratory failure, and may represent reactivation of latent infection (Garcia-Hermoso et al. 1999) or progression of primary infection (MacDougall and Fyfe 2006). Symptoms and radiographic findings are non-specific: patients present with fever, pleuritic chest pain, cough or shortness of breath, and the chest X-ray most commonly shows single or multiple nodules in immunocompetent patients, and focal or diffuse pulmonary infiltrates or cavitating lesions in the immunocompromised, in whom the differential diagnosis includes other opportunistic infections such as pneumocystis and tuberculosis, which may also be present as co-infections (Jarvis and Harrison 2008). In rare patients with underlying chronic lung disease with no acute chest X-ray changes, negative serum cryptococcal antigen and no evidence of disseminated disease, repeated isolation of *C. neoformans* from sputum may represent chronic respiratory colonization (Duperval et al. 1977).

In the immunocompromised, a high index of suspicion is required, as lung infection may herald CNS infection weeks to months later, and provides a window of opportunity for earlier treatment (Driver et al. 1995; Kerkering et al. 1981). A serum cryptococcal antigen is positive in more than 90% of HIV-infected patients with pulmonary disease (Meyohas et al. 1995), compared with around 50% in non-HIV patients (Pappas et al. 2001). Blood cultures and a lumbar puncture are warranted in immunocompromised patients (and may be prudent in apparently immunocompetent patients) to rule out disseminated disease, as treatment is different.

7.2.5 Other Organs

The skin is the third most common site of cryptococcal infection. Rarely, *Cryptococcus* sp. may infect the skin by primary inoculation (Neuville et al. 2003), but usually the observation or culture of *Cryptococcus* sp. in skin samples implies disseminated disease. The most common cutaneous presentation is a papule or

maculopapule with a soft or ulcerated centre, although all types of skin lesions have been reported (Mitchell and Perfect 1995), emphasizing the importance of skin biopsy in high-risk patients. In HIV patients, umbilicated papules must be distinguished from infection with molluscum contagiosum, and in patients from relevant geographical regions, penicilliosis. Similar to lung infection, skin lesions may be a sentinel sign for CNS disease.

The prostate may act as a reservoir of infection following successful treatment, which may lead to relapses after urological procedures (Plunkett et al. 1981). Eye involvement manifests as choroiditis or endopthalmitis, which are sight-threatening (Crump et al. 1992). Rapid or more gradual, often irreversible, visual loss in cryptococcosis most commonly results from compression of the blood supply to the optic nerve by raised intracranial pressure, but may also occur due to optic neuritis secondary to direct yeast invasion of the optic nerve (Rex et al. 1993). Infection of bone and joints can occur, most commonly presenting as spinal osteolytic lesions (Behrman et al. 1990) that must be differentiated from tuberculosis and sarcoidosis. Other rarely reported sites of infection include the heart, gastrointestinal tract, breast, lymph nodes, thyroid, adrenals, head and neck (Chayakulkeeree and Perfect 2006).

7.3 Laboratory Diagnosis

7.3.1 Microscopy

The CSF white cell count ranges from normal to markedly elevated, with a predominance of lymphocytes. CSF protein is usually mildly elevated (0.5–1 g/dL) and CSF glucose may be low. However, in HIV patients all these parameters can be entirely normal (Moosa and Coovadia 1997). The India Ink test, which is simple, cheap and reliable, will detect $\geq 10^4$ CFU of yeast per ml of CSF. Hence in AIDS patients, who tend to present with high fungal burdens (10^5–10^6 CFU/ml) sensitivity is over 80%, but only around 50% or less in non-AIDS patients (Kovacs et al. 1985; Zuger et al. 1986).

The spherical encapsulated yeasts can also be visualized in histology samples using specialized stains such as Gomori methenamine silver, alcian blue and mucicarmine (capsular stains). The size and morphology of fungal cells, and presence of capsule, allows specific identification in most cases.

7.3.2 Serology

Antibodies to *C. neoformans* are not useful in diagnosis (Goldman et al. 2001). However, detection of the cryptococcal polysaccharide antigen in body fluids by enzyme immunoassay and latex agglutination tests has a high sensitivity and specificity (Tanner et al. 1994), best validated in serum and CSF (Snow and

Dismukes 1975). The antigen may also be detectable in urine (Chapin-Robertson et al. 1993) and bronchoalveolar lavage fluid (Baughman et al. 1992). With appropriate pre-treatment (e.g. with pronase or 2-mercaptoethanol treatment), false-positive tests are rare at CSF titres of $\geq$1:4 (Tanner et al. 1994). False-positive latex agglutination tests are usually negative by enzyme immunoassay, and vice versa. False-positives may rarely occur due to cross-reacting antigens in the specimen, including the yeast *Trichosporon beigelii* (Mcmanus and Jones 1985). False-negative tests may occur in early asymptomatic meningitis, and in immunocompetent patients (Berlin and Pincus 1989).

In asymptomatic HIV-infected patients, serum antigenaemia identifies early cryptococcal disease, requiring CSF examination and treatment (Feldmesser et al. 1996). High initial CSF titres ($\geq$1:1024) correlate with a high organism burden by quantitative culture (Brouwer et al. 2005), and are a marker of poor prognosis. CSF antigen titres do fall with successful treatment, but are of little value in management as they may remain elevated for months after successful treatment of cryptococcosis (Powderly et al. 1994).

7.3.3 Culture and Sensitivity

C. neoformans from CSF, blood, or other sites produces cream-colored smooth or mucoid colonies within 48-72 hours on most bacterial and fungal (Sabouraud's dextrose) media. Although *C. neoformans* grows at 37°C, 30–35°C is optimal. Standard automated blood culture systems will detect cryptococcaemia. Identification is based on biochemical tests (e.g. production of urease), or molecular methods. Serotyping (A-D) is possible with commercial kits using monoclonal antibodies (Dromer et al. 1993). Canavanine-glycine-bromothymol blue agar can be used to discriminate *C. gattii* from *C. neoformans* (Kwon-Chung et al. 1982). Multilocus sequence typing separates strains into at least 8 major genotypes (Litvintseva et al. 2006).

Primary resistance to first-line antifungals is not currently a significant clinical problem (Pfaller et al. 2005), and there is a paucity of data correlating MIC with clinical response on which to base susceptibility/resistance breakpoints (Aller et al. 2000; Witt et al. 1996). Therefore, susceptibility testing of *C. neoformans* isolates is currently only recommended for those patients who relapse or fail primary therapy. However, initial isolates should be stored so that they can be tested in parallel with any subsequent isolates if secondary resistance is suspected.

7.4 Treatment

Whilst antifungal drugs are the mainstay of therapy for patients with cryptococcosis, optimal management also involves early recognition and management of complications, and restoration of host immunity, if possible. In patients with late

stage HIV infection in areas of high cryptococcal incidence, there is also a strong rationale for screening for early sub-clinical infection and pre-emptive treatment. Antifungal drug regimens for cryptococcal meningitis have been some of the best studied in trials of invasive mycoses, using both mycological (drug early fungicidal acitivity and rate of CSF sterilization) and clinical outcome measures (resolution of symptoms and mortality). The current paradigm, based on the last large Mycoses Study Group trial (van der Horst et al. 1997), is to use the most rapidly fungicidal, amphotericin B-based drug combination initially, to gain control of the infection, and then consolidate and maintain with long-term therapy with less fungicidal but also less toxic azoles. To date, the treatment of *C. gattii* and *C. neoformans* infection is not substantially different, although the mass lesions seen more frequently with *C. gattii* infection may need longer intensive therapy.

7.4.1 Antifungal Therapy

Cryptococci are generally susceptible to polyenes (amphotericin B), flucytosine and triazoles (itraconazole, fluconazole, voriconazole, posaconazole), but are naturally resistant to echinocandins (caspofungin, micafungin, anidulafungin). The most rapidly fungicidal drug combinations are all based on amphotericin B deoxycholate (AmBd). Flucytosine should not be used alone due to rapid emergence of drug resistance (Hospenthal and Bennett 1998). Fluconazole has excellent CSF pharmacokinetics and safety, but at conventional dosage is essentially a fungistatic agent best reserved, when used alone, for treatment of mild disease or later treatment stages of disseminated disease, when there are lower fungal burdens (Bicanic et al. 2007; Larsen et al. 1990; Saag et al. 1992). Treatment of meningoencephalitis and disseminated disease is generally divided into three stages: induction, consolidation and maintenance.

7.4.1.1 Cryptococcal Meningoencephalitis/Disseminated Disease

HIV-Infected Patients

Induction therapy is with amphotericin B deoxycholate (AmBd) 0.7–1.0 mg/kg/day i.v. plus flucytosine 100 mg/kg/day p.o. or i.v. for at least 2 weeks (Bicanic et al. 2008; de Lalla et al. 1995; van der Horst et al. 1997), a combination which results in CSF sterilization in 60–75% of patients (Dromer et al. 2008; van der Horst et al. 1997). Many experts recommend a lumbar puncture at 2 weeks to check for CSF sterility, and consideration of prolonged combination therapy if the CSF is still culture positive. The addition of flucytosine to AmBd results in faster fungal clearance (Brouwer et al. 2004) and has been shown to be an independent predictor of CSF sterilization at 2 weeks, treatment success at 3 months and lower incidence of relapse (Dromer et al. 2008; Robinson et al. 1999).

AmBd is nephrotoxic and associated, during a 2-week course, with a fall in haemoglobin of around 20% (Gallis et al. 1990; Brandriss et al. 1964). Flucytosine can suppress bone marrow function, in particular neutrophil counts, and is renally excreted, necessitating dose adjustment according to creatinine clearance. Fluid and sodium supplementation, equivalent to 1-L normal saline per day, is essential to minimize amphotericin B nephrotoxicity (Branch 1988). All patients require frequent monitoring of renal function and full blood count. However, these toxicities are predictable, dose-dependent, and reversible on stopping therapy (Bicanic et al. 2008). Some experts recommend monitoring of flucytosine levels, if available, although in published trials at the 100 mg/kg/day dose for 2 weeks in HIV-infected patients, serious toxicity has been uncommon (Brouwer et al. 2004; Brouwer et al. 2007b; van der Horst et al. 1997). Lipid formulations of AmB, such as liposomal AmB (dose 3–6 mg/kg/day i.v.) (Leenders et al. 1997; Hamill et al. 1999) and amphotericin B lipid complex (dose 5 mg/kg/day i.v.) (Sharkey et al. 1996) may be substituted for AmBd among patients with or predisposed to renal dysfunction (Deray 2002). Flucytosine is not available in many countries, so alternatives, using AmBd monotherapy at the higher, 1 mg/kg/day dose (Bicanic et al. 2007) or in combination with fluconazole, 800 mg/day (Larsen et al. 2004; Pappas et al. 2009), are suggested in Table 7.1. For more remote hospitals in developing countries where AmBd cannot be safely administered or monitored, the best oral regimen is a combination of high-dose fluconazole (800–1,200 mg/day), combined with flucytosine, if available (Haubrich et al. 1994; Longley et al. 2008; Menichetti et al. 1996; Larsen et al. 1994; Mayanja-Kizza et al. 1998; Milefchik et al. 2008).

The newer extended spectrum triazoles, voriconazole and posaconazole, have good in vitro activity against C. neoformans (Pfaller et al. 2004). Voriconazole has excellent CSF penetration (Andes 2006) and high levels have been demonstrated in

Table 7.1 Treatment recommendations for cryptococcal meningoencephalitis in HIV-infected patients

Initial antifungal regimen: induction	Duration
AmBd 0.7–1.0 mg/kg/day + 5FC 100 mg/kg/day	2 wks
Alternatives:	2 wks
1. *Renal dysfunction*: Liposomal AmB 3–6 mg/kg/day	
2. *Flucytosine not available*:	2 wks
AmBd 1 mg/kg/day i.v.	
AmBd 0.7–1 mg/kg/day i.v. plus fluconazole 800 mg/day p.o.	
3. *AmBd not available*:	2–10 wks
fluconazole 1,200 mg/day p.o. ± flucytosine 100 mg/kg/day p.o.	
Consolidation	
Fluconazole 400 mg/day	8 wks
Alternative: itraconazole 400 mg/day (van der Horst et al. 1997)	
Maintenance	
Fluconazole 200 mg/day, start ART at 2–10 weeks	>1 yr
Alternative: itraconazole 200–400 mg/day (Saag et al. 1999)	

Adapted from: Clinical practice guidelines for the management of cryptococcal disease: 2009 Update by the Infectious Diseases Society of America [Clin Infect Dis 2009; in press]

brain tissue (Lutsar et al. 2003). Despite achieving only low levels in CSF, posaconazole has also been shown to have in vivo efficacy. Both drugs, thus far, have only been evaluated clinically in a salvage setting (Perfect et al. 2003; Pitisuttithum et al. 2005). They are therefore not recommended as part of primary treatment regimens, but may be acceptable as alternatives in case of fluconazole intolerance, toxicity or treatment failure. Interactions of both agents with antituberculous and antiretroviral drugs needs careful attention.

The 2-week induction period is followed by consolidation therapy with fluconazole 400 mg p.o. daily for a minimum of 8 wks (van der Horst et al. 1997) (alternative itraconazole, see Table 7.1). Thereafter, patients should have maintenance therapy with fluconazole 200 mg/day p.o. for a minimum of 12 months, and until there is evidence of persistent immune reconstitution on antiretroviral therapy (ART), with a CD4 count >100 cells/μL and suppression of HIV viral load for ≥3 months (Mussini et al. 2004; Vibhagool et al. 2003; Aberg et al. 2002).

Organ Transplant Recipients

As renal dysfunction is common in this group of patients, recommended induction therapy for CNS disease or severe pulmonary infection is liposomal AmB 3–6 mg/kg/day i.v. plus flucytosine 100 mg/kg/day for at least 2 weeks; consolidation with fluconazole 400–800 mg/day p.o. for 8 weeks; then maintenance with fluconazole 200–400 mg/day p.o. for 6–12 months (Singh et al. 2005b). Immunosuppression with corticosteroids or calcineurin inhibitors (cyclosporin, tacrolimus) should be carefully reduced where possible (Singh et al. 2008). Abrupt reduction in immunosuppression has been associated with immune reconstitution reactions and graft loss (Singh et al. 2005a).

Immunocompetent Patients

Induction therapy is as for HIV-infected patients, but some experts recommend the duration is prolonged to 4–6 weeks, based on earlier trials in the pre-HIV era (Dismukes et al. 1987). Of note, those trials were done with lower AmBd doses than currently used, and liposomal AmB may need to be substituted for AmBd in the event of nephrotoxicity from prolonged use. Consolidation is with fluconazole 400 mg daily for 8 weeks; followed by maintenance on fluconazole 200 mg daily for 6–12 months.

7.4.1.2 Cerebral Cryptococcomas

Induction therapy may need to be prolonged, as for cryptococcal meningoencephalitis in immunocompetent patients, followed by a prolonged fluconazole 400–800 mg/day maintenance phase of 6–18 months. Corticosteroids may be used for significant associated oedema, and surgery considered for large lesions (≥3 cm) causing a mass

effect. Occasionally, a cryptococcoma may cause obstructive hydrocephalus necessitating the placement of a ventriculo-peritoneal shunt (Speed and Dunt 1995).

7.4.1.3 Pulmonary Disease

For treatment of mild or moderate disease, fluconazole 400 mg/day p.o. is given for 6–12 months. In HIV-infected patients, a minimum of one year is recommended, and, in addition, the CD4 cell count should be >100 cells/μL on ART. Severe disease (diffuse infiltrates, acute respiratory distress syndrome, evidence of dissemination) has a high mortality (Visnegarwala et al. 1998) and should be treated as for meningoencephalitis. For *C. gattii* pulmonary cryptococcomas that are large and multiple, induction therapy should be given for 4–6 weeks. Surgery may be considered for symptomatic, focal radiographic abnormalities not responsive to antifungal therapy.

7.4.2 Isolated Cryptococcal Antigenaemia and Screening for Subclinical Infection

In HIV-infected patients, a positive serum cryptococcal antigen is predictive of the development of disseminated disease (Feldmesser et al. 1996; Jarvis et al. 2009). A lumbar puncture should be performed on such patients to exclude meningitis. If the CSF is normal, and there is no evidence of clinical infection elsewhere, patients can be treated with fluconazole 400 mg/day, then 200 mg/day, until immune restoration with ART (see below).

In parts of sub-Saharan Africa, around 10% of HIV-infected patients with a CD4 count <100 cells/μL, screened prior to initiation of ART, have positive serum antigen tests (Liechty et al. 2007; Desmet et al. 1989; Jarvis et al. 2009). Antigenaemia is associated with an increased risk of death and of developing clinical cryptococcal disease in the first year of ART, with the risk being higher at higher titres. These data provide a strong rationale for screening of patients with a low CD4 cell count prior to initiation of ART, and pre-emptive treatment for those with a positive test, as an alternative to across-the-board primary prophylaxis with fluconazole (Jarvis et al. 2009).

7.4.3 Management of Complications

7.4.3.1 Raised Intracranial Pressure

Raised CSF opening pressure (>25 cm H_20), measured at lumbar puncture using a manometer attached to a three-way tap, occurs in more than 50% of cases of HIV-associated cryptococcal meningitis, and can cause severe headaches, visual and hearing loss, decreased conscious level, and death (Graybill et al. 2000; Denning

et al. 1991). The mechanism leading to increased pressure is debated, but is most likely to be due to obstruction of CSF re-absorption at the arachnoid villi by organisms and shed polysaccharide (Hussey et al. 1970; Katzman and Hussey 1970), producing a communicating hydrocephalus. This is consistent with the association of raised pressure with higher CSF polysaccharide antigen titres and higher fungal burdens, and the apparent efficacy of mechanical drainage in patients with high CSF pressure. Analysis of data from a combined cohort of patients studied prospectively in Thailand and South Africa, suggests a high organism load is necessary but not sufficient for the development of high CSF pressure (Bicanic et al. 2009a). Cryptococcal capsular phenotype has also been shown to play a role in animal models (Fries et al. 2005).

Management consists of daily lumbar punctures for all patients with elevated opening pressure (>25 cm H_20), with removal of sufficient CSF to reduce opening pressures by 50% or to <20 cm H_2O, continued until opening pressure has been normal for several days (Saag et al. 2000). For cases of persistently raised and symptomatic CSF pressure, CSF diversion using a temporary lumbar drain, or permanent shunting, is recommended (Bach et al. 1997; Fessler et al. 1998; Macsween et al. 2005; Park et al. 1999). Temporary lumbar drains are not without risk and staff need to be familiar with or carefully trained in their use, but they have been successfully used even in resource-limited settings (Manosuthi et al. 2008). Managing opening pressure aggressively with serial large volume therapeutic lumbar punctures may attenuate the apparent effect of raised CSF pressure on mortality (Bicanic et al. 2009a). The use of steroids is not recommended (Graybill et al. 2000).

7.4.3.2 Relapse

In HIV-infected patients representing with symptomatic relapse, fluconazole adherence should be assessed, and response to ART by CD4 cell count and HIV viral load. Induction therapy should be re-started pending results of investigations. Opening pressure should be checked by lumbar puncture and managed as above, if elevated. CSF specimens should be sent for repeat culture and, if the culture is positive, paired susceptibility testing using the relapse and original isolate (if available). In the event of fluconazole resistance, weekly amphotericin B 1 mg/kg/day, is an alternative, though inferior, maintenance therapy (Powderly et al. 1992). Voriconazole or posaconazole could also be considered, although drug interactions may be a major issue. For those with symptomatic relapses after start of ART, a diagnosis of IRIS should be considered (Bicanic et al. 2006), especially if the CSF culture is negative or the organism load low.

7.4.3.3 Immune Reconstitution Inflammatory Syndrome (IRIS)

In HIV-infected patients starting ART, cryptococcal immune reconstitution syndrome can occur in one of two forms: (1) “unmasking” IRIS in patients with subclinical but previously undiagnosed cryptococcal disease, with first appearance

of symptoms after the start of ART and positive cultures and (2) "paradoxical" IRIS in patients previously diagnosed and treated for cryptococcal infection, with recurrence of symptoms after start of ART and (usually) negative cultures (Shelburne et al. 2005; Bicanic et al. 2009b). IRIS is also recognized on decrease of immunosuppression in organ transplant recipients (Singh et al. 2005a).

IRIS has been reported in 8–30% of patients with cryptococcal meningoencephalitis after starting ART (Lortholary et al. 2005; Shelburne et al. 2005; Bicanic et al. 2009b). Risk factors include a high fungal burden (cryptococcaemia and high CSF cryptococcal antigen), failure to sterilize the CSF at 2 weeks, a low CD4 count and rapid rise in CD4 count on ART, and early institution of ART (less than 1–2 months from diagnosis of cryptococcal meningitis). In a cohort of HIV-infected patients from South Africa, both unmasking and paradoxical IRIS were associated with a positive serum cryptococcal antigen prior to the start of ART, and the risk was increased at higher titres (Jarvis et al. 2009). It occurs within days to months of immune restoration and manifests most commonly as lymphadenitis or central nervous system symptoms or signs. IRIS is a diagnosis of exclusion, for which there is so far no laboratory diagnostic test, and must be distinguished from treatment failure or alternative infections. ART and antifungal therapy should be continued, with consideration of the use of steroids (prednisolone 0.5–1 mg/kg/day for 2–6 weeks, tapered dose) for patients with severe or progressive manifestations.

7.4.4 Restoration and Boosting of Host Immunity

In HIV-infected patients with cryptococcosis, introduction of ART may restore cell-mediated immune responses against cryptococci and enhance clearance of infection. However, early restoration of immune responses in the presence of a large burden of viable organisms or polysaccharide antigen in severely immunosuppressed patients may result in IRIS with potentially fatal consequences (Lawn et al. 2005a). The risk of IRIS has to be balanced against the risk of death from other HIV-related opportunistic infections if initiation of ART is delayed (Lawn et al. 2005b). The optimum time to start ART after the start of antifungal treatment is uncertain: a recent randomized trial (which included mainly patients with *Pneumocystis* infection, but also 37 patients with cryptococcal meningitis) suggested that starting ART at 2 weeks compared to 6 weeks into therapy for the opportunistic infection was associated with fewer combined endpoints of death or AIDS progression, without an increase in IRIS (Zolopa et al. 2008). Importantly this trial was in the context, for the cryptococcal patients, of initial treatment with amphotericin B. If initial treatment is with the much less rapidly active fluconazole, IRIS may be more of a concern and a study reported in abstract from Zimbabwe found that starting ART concurrently with fluconazole (800 mg/day) was associated with much higher mortality than deferring ART for 10 weeks (Makadzange et al. 2009). Further studies are planned in the African setting to help address this issue.

Given ongoing high mortality despite best current antifungal therapy, there is continued interest in adjunctive immunotherapies. Treatment with anti-capsular monoclonal antibodies has been used in animal models (Casadevall et al. 1998) and has been shown to be safe in humans (Larsen et al. 2005), but has not progressed beyond phase I trials. Interferon-γ, a cytokine produced by T-cells, is important in cryptococcal clearance from the CSF (Siddiqui et al. 2005), and when used as an adjunct to antifungal therapy in trials of cryptococcal meningitis, showed a trend towards improved mycological and clinical success at 10 weeks (Pappas et al. 2004). Pending further studies, its use (dose 100 μg sc 3 times/week) may be considered in patients refractory to standard antifungal therapy, especially if there is evidence for a poor or dysfunctional immune response (Brouwer et al. 2007a).

7.5 Prognostic Factors and Outcome

Without antifungal therapy, cryptococcal meningoencephalitis is uniformly fatal (French et al. 2002). In HIV-infected patients, 10-week mortality ranges from 10% to 26% (Larsen et al. 1990; Saag et al. 1992; van der Horst et al. 1997; Dromer et al. 2007) in developed countries, and from around 25% to over 50% in developing countries (Bicanic et al. 2007; Imwidthaya and Poungvarin 2000; Kambugu et al. 2008), where delays in presentation to hospital are common and access to treatment may be limited (Bicanic et al. 2005).

Abnormal mental status (confusion, seizures or depressed conscious level) and high fungal burden at diagnosis, measured by quantitative CSF culture or CSF antigen titre, are the most important determinants of early death (Brouwer et al. 2004; Saag et al. 1992). Raised CSF opening pressure and low CSF white cell count are also associated with poor outcome. Long-term outcomes are determined by the ability to control the underlying disease. In HIV-infected patients, patients surviving to 6 months on ART have an excellent long-term prognosis, whether in the developed or developing country setting (Bicanic et al. 2007; Lortholary et al. 2006).

In addition to refinements of existing antifungal treatment strategies and restoration of host immunity, future efforts to decrease the high mortality from cryptococcal meningoencephalitis must be directed at the prevention of severe disease, with expansion of screening for subclinical cryptococcal infection in the immunosuppressed (Jarvis et al. 2009); and earlier diagnosis, with the development of rapid, non-invasive, outpatient diagnostic tests that are more applicable to and affordable for resource-limited settings than current assays (Tassie et al. 2003).

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Chapter 8
Typing Species of *Cryptococcus* and Epidemiology of Cryptococcosis

Thomas G. Mitchell and Anastasia P. Litvintseva

Abstract This article reviews the more common DNA-based molecular markers used to genotype species and strains of *Cryptococcus*. The current schemata for molecular epidemiological typing of *C. neoformans* and *C. gattii* are defined and summarized. Common methods of assessing the population genetics of the *C. neoformans*–*C. gattii* species complex are described and exemplified. In addition, the epidemiology, ecology and geographic distribution of *Cryptococcus* species and subpopulations are reviewed.

8.1 Introduction

As described in the previous chapter, cryptococcosis is caused by two species of *Cryptococcus*, *C. neoformans* and *C. gattii*. These basidiomycetous sibling species diverged approximately 37 million years ago (Xu et al. 2000). Based on genetic and phenotypic differences, two varieties of *C. neoformans* are recognized, *C. neoformans* var. *grubii* (serotype A) and *C. neoformans* var. *neoformans* (serotype D), although AD hybrids also exist. Strains of *C. gattii* possess either serotype B or C. Clinical and environmental isolates are usually haploid, possessing one of two mating type alleles, *MAT*α or *MAT**a***. These species differ in their ecology, geographic distribution, serotypes, pathogenicity, clinical manifestations, epidemiology, several biochemical phenotypes, and clinically relevant properties. In addition, *C. gattii* may be more resistance to antifungal drugs, such as fluconazole (de Bedout et al. 1999; Gomez-Lopez et al. 2008; Torres-Rodríguez et al. 2008).

The proper taxonomy and classification of the *C. neoformans*–*C. gattii* species complex is a work in progress. Researchers have collected clinical and environmental isolates from numerous disparate locations, and collectively, a variety of

T.G. Mitchell (✉) and A.P. Litvintseva
Department of Molecular Genetics and Microbiology, Duke University Medical Center, Durham, NC 27710, USA
e-mail: Tom.Mitchell@duke.edu

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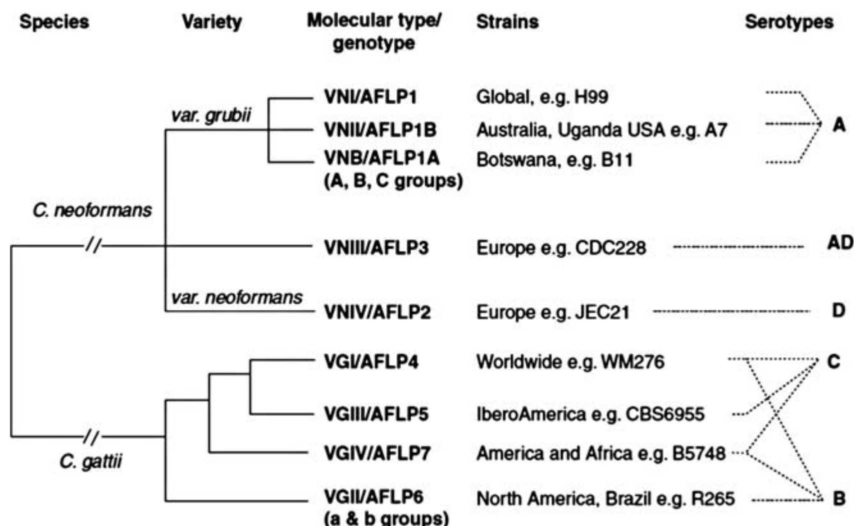

Fig. 8.1 A schematic depiction of the *C. neoformans-C. gattii* species complex. Phylogenetic analyses of AFLP and MLST genotypes have identified three genetic subpopulations of *C. neoformans* var. *grubii* (serotype A), designated VNI, VNII and VNB. VNIV consists of strains of *C. neoformans* var. *neoformans* (serotype D), and strains of VNIII are diploid or aneuploid hybrids of serotypes A and D. Members of the four monophyletic lineages of *C. gattii* are designated as VGI-VGIV. Strains of VGII are serotype B, and VGIII, serotype C, but strains of VGI and VGIV may possess serotype B or C. For VGII, an additional subgroup, VGIIc, was recently discovered in the northwestern USA (Byrnes et al. 2009). The indicated geographical distributions of these lineages denote regions with relatively high prevalence, but they are not restricted to these areas. From: Ma and May (2009), used with permission

molecular markers have been used to genotype them. The application of phylogenetic analyses to these markers and the utilization of sophisticated software programs to estimate the relationships among these genotypes have generated the schema depicted in Fig. 8.1. These subpopulations have been confirmed by multiple methods and datasets (Boekhout et al. 2001; Bovers et al. 2009; Findley et al. 2009; Litvintseva et al. 2006). This review will focus on molecular typing methods that involve nucleic acids, which may include a variety of genomic regions but most commonly, chromosomal DNA.

8.2 Molecular Methods

The more common DNA-based methods and their applications to fungi have been compared in several reviews (Mitchell and Xu 2003; Mitchell 2005; Taylor et al. 1999; Xu and Mitchell 2002, 2003). In general, to genotype individuals for comparison with other isolates of the same species, the most reliable DNA markers identify differences in their nucleotide sequences. Typical DNA markers recognize single nucleotide polymorphisms (SNPs), insertions or deletions (indels), and length

variations in tandem oligonucleotide repeats (e.g. microsatellites or minisatellites). These markers can be detected by a range of molecular techniques, including PCR amplicon length polymorphisms, randomly amplified polymorphic DNA (RAPD) generated by a single oligonucleotide primer, detection of the presence or absence of an endonuclease site (i.e., restriction fragment length polymorphism or RFLP). More elaborate methods include amplified fragment length polymorphisms (AFLP), hybridization of nucleotide probes to electrophoretic blots of digested genomic DNA, and direct DNA sequencing of homologous genomic regions of the strains or species of interest (MLST or multilocus sequence typing). The prevalent DNA-based markers applied to studies of *Cryptococcus* were recently reviewed (Mitchell 2008).

Using adequate controls and standardized protocols, most of these typing methods can be used to identify individual strains. However, the methods vary in stability, reproducibility and cost. For example, AFLP genotyping is relatively inexpensive, rapid, and capable of generating numerous polymorphic bands. However, AFLP markers are laboratory-specific and scoring them can be rather subjective. Furthermore, the genetic linkages among the AFLP loci cannot be determined. In contrast, MLST markers such as SNPs, reflect specific DNA sequence polymorphisms, are absolute and can be reproduced in other laboratories. The major disadvantage is the expense of sequencing multiple genes and strains, but the resulting data are much more useful. In one recent study, a subset of AFLP-typed strains of *C. neoformans* was also genotyped and analyzed by MLST. There was congruency among the gene genealogies and both datasets produced similar Index of Association values (Litvintseva et al. 2003, 2006).

Phylogenetic analyses are typically used to develop a classification of distantly related taxa that reflects their evolutionary relationships. For these applications, the most comprehensive phylogenies are constructed from comparisons of highly conserved nucleotide sequences, and therefore, it is imperative to select gene(s) or genomic sequences that are relatively conserved and alignable across the taxa of interest. Ribosomal RNA, especially alignable regions of the small (18S) and large (26S) subunits (SSU and LSU) of the rDNA genes have proven to be exceptionally useful in phylogenetic studies of fungi as well as other eukaryotes.

Some markers are better at discriminating individual strains or evaluating phylogenetic relationships. For certain studies, it is desirable to use markers in specific genes, and for other purposes, markers in noncoding, usually anonymous portions of the genome are preferable because they are assumed to be neutral or unaffected by selective pressure. In general, DNA sequencing methods are more suitable for population genetics and phylogenetic studies of closely related taxa and strains of a species (Berbee and Taylor 1999; Mitchell 2005; Taylor et al. 2000; Taylor and Fisher 2003; Xu and Mitchell 2003).

8.2.1 Molecular Markers

The DNA markers used for studying the pathogenic *Cryptococcus* species are similar to those developed for other yeasts and described in previous chapters.

Table 8.1 Common methods to genotype strains of the *C. neoformans-C. gattii* complex

Method	Selected references
Electrophoretic karyotype	Boekhout and van Belkum (1997), Fries et al. (1996), García-Bermejo et al. (2001), Perfect et al. (1993), Pfaller et al. (1998)
Random amplified polymorphic DNA (RAPD)	Cavalcante et al. (2007), Martins et al. (2007), Meyer et al. (1999), Poonwan et al. (1997), Sorrell et al. (1996)
PCR fingerprinting	Meyer et al. (2003)
PCR-RFLP	Chaturvedi et al. (2000), Feng et al. (2008a), Meyer et al. (2003)
Amplified fragment length polymorphism (AFLP)	Bovers et al. (2008), Forche et al. (2000), Halliday and Carter (2003), Litvintseva et al. (2005a), Trilles et al. (2008)
Multilocus microsatellite typing (MLMT)	Hanafy et al. (2008)
Multilocus sequence typing (MLST)	Bovers et al. (2008), Byrnes et al. 2009, Feng et al. (2008b), Litvintseva et al. (2006)
Hybridization with retrotransposons	Jain et al. (2005), Keller et al. (2006), Litvintseva and Mitchell (2009)
Hybridization with other probes	Chen et al. (1995), Diaz and Fell (2005), Garcia-Hermoso et al. (1999, 2001), Jain et al. (2005), Spitzer and Spitzer (1992, 1994), Varma et al. (1995)

Although RAPDs and electrophoretic karyotypes are capable of distinguishing cryptococcal strains, these methods frequently exhibit excessive variability (Boekhout and van Belkum 1997). The most popular methods for typing *Cryptococcus* use markers that are obtained by PCR fingerprinting, microsatellites and similar repetitive DNA elements, RFLP, AFLP, DNA sequencing, such as MLST. Table 8.1 lists the more prevalent methods and cites representative examples of their applications.

8.2.2 *Randomly Amplified Polymorphic DNA (RAPD)*

In RAPD analysis, genomic DNA is amplified at a low-annealing temperature (30–38°C) with a single short oligonucleotide (ca.10 nucleotides). This PCR typically generates multiple PCR products of different electrophoretic mobilities, and in comparing species or strains, each isolate will often yield bands of different sizes. RAPD analysis detects variations in the length between two primer binding sites, or sequence length polymorphisms in the fragments between PCR priming regions (Welsh and McClelland 1990; Mitchell and Xu 2003). Similarities in banding profiles among strains (i.e. the number and mobility, but not the density of the bands) can be calculated and used to infer epidemiological relationships. When multiple primers are employed, the RAPD banding patterns are sufficiently sensitive to detect variation among isolates. However, combining RAPDs with other methods provides optimal discrimination (Boekhout et al. 1997; Brandt et al. 1995; Mondon et al. 1997).

Despite being technically fast and simple, there are some disadvantages to RAPD. The major drawback is reproducibility. Small differences in PCR conditions

may affect binding of the primer. This problem is minimized when the protocol and reagents are carefully standardized. For this reason, the RAPD banding patterns of individual strains are generally specific to each laboratory. RAPDs can also be problematic because bands with the same electrophoretic mobility may not share the same sequence. Another concern with the interpretation of RAPD profiles is the difficulty of assigning alleles, which often precludes the use of RAPD markers for studies of population genetics (Clark and Lanigan 1993). Nevertheless, for comparing the similarities among strains and developing fingerprints for molecular epidemiology, RAPD analyses have been widely applied to a number of medical fungi.

8.2.3 PCR Fingerprinting

Similar to RAPDs, PCR fingerprinting employs a single, but longer (> 15 nucleotides) primer, such as a minisatellite, microsatellite or di- or tri- or tetranucleotide repeats, as well as rigorous PCR conditions and known reference strains to identify signature amplicons. Due to the increased stringency, PCR fingerprinting is generally more reproducible than RAPD. Although it suffers the same problems of interpretation, under standardized conditions, PCR fingerprinting has proven most reliable for the identification of species and strains (Mitchell et al. 1993; Meyer and Mitchell 1995; Meyer et al. 2003).

Wieland Meyer and colleagues used the M13 primer to develop a standard PCR fingerprinting method to identify the most common species and strains of *Cryptococcus* (Meyer et al. 1993; Meyer and Mitchell 1995). The current protocol is simple, relatively fast and inexpensive, and it has been implemented in numerous laboratories around the world (Meyer et al. 2003). Because the PCR fingerprinting patterns may vary in different laboratories, the use of eight reference strains, representing the major subpopulation of the *C. neoformans*-*C. gattii* complex (*viz*., VNI-IV and VGI-IV), are required as controls and readily available to researchers. This PCR method has been refined by the addition of a PCR-RFLP step. A portion of the *URA5* gene is amplified, and the amplicon is digested with two endonucleases, *Sau*961 and *Hha*1 (Meyer et al. 2003). This digestion is run for three or more hours and the resulting electrophoretic DNA fragments are compared with reference strains (VNI-IV and VGI-IV) that are treated similarly (Meyer et al. 2003). This PCR fingerprinting procedure will allow most isolates to be placed in one of the global subgroups depicted in Fig. 8.1. However, it does not recognize members of the unique VNB clade found in sub-Saharan Africa.

8.2.4 Microsatellites

The amplification of microsatellite repeats exploits the hypervariability of DNA regions composed of tandem repeats of di-, tri- or multiple nucleotides. This

hypervariability can be caused by slippage during DNA replication or unequal crossing-over during meiosis. Polymorphisms among the microsatellites of isolates of the same species have provided superb molecular markers. Microsatellites can be discovered by probing a genomic library or searching databases of gene sequences (Marra et al. 2004). PCR primers flanking these repeat regions can be designed, and the PCR products are typically sequenced or electrophoresed to detect size differences in a single microsatellite. Many microsatellites do not display intraspecies variation, but others may exhibit one or more polymorphisms or alleles. Concerns about these markers relate to the occasional instability of some microsatellites and PCR artifacts (de Valk et al. 2007). Recently, scrutiny of the genomic sequence of strain H99 (*C. neoformans* var. *grubii*) permitted the researchers to identify and design PCR primers for several microsatellites, three of which were polymorphic and used to genotype 87 isolates of *C. neoformans* (Hanafy et al. 2008). As noted above, microsatellite sequences have also been used as primers in PCR fingerprinting (Trilles et al. 2008).

8.2.5 Amplified Fragment Length Polymorphisms (AFLP)

Detection of AFLP is a powerful method for fingerprinting genomic DNA, as well as generating a large number of dominant markers for genotypic analysis (Vos et al. 1995; Vos and Kuiper 1997). With AFLP, genomic DNA is first digested with two endonucleases (usually a frequent and a rare cutting enzyme), and dsDNA adapters are ligated to the ends of the DNA fragments to create template DNA for the PCR. AFLP adapters are comprised of a core sequence and an enzyme-specific sequence. The AFLP amplification primers consist of the core sequence, the enzyme-specific sequence and a selective extension of one to three nucleotides, which will amplify a subset of the restriction fragments. AFLP involves two PCR steps: The preamplification uses unlabelled primers with a single selective nucleotide. After this PCR, the reactions are diluted ten-fold and used as templates for the second or selective PCR, which uses a longer extension and labeled primers. AFLP has several advantages over other methods. Many more fragments can be generated and analyzed. By varying the restriction enzymes and the extension nucleotides, 30 to >100 fragments can be produced. In addition, the fragments are stable and highly reproducible as they are amplified with two specific primers under stringent conditions. Like RAPD markers, AFLP markers are amplified using arbitrary sequences but with greater reproducibility and fidelity. However, as noted above, AFLP data are also laboratory-specific (Litvintseva et al. 2005a; Forche et al. 2000).

8.2.6 PCR-Restriction Fragment Length Polymorphism (RFLP)

In the most common utilization of RFLP to discriminate species and strains of *Cryptococcus*, one or more four-base or other frequently cutting restriction

endonuclease is allowed to digest a genomic amplicon, and the resultant bands are examined on an electrophoretic gel. This approach was used to develop markers for cryptococcal strain differentiation by detecting RFLPs in several gene sequences, including nuclear rDNA (Vilgalys and Hester 1990), *URA5* (Velegraki et al. 2001), and *PLB1* (Latouche et al. 2003), as well as mitochondrial rDNA and *ND2* (Xu 2002). In addition, the mating type alleles of *C. neoformans* and *C. gattii* can be distinguished by PCR-RFLP analysis of two genes located within the mating type locus, *CAP1* and *GEF1* (Feng et al. 2008a).

8.2.7 DNA–DNA Hybridization

In the standard Southern blot hybridization method, genomic DNA is digested with one or more restriction endonucleases and electrophoresed on an agarose gel to separate the fragments according to their length. The gel is transferred to an alkaline solution to denature the DNA and produce single-stranded DNA fragments, which are then transferred to a negatively charged nitrocellulose (or nylon) membrane by blotting and drying (i.e., capillary action and ionic exchange). The membrane is then heated at 80°C to permanently fix the single-stranded DNA fragments to the membrane. After washing and treating the membrane with unrelated DNA to block nonspecific DNA binding, the membrane is treated with a single-stranded DNA probe that is end-labeled with a fluorescent or chromogenic dye. The probe will bind to the complementary DNA on the membrane and can be detected by visualization of the label. If multiple lanes are electrophoresed with template DNA from different strains, the probe will not bind to strains that possess polymorphic DNA with a different, noncomplementary sequence. Genomic RFLPs detected by hybridization with single-copy probes are uncomplicated, and the data can be used for multiple purposes. However, bands generated by hybridization with probes to multi-copy loci may be difficult to interpret because of the large number of bands and/or the conditions of electrophoresis. These banding patterns can often identify species or strains, but it is difficult for them to distinguish the allelic status of individual loci (Xu and Mitchell 2002).

8.2.8 DNA Sequencing

The most precise method to identify differences at the DNA level is the direct sequencing of PCR products, cloned genes, or whole genomes. This approach provides the most accurate data for phylogenetic analyses. As with other procedures, several investigations have analyzed medical fungi by direct DNA sequencing. For comparing related taxa, robust approaches involve whole genome sequencing and comparative genomics (Galagan et al. 2005). The more amenable strategy, especially for molecular epidemiology and population genetics, is multilocus

sequence typing (MLST), whereby sequences of several genes are compared across members of the species (Mitchell 2008; Odds and Jacobsen 2008; Taylor and Fisher 2003). MLST data are reproducible and the protocols can be implemented by any laboratory with DNA sequencing capability. Matthew C. Fisher and colleagues have created a web site that catalogs protocols and data for many pathogenic bacteria and fungi, including *C. albicans, C. glabrata, C. krusei, C. tropicalis*, and *C. neoformans* var. *grubii* (http://www.mlst.net/databases/default.asp).

8.2.9 Method Selection

There is no single or ideal molecular method for every application. As mentioned above, different typing methods are appropriate for different purposes. For example, rDNA sequences are highly conserved at the species level, and, therefore, they provide poor markers for strain identification, but they are excellent for differentiating higher taxa. In contrast, stable mutations are more common in noncoding sequences and also among the third bases of the codons of protein encoding genes. Consequently, for comparisons among strains of a species, MLST using protein encoding genes or nonfunctional DNA are usually more informative than rDNA. Thus, depending on the goal, molecular methods can be selected to permit the identification of species, molecular epidemiology, detection of key phenotypic differences (e.g. resistance to antifungals), or analysis of genetic diversity and evolution.

8.2.10 Applications of Molecular Typing

There are multiple reasons to genotype clinical and environmental isolates of *Cryptococcus* and to determine their population structure and geographic distribution. Molecular typing has the potential to (1) confirm the identification of clinical isolates, (2) resolve the taxonomic status of members of the pathogenic *Cryptococcus* species complex, (3) determine the source of cryptococcal infections in local areas of high endemicity, (4) improve the accuracy of identifying subpopulations of *Cryptococcus* in clinical specimens, (5) recognize strains with clinically important phenotypes (e.g., virulence factors, resistance to antifungal drugs), (6) analyze the genetics of recognized subpopulations of *C. neoformans* and *C. gattii*, (7) investigate the evolution of pathogenic species of *Cryptococcus*, (8) validate the medical relevance of well-characterized strains that are used for basic research, studies of pathogenesis and comparative genomics, and the development of new antifungal antibiotics and diagnostic tests, and (9) discover environmental reservoirs of *Cryptococcus*, which may lead to public health recommendations to minimize exposure. These overlapping advantages of molecular typing are summarized in Table 8.2, and a few of them warrant explication.

Table 8.2 Utility of typing strains of the *C. neoformans-C. gattii* species complex

- Confirm phenotypic identification
- Resolve taxonomy
- Molecular epidemiology
- Improve the diagnosis of cryptococcosis
- Identify any genotype-phenotype associations to investigate pathogenicity;
- Investigate mechanisms of genotypic or phenotypic variation
- Validate strains for basic and applied research
- Determine the ecology of prevalent clinical genotypes
- Investigate the evolution of pathogenic species of *Cryptococcus*

8.2.11 Identification

Numerous molecular methods have been developed to type or identify pathogenic microbes, including species and strains of *Cryptococcus*. Conventional methods of identifying fungi utilize phenotypic differences in vegetative and reproductive morphology, physiology, and the presence of structural macromolecules. Unlike molds, yeasts exhibit less distinctive reproductive structures, and consequently, biochemical tests are generally more useful than morphological criteria. These phenotype-based methods are practical for the routine identification of medical yeasts, and algorithms are designed to provide a rapid and presumptive, if not definitive, identification. Species of *Cryptococcus* are nonpigmented and usually produce mucoid colonies. In the clinical microbiology laboratory, the isolation of a hyaline, encapsulated and spherical yeast that lacks pseudohyphae, is nonfermentative and produces urease will be identified presumptively as a species of *Cryptococcus*. Although few of the many species of *Cryptococcus* are able to infect mammals, two additional properties will identify the major pathogenic species. If a clinical isolate also grows at 37°C and produces laccase, which is demonstrated by the deposition of melanin in the cell walls after growth on an appropriate diphenolic substrate, it is almost certainly *Cryptococcus neoformans* or *C. gattii* (or extremely rarely, *C. laurentii*). The color reaction of the isolate on canavanine-glycine-bromthymol blue (CGB) agar will distinguish most isolates of *C. gattii* (blue) from *C. neoformans* (greenish yellow) (Kwon-Chung and Bennett 1992). This information is sufficient to establish a diagnosis of cryptococcosis and initiate appropriate therapy. The diagnosis can also be demonstrated or corroborated by a positive test for the cryptococcal capsular antigen in the patient's serum or cerebrospinal fluid (see Chap. 7).

However, not all strains of *C. neoformans* and *C. gattii* exhibit typical properties. A clinical isolate may lack a capsule, and a small percentage of isolates do not give the typical species-specific reaction on CGB agar. As noted above and in Table 8.2, if the morphological or biochemical phenotypes of an isolate are atypical, molecular methods can be used to establish the identification. Furthermore, as molecular strain typing becomes more discriminating, this approach may soon be able to detect clinically relevant information, such as resistance to fluconazole, as well as track the source of the infection.

8.2.12 *Pathogenesis and Phenotypic Strain Variation*

C. neoformans and *C. gattii* are exogenous and acquired by inhalation of desiccated yeast cells or basidiospores. When these infectious propagules reach the alveoli, they become encapsulated yeasts and proliferate. This primary infection may become latent or progress to active disease, often with dissemination to the skin, reticuloendothelial tissues, and numerous other sites, but preferentially to the central nervous system. The myriad clinical manifestations of cryptococcosis are determined by interactions that involve the immune responses of the host and the number of cells of the infecting strain and their virulence. These clinical differences include variations in the histopathology, site(s) of dissemination, rate of progression, host immune responses, and response to treatment, as well as co-infections and other predisposing conditions (Casadevall and Perfect 1999; Mitchell and Perfect 1995). With regard to this dynamic engagement with the host, strain variation in cryptococcal pathogenicity affects the clinical progression of disease.

Many determinants of pathogenicity have been identified, and isolates of *Cryptococcus* vary extensively in the expression of these phenotypes, which include the serotype and properties known to affect the clinical outcome. Some attributes have been proven essential, but not necessarily sufficient for pathogenicity, such as encapsulation, growth at 37°C, and the production of laccase (Casadevall and Perfect 1999; Mitchell and Perfect 1995). Using well-characterized laboratory strains, specific gene disruption experiments have confirmed the importance of numerous genes and signal transduction pathways, and undoubtedly many more pivotal genes and genetic networks affecting virulence will be discovered (Perfect 2005).

Multiple experimental reports have documented variation in pathogenicity among both clinical and environmental strains of *C. neoformans* (Louria 1960; Fromtling et al. 1989; Kagaya et al. 1985; Kwon-Chung et al. 1992; Litvintseva and Mitchell 2009;Clancy et al. 2006) and the expression of virulence phenotypes, such as the size (Small et al. 1986; Bottone and Wormser 1986; Dykstra et al. 1977), composition (Cherniak et al. 1993; Small et al. 1986) and the biological activity of the capsule (Small and Mitchell 1989), the production of laccase (Kwon-Chung et al. 1982; Jacobson and Tinnell 1993) and phospholipases (Chen et al. 1997), susceptibility to antifungal drugs (Casadevall et al. 1993; Chin et al. 1989; Velez et al. 1993; Iwata et al. 1990), and resistance to phagocytosis and phagocytic killing mechanisms (Bolaños and Mitchell 1989; Miller and Mitchell 1991; Kagaya et al. 1985; Xie et al. 1997). In addition, the phenotypes of individual strains are known to change, and one mechanism involves switching (Goldman et al. 1998; Fries et al. 2002; Pietrella et al. 2003; Jain et al. 2006). Another may be the activity of retrotransposons, which are manifold in *C. neoformans* (Litvintseva and Mitchell 2009;Keller et al. 2006; Loftus et al. 2005). A major goal of molecular typing and phylogenetics is to discover genotypes that identify strains with enhanced virulence, clinically relevant phenotypes and predictable patient outcomes. However, consistent genotype-phenotype associations have not yet been defined for *Cryptococcus* strains.

8.2.13 *Elucidate the Epidemiology and Ecology of* Cryptococcus *Species*

Compared to cataloging phenotypic differences among strains, molecular markers provide a more definitive and stable method to characterize strains. In general, several categories of molecules have been utilized for the identification of medical fungi and the diagnosis of mycotic infections. They include fungal antigens, secreted enzymes and proteins, fungal byproducts and secondary metabolites, cell wall mannoproteins and other compounds. For the pathogenic species of *Cryptococcus*, antibodies to the major epitopes of the predominant capsular polysaccharide, glucuronoxylomannan (GXM), have been used to classify five serotypes of *C. neoformans* and *C. gattii*, which are designated A, B, C, D or AD. Commercial antisera that recognize all these serotypes of GXM have been developed for the clinical, diagnostic detection and titration of cryptococcal GXM in serum, spinal fluid and other body fluids (Hung et al. 2008; Stevens 2002). However, these diagnostic tests do not distinguish the serotypes or species of *Cryptococcus*.

Similar to other systemic mycoses, cryptococcosis is often asymptomatic, at least initially, although infected subjects may retain viable but dormant organisms in quiescent lesions with the potential for subsequent reactivation and disease (Mitchell 2004). With the endemic mycoses, asymptomatic infections can be detected by the development of cell-mediated immune responses or antibodies to the fungal antigens as well as histopathological evidence of infection. However, there is limited serological and histopathological evidence to suggest that many people are infected with *Cryptococcus* and only a subset develop serious disease. Unlike the endemic mycoses, facile tests to detect exposure among the human population are lacking. There are no commercial *Cryptococcus*-specific skin test antigens with which to conduct inexpensive, large-scale testing to determine the extent of specific immune responses and latent infection in the healthy human populations. Furthermore, after a primary cryptococcal episode, the lungs usually do not retain fibrotic or calcified lesion(s) that are visible on chest radiographs. Nevertheless, from reviews of histopathological data (Baker and Haugen 1955) as well as a few studies of specific antibodies to cryptococcal antigens (Davis et al. 2007; Goldman et al. 2001), it is likely that an undetermined portion of the population develops latent cryptococcal infection, often in childhood.

Lacking definitive public health data on the prevalence of cryptococcosis, widespread and rigorous genetic and phenotypic analyses of clinical and environmental isolates of *Cryptococcus* will help define the global dynamics of these species and regional pockets of endemicity. For example, environmental surveillance studies have confirmed that strains of serotype A are much more prevalent in pigeon guano collected in the southeast and east coast of the USA than the western states (Littman and Borok 1968; Currie et al. 1994; Litvintseva et al. 2005a). A large comparison of more than 800 clinical and environmental isolates revealed that strains with certain AFLP genotypes are significantly more prevalent in patients than the environment, and vice versa (Litvintseva et al. 2005a). Strain genotyping

has been used to track the spread of an ongoing outbreak of VNII strains of *C. gattii* that started in 1999 and has since expanded from Vancouver Island to mainland British Columbia, Canada and the Pacific northwest of the USA (Kidd et al. 2004, 2005; Fraser et al. 2005). The outbreak strains are predominantly one of two MLST genotypes of VNII, but a third MLST genotype recently emerged in the US state of Oregon (Byrnes et al. 2009).

8.2.14 Population Genetics of Cryptococcus

Molecular markers can be used to determine the structure and mode(s) of reproduction of populations and subpopulations of *C. neoformans* and *C. gattii*. As with other pathogenic microbes, it is useful to determine the life cycle, reproductive options and population structure because these features propel the evolution of *Cryptococcus*. This information has public health significance by predicting a microorganism's capacity to adapt quickly to changing environments, host species, and antimicrobial antibiotics.

Many of the same molecular markers used to identify strains can be applied to dissect these processes. Species of *Cryptococcus* normally reproduce vegetatively by asexual, mitotic budding, which yields clonal populations. In the laboratory and perhaps in nature, they are also capable of sexual reproduction. DNA-based markers can be used to analyze the extent of recombination and clonality within a population of strains. The usual evidence supporting a clonal population structure are (1) over-representation of one or more genotypes; (2) nonrandom segregation at individual loci (e.g., deviation of genotypic frequencies from Hardy–Weinberg expectations in diploids); and (3) absence of recombination between loci (linkage disequilibrium). Phylogenetic criteria for clonality include evidence of phylogenetic structure among the individuals and congruence among multiple trees constructed from different datasets. Studies of serotype A have determined that most populations are predominantly clonal, but localized samples exhibit evidence of recombination (Litvintseva et al. 2003). Similar results have been reported for *C. gattii* (Campbell and Carter 2006; Halliday and Carter 2003). *C. neoformans* is also capable of same-sex mating (Lin et al. 2005), and this novel mechanism of genetic exchange has been shown to occur in nature and affect pathogenicity and population structure (Lin et al. 2008, 2009).

8.3 Ecology and Epidemiology of Cryptococcosis

Cryptococcosis is acquired by the inhalation of an exogenous species of *Cryptococcus*, and the incidence of cryptococcosis tends to be greater in geographical areas with a relatively high prevalence of *Cryptococcus* in the environment. Veterinary reports have confirmed that both species may infect other mammals,

including domestic pets (dogs, cats), livestock (alpacas) and wild animals (koala) (Krockenberger et al. 2003; MacDougall et al. 2007; O'Brien et al. 2004). However, pediatric cases are unaccountably rare (Othman et al. 2004; Sweeney et al. 2003; Abadi et al. 1999).

8.3.1 C. neoformans

In patients and the natural world, both varieties of *C. neoformans* are globally distributed, but serotype A is ubiquitous, and serotype D is more frequent in Europe. Serotype A strains are responsible worldwide for most cases of cryptococcosis and more than 95% of cases in patients with AIDS (Mitchell and Perfect 1995; Casadevall and Perfect 1999). This cosmopolitan species occurs more frequently in tropical to temperate regions, but it is less prevalent in northern Russia, Canada and Scandinavia (Knudsen et al. 1997; Torfoss and Sandven 2005). A significant natural reservoir for *C. neoformans* is avian habitats, especially pigeon roosting areas, where the yeast is enriched by aged avian excreta. Birds may inadvertently spread the yeast cells, but they are rarely infected because the avian body temperature ranges from 41.5 to 43°C, which is too high to support the growth of *Cryptococcus* (Emmons 1955; Littman and Borok 1968). Nevertheless, at least 26 avian cases of cryptococcosis due to both species have been documented, but the temperature tolerance of these strains was not reported (Malik et al. 2003). In many tropical and semitropical climates, *C. neoformans* can be readily isolated from avian roosting areas that are shielded from direct sun and ultraviolet light, and in this milieu, the yeasts secrete urease and apparently thrive on urea and other nitrogenous substrates. They also produce airborne yeast cells that are small enough to reach the alveoli if inhaled (Swinne-Desgain 1975; Powell et al. 1972). Both *Cryptococcus* species grow well on pigeon guano in the laboratory and become melaninized (Nielsen et al. 2007; Nosanchuk et al. 1999), and *C. neoformans* can also produce potentially infectious basidiospores (Nielsen et al. 2007).

The prevalence of *C. neoformans* in avian environments corresponds with the endemic areas for cryptococcosis, and isolates with the same genotype have been recovered from patients and pigeon guano (Litvintseva et al. 2005a; Franzot et al. 1997; Currie et al. 1994). Therefore, *C. neoformans* in avian habitats has been considered a major source of human cryptococcosis. However, this consensus was challenged by a recent report that pigeon isolates were less virulent for mice than human isolates with the same AFLP and MLST genotypes (Litvintseva and Mitchell 2009). This observation may be an artifact of the infection model or a limitation of the genotyping methods. However, it could also indicate that *C. neoformans* has other significant reservoirs in nature. In recent years, there have been a growing number of reported isolations of *C. neoformans* from a variety of trees in various parts of the world, including Colombia (Granados and Castañeda 2006; Nishikawa et al. 2003), Brazil (Reimao et al. 2007; Kobayashi et al. 2005; Reimao et al. 2007), Thailand (Sriburee et al. 2004) and India (Randhawa et al. 2008; Gugnani et al. 2005;

Grover et al. 2007). Thus, *C. neoformans* can be found in arboreal as well as avian environs.

Over the past three decades, the incidence of cryptococcosis due to *C. neoformans* has been increasing. Most cases occur in immunocompromised patients, and the major risk factors include HIV/AIDS, hematological malignancies and treatment with systemic corticosteroids. This global increase can be attributed to the advent of AIDS and the growing population of patients with compromised host defenses, especially those with cellular immunodeficiencies that often result from the administration of immunosuppressive or cytotoxic drugs to combat cancer or the rejection of hematopoietic stem cell or solid organ transplants. Since the institution of highly active antiretroviral therapy (HAART) for the treatment of HIV/AIDS, the incidence of co-infection with *C. neoformans* and HIV has abated in the developed nations (Antinori et al. 2009), but some of these patients may subsequently acquire immune reconstitution inflammatory syndrome (Shelburne et al. 2005). However, in Africa, Asia, and South America, the burden remains high because therapy is less available and patients are usually not treated until the late stages of disease. According to the latest data from the UN, there are approximately one million new cases of cryptococcal meningitis annually, and the mortality is estimated to exceed 50% within three months after diagnosis (Park et al. 2009). Both the incidence and mortality are highest in sub-Saharan Africa.

The vast majority of these infections with *C. neoformans* are caused by the cosmopolitan population of VNI strains, which are readily isolated from the environment. Strains of VNII have apparently also infected people on every continent but they are much less frequent, and there have been very few isolations of VNII from the environment. To date, all the clinical cases with VNB strains have occurred in sub-Saharan Africa or Brazil (Ngamskulrungroj et al. 2009; Litvintseva et al. 2006). Strains of VNIV (serotype D) and VNIII (AD hybrids) have been isolated globally from the environment and patients. Infections with these genotypes are more prevalent than VNII or VNB, and more concentrated in Europe (Lizarazo et al. 2007; Viviani et al. 2006). A rigorous clinical and epidemiological analysis of 230 patients with cryptococcosis due to serotype A or D concluded that the disease was significantly more severe in patients who were male, seropositive for HIV, and infected with serotype A (Dromer et al. 2007).

Despite the strong association with HIV/AIDS and other immunocompromising, underlying conditions, there are numerous reports of *C. neoformans* infections in immunocompetent persons. In these "normal" hosts, pulmonary infection is more common, but dissemination also occurs (Baddley et al. 2008; Othman et al. 2004; Nadrous et al. 2003).

8.3.2 C. gattii

As mentioned earlier, *C. gattii* differs from *C. neoformans* in geographical distribution, serotypes, ecology, clinical manifestations and several in vitro phenotypes.

The prevalence of *C. gattii* is more limited in patients and the environment. In the environment, *C. gattii* has long been associated with trees, tree hollows and decayed wood, and this species has been isolated from *Eucalyptus camaldulensis* and other varieties of *Eucalyptus* as well as other species of trees in Australia and elsewhere (Sorrell 2001). *Eucalyptus* trees have been sampled more than other trees, but it is clear that *C. gattii* populates native trees wherever it becomes established, and the tree species harboring *C. gattii* differ in Argentina, Brazil, Canada, Colombia, India and the USA. Historically, *C. gattii* was thought to be restricted to tropical areas of Australia, Asia, Africa and the Americas, but it emerged this millennium in southwestern Canada and the northwestern USA (Kidd et al. 2004; Fraser et al. 2005; Kidd et al. 2005;Byrnes et al. 2009). In extensive environmental studies associated with this outbreak of *C. gattii* in North America, the predominant clinical genotypes (VGIIa and VGIIb) were isolated from regional trees (Douglas fir, alder, red cedar, arbutus, Garry oak et al.), soil, air, fresh and sea water, and fomites (shoes, auto tires) (Kidd et al. 2007a,b).

In both the environment and among patients, the geographical distribution of the subpopulations of *C. gattii* varies considerably, and more detailed genotyping surveys are needed to confirm and extend the current findings. Overall, VGI and VGII are far more common than VGIII or VGIV. In Australia, VGI isolates of serotype B are most prevalent in both clinical and environmental samples (Sorrell et al. 1996), but in South America, VGII isolates of serotype B predominate (Lizarazo et al. 2007; Escandon et al. 2006; Granados and Castañeda 2006; Trilles et al. 2008; Nishikawa et al. 2003). Nearly all environmental isolates of *C. gattii* have been serotype B (VGI or VGII). The ecology of serotype C remains a mystery, although two isolates were recovered from almond trees in Colombia (Callejas et al. 1998).

Overall, most cases of *C. gattii* occur in apparently immunocompetent humans and other mammals. However, there are many confirmed cases in AIDS patients (Lindenberg et al. 2008; Bogaerts et al. 1999), including African patients with AIDS and serotype C (Litvintseva et al. 2005b; Karstaedt et al. 2002).

8.4 Conclusions

Over the past two decades, there have been manifold studies of the phylogenetics and population structure of these species, and a plethora of DNA markers have been employed (Mitchell 2008). Quite recently, several laboratories involved in this research have developed standard MLST markers for genotyping strains of *C. neoformans* var. *grubii*, and a similar consensus is being developed for *C. gattii*. This collective effort has defined several distinct genetic subpopulations of *C. neoformans* and *C. gattii*, designated VNI-VNIV, VNB and VGI-VGIV, respectively (Bovers et al. 2008; Campbell et al. 2005; Kidd et al. 2005; Kwon-Chung and Varma 2006; Litvintseva et al. 2006; Meyer et al. 2003; Meyer et al. 2009; Ngamskulrungroj et al. 2009; Xu et al. 2000;). These methods are resolving the

phylogeny of this species-complex. They enabled the discovery that the most diverse isolates of *C. neoformans* var. *grubii* exist in sub-Saharan Africa (Litvintseva et al. 2003, 2006, 2007). In recent years, human and veterinary cases of cryptococcosis due to *C. gattii* emerged on Vancouver Island and have spread to mainland British Columbia and the northwestern USA (Kidd et al. 2004). This major, ongoing outbreak has been tracked by genotyping environmental, clinical and veterinary strains, and MLST markers are being used to determine the origin and evolution of the responsible strains (Kidd et al. 2004, 2005;Byrnes et al. 2009). Clearly, the genotyping methods are in place to investigate the next emergent strains of *Cryptococcus*.

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Chapter 9
Environmental Stress-Sensing and Pathogenicity in *Cryptococcus neoformans*

Man-Shun Fu, Rebecca A. Hall, and Fritz A. Mühlschlegel

9.1 Introduction

Cellular stress can be defined as the damage caused to macromolecular systems when the cell is exposed to acute environmental changes, while a stress response is a conserved mechanism of resistance to these damages (Kültz 2003). *Cryptococcus neoformans*, like most pathogens, not only has to cope with substantial changes in its natural environment, but must also respond and proliferate in a variety of conditions found within the host. As with most pathogenic microbes therefore, appropriate responses to stress are key elements for survival in the host. The stresses *C. neoformans* encounters within its host include oxidative stress, nitrosative stress, osmotic shock, high temperature, hypoxia, nutrient deprivation, changes in pH, low- calcium and iron deprivation (Brown et al. 2007). Several signaling pathways mediated by the Hog1p, protein kinase C (pkC) and calcineurin/calmodulin allow this fungus to sense and respond to stress. However, the mechanisms underlying stress responses in *C. neoformans* are not completely understood. Recent genomic and proteomic approaches have allowed us to gain further understanding of the stress responses in *C. neoformans*. In this chapter, the current knowledge on individual genes, pathways and transcription factors which are essential for stress resistance in *C. neoformans* are discussed.

9.2 The Main Phenotypes Elaborating *C. neoformans* Stress Responses

Although *C. neoformans* is able to survive in the mammalian host, where it causes infection, it is naturally found in soil environments contaminated with bird excreta (Hull and Heitman 2002; Bose et al. 2003). In order to adapt to both external and

M-S. Fu, R.A. Hall and F.A. Mühlschlegel (✉)
Department of Biosciences, University of Kent, Canterbury CT2 7NJ, United Kingdom
e-mail: F.A.Muhlschlegel@kent.ac.uk

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internal hostile environments, *C. neoformans* has evolved multiple mechanisms, including capsule enlargement, melanin biosynthesis and biofilm formation, which function in stress resistance.

9.2.1 Capsule Enlargement

The *C. neoformans* capsule is considered one of its main virulence factors. The capsule is formed from two main polysaccharide components, glucuronoxylomannan (GXM) and galactoxylomannan (GalXM), together with one minor component, mannoprotein (Bose et al. 2003; Janbon 2004). The capsule is considered a virulence factor as it not only protects the cell, by providing a physical barrier, but it also interferes with normal phagocytosis by effector cells of the host's immune system (Bose et al. 2003; Janbon 2004). Moreover, *in vitro* assays suggest that the capsule plays a role in resistance to oxidative stress. In fact, *C. neoformans* strains that exhibit larger capsules can survive for longer periods when exposed to reactive oxygen species (ROS) when compared to cells with smaller capsules. This resistance is due to increased capsule polysaccharides and not higher catalase activity (Zaragoza et al. 2008). The addition of purified capsular polysaccharides not only protects *C. neoformans* with small capsules, but notably also protects other species such as *Saccharomyces cerevisiae* against oxidative stress (Zaragoza et al. 2008). The molecular mechanism of the capsular antioxidant mechanism is still unknown, but it is thought that the polysaccharide scavenges oxygen-related oxidants (Zaragoza et al. 2008). Therefore, capsule enlargement is suggested to be one of the key mechanisms to enhance survival of *C. neoformans* in phagocytes, where the production of ROS like hydrogen peroxide and nitric acid are essential to phagocytosis. Furthermore, capsule enlargement can increase resistance to antimicrobial peptides and the antifungal drug, Amphotericin B (Zaragoza et al. 2008). This enhanced resistance may result from charge repulsion and/or steric hindrance generated by high cross-linking of the polysaccharide fibers. However, it does not have any effect against other antifungal drugs, such as voriconazole, posaconazole, itraconazole and fluconazole (Zaragoza et al. 2008).

9.2.2 Biofilm Formation

Biofilms are complex aggregations of microorganisms that form on surfaces and are held together by an extracellular matrix (Blankenship and Mitchell 2006). Cryptococcal biofilms are formed as a complex of yeast cells and polysaccharide matrix on the surface of plastics after GXM shedding (Martinez and Casadevall 2005). The organism's ability to adhere to the prosthetic medical devices used during the course of treatment of cryptococcal meningoencephalitis infections means that, it is crucial to investigate the role of biofilm formation in *C. neoformans*.

Biofilm formation can enhance resistance to several environmental stresses such as oxidative stress, nitrosative stress, heat, cold and UV light. Metabolic activity and cell viability of *C. neoformans* in biofilms and planktonic cells have been investigated using XTT reduction and CFU assays. Individual cells in Cryptococcal biofilms are less susceptible to relatively high (37°C) or low temperatures (−20°C) (Martinez and Casadevall 2007). Moreover, cells in biofilms are more resistant to UV irradiation than planktonic cells. Furthermore, these cells are more resistant to oxidative stress when incubated with chemically generated oxygen-, nitrogen- and chlorine derived oxidants. The exopolymeric matrix of the biofilm is believed to provide a shield to resist the stress conditions and protect the organism from environmental changes.

9.2.3 Melanin Formation

Melanin, a black or brown pigment, is considered another virulence factor of *C. neoformans*. In 1982, Rhodes and colleagues demonstrated that *C. neoformans* melanin mutants are less virulent in mice when compared to wild-type strains, thus melanin is essential for the virulence of *C. neoformans* (Buchanan and Murphy 1998; Pukkila-Worley et al. 2005). Melanin is considered a free-radical scavenger and protects *C. neoformans* against oxidants *in vitro* (Wang and Casadevall 1994). *C. neoformans* cells which form melanin are more resistant to nitrogen- and oxygen-derived oxidants than nonmelanized cells (Wang and Casadevall 1994). Furthermore, melanin has been shown to be protective against microbiocidal proteins and antibiotics. Therefore, melanin biosynthesis is of substantial interest for pathogenesis studies of *C. neoformans* (Zhu and Williamson 2004).

9.3 The Genes Involved in the *C. neoformans* Stress Responses

The association of specific phenotypes to certain types of environmental stress, and their involvement in pathogenicity has led researchers to identify the genes involved in regulating the respective phenotype. This section will provide a brief introduction to some of the most important genes which have been identified to date. A summary of genes and their functions is shown in Table 1.

9.3.1 Oxidative and Nitrosative Stress Responses

C. neoformans encounters cells from the mammalian host innate immune system immediately after penetrating the lung parenchyma, and entering the alveolar space following inhalation (Giles et al. 2005). Phagocytosis is a major mechanism of the

innate immune system and elicits reactive oxygen and/or nitrogen species to remove the invading organism (Missall and Lodge 2005). In order to survive in the host, *C. neoformans* has evolved an antioxidant defense system to protect the pathogen against reactive species generated from host immune cells. This antioxidant defense system is also utilized for aerobic growth and energy production from oxidative phosphorylation (Giles et al. 2005; Missall and Lodge 2005).

9.3.1.1 Superoxide Dismutases

Superoxide dismutase enzymes are present in all organisms and protect the cells against superoxide radicals by catalyzing their conversion to hydrogen peroxide and oxygen. Superoxide dismutases are metalloenzymes, and normally form a complex with metal ions such as iron, manganese, copper or zinc (Cox et al. 2003). Copper-zinc superoxide dismutase (Sod1p) is located in the cytoplasm of eukaryotic cells. It is predicted that the gene *SOD1* encodes Sod1p in *C. neoformans* (Cox et al. 2003). Deletion of *SOD1* results in higher susceptibility of cells to ROS *in vitro*. The *sod1* mutant strain is less virulent, but still pathogenic in the mouse inhalational model (Cox et al. 2003). Moreover, the *sod1* mutant is able to grow within macrophages, suggesting that Sod1p is important for antioxidant resistance, but not essential for pathogenesis in *C. neoformans* (Cox et al. 2003).

Manganese superoxide dismutase (Sod2p) is an essential component of the mitochondrial antioxidant defense system in many eukaryotes. In fact, mitochondria are the major source of endogenous ROS in the cell. Manganese superoxide dismutase is located in the mitochondrial matrix, where its function is to detoxify oxygen radicals. In contrast to the phenotypes of the *sod1* mutant described above, *C. neoformans sod2* mutants are significantly more susceptible to oxidative stress. Furthermore, they are strongly attenuated in virulence in an inhalational mouse model of systemic cryptococcosis (Giles et al. 2005). Besides, Sod2p is also essential for growth at high temperature (37°C). These findings suggest that Sod2p has more crucial roles than Sod1p in oxidative stress resistance and pathogenesis of *C. neoformans* (Giles et al. 2005).

9.3.1.2 Glutathione Peroxidases

The glutathione-mediated antioxidant system is one of the most important antioxidant mechanisms in all organisms. Glutathione peroxidases (Gpx) are the main components of the glutathione system, which catalyze the reduction of hydrogen peroxide to glutathione disulfide.

Two Gpx, Gpx1p and Gpx2p, have been identified in *C. neoformans* and show different expression patterns (Missall et al. 2005a). Although, both *GPX1* and *GPX2* are highly expressed in response to *t*-butylhydroperoxide and cumene hydroperoxide, only *GPX2* is up-regulated upon hydrogen peroxide stresses.

Therefore, there may be subtle differences in the functions of Gpx1P and Gpx2P. Interestingly, however, both genes are repressed in response to nitric oxide stress, suggesting that neither protein functions in this response. Deletion of *GPX1p* and *GPX2p* confirms that the gene products are required for resistance to *t*-butylhydroperoxide and cumene hydroperoxide, but not superoxide, hydrogen peroxide or nitric oxide (Missall et al. 2005a). Moreover, Gpx are important for resistance to macrophage killing, but they are not essential for virulence in mouse models of cryptococcosis (Missall et al. 2005a).

9.3.1.3 Thiol Peroxidase

Thiol peroxidase, a peroxide-removing enzyme, is another common enzymatic antioxidant element in most organisms. Three thiol peroxidases, Tsa1p, Tsa3p and Tsa4p, have been identified and characterized in *C. neoformans*. Tsa1p and Tsa3p are expressed differentially at 37°C, but both of them are induced in response to hydrogen peroxide (Missall et al. 2004). Deletion of *TSA1* shows high sensitivity to hydrogen peroxide, *t*-butylhydroperoxide and nitric oxide, moreover, Tsa1p is essential for virulence in the mouse infection model (Missall et al. 2004). In contrast, both the *tsa3* and *tsa4* mutants are not sensitive to any of these oxidants. However, the *tsa3* mutant is slightly more resistant to *t*-butylhydroperoxide when compared to wild type strains (Missall et al. 2004).

9.3.1.4 Thioredoxin

Thioredoxin is a component of the thioredoxin system which provides protection against oxidative stress. Thioredoxin is also a cofactor for other essential enzymes and is responsible for the reduction of protein disulphides (Missall and Lodge 2005; Stewart et al. 1998). The disulphide reducing system in *C. neoformans* consists of two small dithiol thioredoxin proteins and one thioredoxin reductase. Two thioredoxin genes, *TRX1* and *TRX2*, have been described in *C. neoformans*. Both these genes are highly expressed when cells are exposed to oxidative and nitrosative stresses, although, different expression patterns are shown in response to some specific peroxides (Missall and Lodge 2005). The functional analysis of Trx1p and Trx2p also indicates that these two enzymes contribute a different degree to various stresses. Trx1p is more important for both oxidative, nitrosative stress responses and growth of *C. neoformans,* while Trx2p contributes specifically to nitric oxide resistance. Moreover, Trx1p is essential for survival in macrophages and virulence of *C. neoformans* (Missall and Lodge 2005).

9.3.1.5 Laccase

Laccases are present in many organisms, and have broad biological functions, such as lignification of cell walls in higher plants, detoxification of hostile toxic

metabolites in fungal pathogens and pigmentation of insect cuticles (Mayer and Staples 2002; Arakane et al. 2005). In *C. neoformans*, laccases are mainly responsible for the production of melanin, which is an antioxidant, and thus contributes to oxidative stress resistance (Sales et al. 1996; Zhu and Williamson 2004). Laccases also contribute to melanin-dependent virulence in mouse models of fungal virulence (Sales et al. 1996; Zhu and Williamson 2004).

Two laccases, encoded by *LAC1* and *LAC2*, have been identified in *C. neoformans*. These homologues share 72% amino acid sequence identity within the conserved laccase copper-binding motifs (Zhu and Williamson 2004). Interestingly, the promoter regions of these two gene copies have low similarity, suggesting that these homologues may be differentially expressed in response to changing environmental conditions (Zhu and Williamson 2004). Indeed, both genes are induced upon oxidative stress and down-regulated in nitrosative stress (Missall et al. 2005b). However, Lac1p is shown to be more important for survival in the oxidative environment of macrophages while Lac2p plays a dominant role against nitrosative stress in strains lacking *TSA1* (Missall et al. 2005b). These findings not only underline the differences in functions between two laccases, but also the crosstalk between the laccase and thioredoxin system. When the thioredoxin system is absent, laccase acts as compensation mechanism to protect the cell from nitric oxide stress.

9.3.1.6 Alternative Oxidases

An alternative oxidase system can be found in most eukaryotic organisms and is the main component of the cyanide-resistant electron transport chain, catalyzing the electron transfer from reduced ubiquinone to oxygen. The *C. neoformans* alternative oxidase encoding gene, *AOX1*, has been identified and provides an antioxidant defense system within mitochondria (Akhter et al. 2003). In fact, the *C. neoformans aox1* mutant strain is more sensitive to *t*-butylhydroperoxide, when compared to the wild-type strain, but it is not temperature sensitive (Akhter et al. 2003). Moreover, this strain shows growth defects in a macrophage-like cell line when stimulated by interferon and lipopolysaccharide, which induces oxidative products (Akhter et al. 2003). Like other antioxidant enzymes, Axo1p is not only involved in the protection against oxidative stresses, but also allows the cells to grow in activated macrophages, and thus is important for the virulence of *C. neoformans* (Akhter et al. 2003).

9.3.1.7 Flavohemoglobin Denitrosylase

Flavohemoglobin denitrosylase reduces nitric oxide and converts it to nitrate in most organisms. The *C. neoformans* flavohemoglobin denitrosylase, Fhb1p, is essential for nitric oxide resistance (de Jesus-Berrios et al. 2003). Moreover, deletion of *FHB1* results in growth defects of *C. neoformans* in response to nitric

oxide stress and within macrophages (de Jesus-Berrios et al. 2003). Indeed an *fhb1* mutant is avirulent, demonstrating that, Fhb1p contributes to the pathogenesis of *C. neoformans* (de Jesus-Berrios et al. 2003).

9.3.2 High Temperature Resistance

The temperature of the human body (37°C) is substantially increased, when compared to the temperature of *C. neoformans*' natural environment. In fact, the ability of *C. neoformans* to grow at mammalian body temperature is considered a key virulence factor. The following section outlines some of the enzymes that function during temperature stress in *C. neoformans*.

9.3.2.1 Trehalose (α-glucopyranosyl-α-D-glucopyranoside)

Trehalose is a disaccharide composed of two α-glucose molecules linked by an a, a-1, 1-glucoside bond. Trehalose-6-phosphate is synthesized by trehalose-6-phosphate synthase, while trehalose-6-phosphate phosphatase can convert the trehalose-6-phosphate to trehalose.

Trehalose is believed to play a general role in the resistance to stress. In *C. neoformans*, the genes encoding trehalose-6-phosphate synthase (*TPS1*) and trehalose-6-phosphate phosphatase *(TPS2*) have been isolated and characterized (Petzold et al. 2006). Both genes are up-regulated at 37°C compared to 30°C, but expression of *TPS2* is less pronounced when compared to *TPS1*. However, both *TPS1* and *TPS2* are required for growth of *C. neoformans* at elevated temperatures (37°C) (Petzold et al. 2006). Moreover, a *tps1* mutant is avirulent in both the mouse inhalational model and rabbit cryptococcal meningitis model (Petzold et al. 2006). The precise mechanism of how trehalose increases *C. neoformans* tolerance to high temperatures is still unknown. However, in *Saccharomyces cerevisiae*, trehalose serves as a membrane protectant, and assists molecular chaperones in reactivating denatured proteins (Crowe 2007). Therefore, trehalose might have similar functions in *C. neoformans,* stabilizing protein and/or outer membrane structure during growth at high-temperatures (Petzold et al. 2006).

9.3.2.2 Cyclophilin A

Cyclophilin A, one of the peptidyl-prolyl isomerases, catalyzes peptidyl-prolyl isomerization to stabilize protein folding and assembly of multi-domain proteins in many organisms (Wang and Heitman 2005). *CPA1* encodes one of the two conserved cyclophilin A proteins in *C. neoformans*, and functions in tolerance to high temperatures (39°C). In addition, Cpa1p is important for virulence in the rabbit model for cryptococcal meningitis (Wang et al. 2001). The underlying mechanism

of how cyclophilin A protects *C. neoformans* at high temperature is still unknown. Highly conserved *C. neoformans* cyclophilin A may have a similar chaperone-like function to those of cyclophilins in other organisms, in order to stabilize protein folding at high temperatures (Wang et al. 2001; Wang and Heitman 2005).

9.3.3 Iron Deprivation

Both capsule and melanin biosynthesis are regulated by iron levels (Tangen et al. 2007), thus iron acquisition is crucial for the virulence of *C. neoformans*. The reduction of ferric iron to ferrous iron is the key process in iron acquisition and is regulated by high- and low-affinity uptake systems (Nyhus et al. 1997; Jung et al. 2006). Siderophore is an iron carrier for iron chelating and uptake in some fungi (Howard 1999; Lian et al. 2005). However, *C. neoformans* does not produce siderophores, so iron uptake is mediated by cell surface ferric reductase and secreted nonenzymatic reductants such as 3-hydroxyanthranilic acid (3HAA) and melanin (Howard 1999; Jung et al. 2006; Nyhus et al. 1997; Tangen et al. 2007). The iron-regulated gene *SIT1* encodes a putative siderophore iron transporter and is required for growth of *C. neoformans* in iron deprived environments. Notably, the *sit1* mutant also affects laccase activity, and thus influences melanin formation. Furthermore, the *sit1* mutant displays organizational changes in the cell wall and affects capsule biosynthesis. However, Sit1p is not required for the virulence in *C. neoformans* (Tangen et al. 2007). The iron permease (*FTR1*) and the multi-copper oxidase (*FET3*) form part of the high-affinity iron uptake system, and are essential for growth of *C. neoformans* upon iron deprivation (Lian et al. 2005).

9.3.4 Calcium Deprivation

Calcineurin activity (more detailed information will be shown in Sect. 9.4.1) is required for the growth of *C. neoformans* at mammalian body temperature (37°C). However, calcineurin is inactivated in low-cytosolic calcium environments such as those normally found inside macrophages (Liu et al. 2006). Thus, the survival of *C. neoformans* in the mammalian body, or within macrophages, requires Ca^{2+}-channels for the uptake of external calcium (Liu et al. 2006). The Cch1p protein has been characterized and is predicted to form a Ca^{2+}-permeable channel, controlling the entry of external calcium into *C. neoformans* (Liu et al. 2006). Deletion of *CCH1* shows that it is essential for calcium uptake and consequently for the growth of *C. neoformans* under Ca^{2+}-limiting conditions (Liu et al. 2006). Cch1p is also found to play a role in Li^{+} stress. However, infection studies with *cch1* mutants suggest that Cch1p is not required for virulence of *C. neoformans* (Liu et al. 2006).

9.4 Stress-Sensing and -Signaling Pathways in *C. neoformans*

9.4.1 Calmodulin/Calcineurin Calcium Mediated Signaling

Calcium ions are common second messengers in signal transduction pathways of all organisms, and the calcium mediated signaling pathway is highly conserved among fungi. In fungal pathogens, the calcium signal is required for virulence, stress responses and resistance to antifungal drugs (Fig. 9.1 and Odom et al. 1997; Kraus and Heitman 2003; Sanglard et al. 2003). The main components of this pathway include calcium permeable channels, pumps and transporters to control the intracellular calcium concentration. Calmodulin is a calcium sensor and calcineurin is a downstream target of calmodulin. Calcineurin, a serine-threonine specific phosphatase, consists of a catalytic A subunit and a Ca^{2+}-binding regulatory B subunit (Fig. 9.2) (Kraus et al. 2005). Calcium binds to the four EF hands in calmodulin resulting in a conformational change and release of free energy (Fig. 9.2) (Kraus et al. 2005). The Ca^{2+}/calmodulin complex then binds to the calmodulin-binding

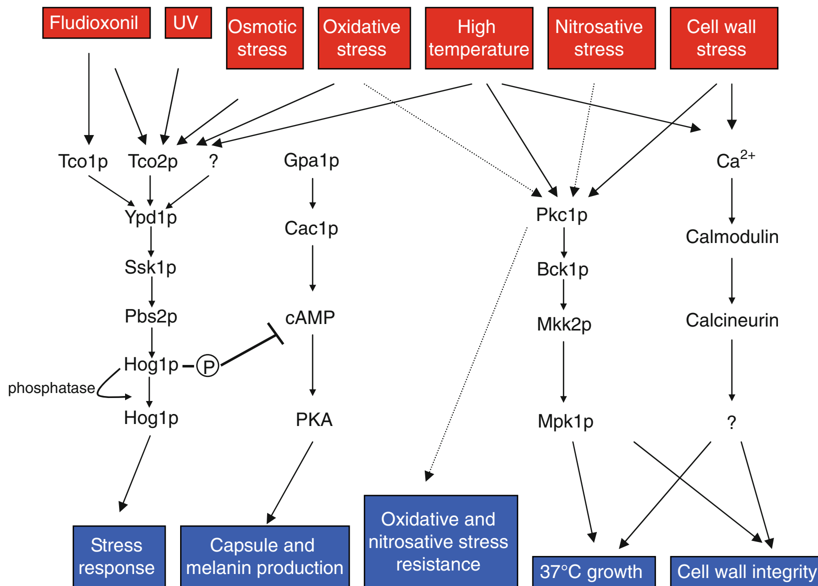

Fig. 9.1 Multiple environmental conditions activate the stress response pathways in *C. neoformans*. Diversified histidine kinases in the Hog1p MAPK pathway allow sensing of a variety of environmental signals. Under stressful conditions, Ssk1p is activated and in turn activates a Hog1p specific phosphatase to dephosphorylate Hog1p into an active form. Under normal conditions, Hog1p is constitutively phosphorylated by Pbs2p. This inactive form of Hog1p represses cAMP-PKA pathway and negatively regulates capsule and melanin production. The calmodulin/calcineurin and PKC1 MAPK pathways independently contribute to stress resistance by promoting cell wall integrity. On the other hand, Pkc1p individually plays a crucial role in resistance to oxidative and nitrosative stresses (shown in *dotted arrow*)

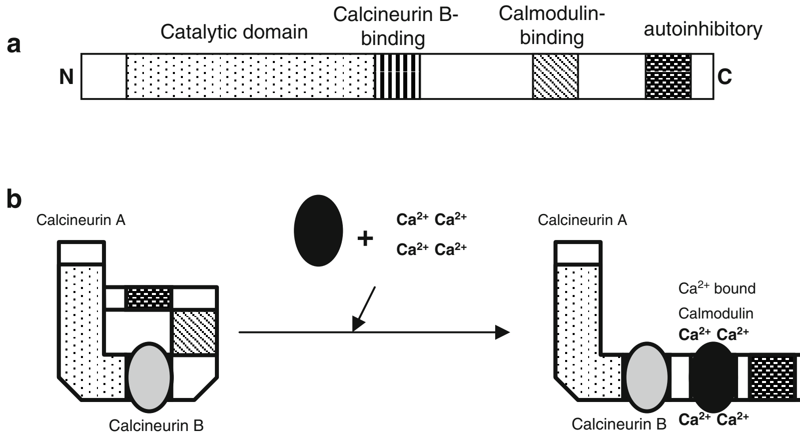

Fig. 9.2 Calcineurin activation in *C. neoformans*. (**a**) Schematic diagram of the calcineurin A subunit. The calcineurin A subunit contains a catalytic domain, a calcineurin B-binding domain, a calmodulin-binding domain and an auto-inhibitory domain. (**b**) Activation of calcineurin: When intracellular Ca^{2+} increases, a Ca^{2+}/calmodulin complex is formed and binds to the calcineurin heterodimer. The binding of calmodulin results in the dissociation of the auto-regulatory domain from the catalytic site. The conformational change activates calcineurin

domain within the catalytic A subunit of calcineurin, resulting in a conformational change of calcineurin and release of its active site from the auto-regulatory domain (Fig. 9.2) (Kraus and Heitman 2003). Activated calcineurin is required for the growth of *C. neoformans* at human body temperature (37°C) and cell wall stabilization. Therefore, the calmodulin/calcineurin signaling pathway is crucial for the virulence of *C. neoformans* (Kraus and Heitman 2003; Kraus et al. 2005).

9.4.2 *The Hog1p MAPK Pathway*

The mitogen-activated protein kinase (MAPK) pathway plays an essential role for stress responses such as high temperature, UV, oxidative and osmotic stresses (Fig. 9.1) (Bahn et al. 2007; Bahn 2008). The upstream signaling cascade of the Hog1p-Pbs2p MAPK pathway is mediated in most fungi by a two-component system initially discovered in bacteria. The fungal two-component-like system actually consists of three components, a hybrid sensor histidine kinase (containing the response regulator), a histidine-containing phosphotransfer (HPt) protein and a response regulator. In response to external signals, the sensor, histidine kinase, is able to auto-phosphorylate a histidine residue (in the histidine kinase domain) and transfers the phosphate to the HPt protein (Posas et al. 1996; Bahn et al. 2006). The response regulators then receive the signal from the histidine kinase through a series of phosphorylations (Bahn et al. 2006, 2007).

So far, seven histidine kinases have been identified in *C. neoformans* and they are thought to each be involved in the sensing of various environmental signals.

Two of them, Tco1p and Tco2p, positively regulate the Hog1 MAPK pathway (Bahn et al. 2006). Tco1p is required for melanin formation and sexual reproduction while Tco2p, is responsible for sensing oxidative and osmotic stresses (Bahn et al. 2006). The HPt protein Ypd1p, which is an orthologue of Ypd1p in *S. cerevisiae*, is required for growth of *C. neoformans* (Bahn et al. 2006). Ssk1p has been identified as a response regulator in *C. neoformans* and functions upstream of the Hog1p-Pbs2p pathway (Bahn et al. 2006). However recent reports suggest that Ssk1p may not be the only response regulator, as osmotic stress signals can bypass Ssk1p and activate downstream components of the pathway (Bahn et al. 2006, 2007).

In *S. cerevisiae* and other fungi, Hog1p is only phosphorylated in response to stress (Bahn et al. 2007). In contrast to this however, *C. neoformans* has developed a unique Hog1p MARK pathway in which Hog1p is constitutively phosphorylated (by Pbs2p MAPKK) in nonstressful conditions, but dephosphorylated under stressful conditions (Bahn et al. 2006). This unique Hog1p phosphorylation pattern is only observed in a majority of serotype A strains and some serotype D strains such as the clinical isolate B-3501. Less virulent laboratory-generated serotype D strains (e.g., JEC21 strain) have no constitutive Hog1p phosphorylation in normal conditions (Bahn et al. 2005, 2007). Interestingly, strains with constitutively phosphorylated Hog1p display a higher tolerance to stress and show increased capsule biosynthesis and melanin production, but also increased sensitivity to the antifungal drug fludioxonil. Fludioxonil activates Hog1p by dephosphorylation and in turn causes intracellular glycerol content accumulation, cell swelling, cytokinesis defects and cell growth inhibition. (Bahn et al. 2006; Kojima et al. 2006).

9.4.3 The PKC1 MAPK Signaling Pathway

Cell wall integrity is essential for fungal growth, stress survival and pathogenesis. The *PKC1*-MAPK signaling pathway mediates cell wall integrity to maintain growth when *C. neoformans* is exposed to several types of stress. The core components involved in the *S. cerevisiae PKC1* pathway have been identified (Gerik et al. 2005; Kraus et al. 2003). Phosphorylation of Pkc1p is initiated when membrane sensors detect cell wall stress. These signals are subsequently transmitted in the pathway through serial phosphorylation of Pkc1p targets that include Bck1p, Mkk1p, Mkk2p and Slt2p) (Gerik et al. 2005). Orthologues of these components are also found in *C. neoformans*, including Pkc1p, Bck1p (serine/threonine MAPK kinase kinase), Mkk2p (threonine/tyrosine MAPK kinase), and Mpk1p MAPK (*S. cerevisiae* Slt2 orthologue) (Fig. 9.1) (Gerik et al. 2005). Bck1p and Mkk2p are considered direct targets of Pkc1p, based on the growth defects and prominent cell wall phenotypes observed in the *bck1* and *mkk2* mutants (Gerik et al. 2005). Mpk1p is the final kinase of the *PKC* pathway and is required for cell integrity in response to high temperature and antifungal drugs (Kraus et al. 2003). However, the upstream stress sensors are still not known (Gerik et al. 2005). More pronounced cell integrity defects and increased susceptibility to oxidative and nitrosative stress is observed in *pkc1* mutants, compared to deletion of other

downstream targets. This finding suggests that *PKC1* not only has a role in activating the MAPK pathway, but also has alternative functions in oxidative and nitrosative stress resistance (Gerik et al. 2008). Moreover, the multiple functions of Pkc1p suggest that the *PKC1* pathway is not always activated in a linear fashion (i.e., from top to bottom). In *S. cerevisiae*, the proteins Lrg1p, Sit4p, Rho1p, Rom1p and Rom2p are considered to be involved in the regulation of the *PKC1* pathway (Gerik et al. 2008). Orthologues of these proteins can be found in *C. neoformans*, but only Lrg1p and Ppg1p (*S. cerevisiae* Sit4p orthologue) are thought to be involved in the regulation of the *PKC1* pathway (Gerik et al. 2005).

In *C. neoformans* the *PKC1* pathway can also interact with the calcineurin pathway with respect to cell wall integrity. This relationship is supported by higher sensitivity to caspofungin in the double mutant strain, that lacks both the calcineurin B regulatory subunit and the Mpk1p kinase, when compared to single mutant of *MPK1* or *CNB1*. Moreover, inhibition of calcineurin by the calcineurin inhibitor FK506 results in phosphorylation of Mpk1p, and induction of its downstream target in *PKC1* pathway (Kraus et al. 2003).

9.4.4 The cAMP Signaling Pathway

The cyclic adenosine 5'-monophosphate (cAMP)-protein kinase A (PKA) signaling pathway is crucial for the regulation of melanin production and capsule formation in *C. neoformans*. Most of the genes involved in this pathway have been identified and characterized. A guanine nucleotide-binding α-subunit (Gα protein) encoded by *GPA1* acts upstream of cAMP (Fig. 9.1) (Alspaugh et al. 1997; Pukkila-worley and Alspaugh 2004; Tolkacheva et al. 1994). Adenylyl cyclase (Cac1p) is stimulated by Gpa1p and thereby leads to the generation of the intracellular secondary messenger, cAMP (Alspaugh et al. 2002). The best-described downstream target of cAMP is protein kinase (PKA), which consists of a homodimer of two regulatory subunits (Pkr1p) and two catalytic subunits (Pka1p and Pka2p) (D'Souza et al. 2001). Activation of PKA leads to the release of its regulatory subunits, which then phosphorylate downstream targets in the pathway (D'Souza et al. 2001). Recent research has revealed that stress response genes are up-regulated in the *pka1* mutant, indicating that there is a relationship between stress and PKA signaling in *C. neoformans*. Interestingly, the *pka1* mutant is more resistant to high temperature stress when compared to controls (Pukkila-Worley and Alspaugh 2004; Hu et al. 2007).

9.5 The Main Transcription Factors Involved in the *C. neoformans* Stress Responses

Generally, most signaling cascades activate specific transcription factors, which then alter the transcriptional profile of the organism. The major transcription factors that function in stress resistance and virulence in *C. neoformans* are discussed below.

9.5.1 Nrg1p

C. neoformans Nrg1p functions downstream in the cAMP signaling pathway. Nrg1p has two H2-type zinc finger DNA-binding domains and contains a consensus sequence for PKA phosphorylation (Cramer et al. 2006). Orthologues of Nrg1p exist in *S. cerevisiae* (Nrg1p/Nrg2p) and *Candida albicans* (Nrg1p) (Cramer et al. 2006; Murad et al. 2001; Vyas et al. 2005). In *S. cerevisiae*, Nrg1p/Nrg2p acts as a repressor, controlling the expression of numerous stress response genes (Vyas et al. 2005). C. *albicans* Nrg1p is important for the down-regulation of hyphae-specific genes (Braun et al. 2001; Murad et al. 2001). In *C. neoformans,* Nrg1p is an inducer of capsule biosynthesis and Nrg1p-mediated capsule production is depended on PKA phosphorylation (Cramer et al. 2006). Putative downstream targets of Nrg1p have been identified using whole-genome microarrays (Cramer et al. 2006). Most Nrg1p target genes are involved in carbohydrate metabolism, sugar transport and oxidative stress responses. It is worth while noting that, *UGD1* (encoding a UDP-glucose degydrogenase for capsule synthesis and cell wall integrity) is down-regulated in the *nrg1* mutant (Cramer et al. 2006). Reduced capsule size in the *nrg1* mutant might therefore be a consequence of a change in carbohydrate metabolism.

9.5.2 Atf1p and Yap4p

Two putative transcription factors, Atf1p and Yap4p, have been identified in *C. neoformans* as being involved in the regulation of thioredoxin in response to either oxidative or nitrosative stress (Missall and Lodge 2005). Atf1p, an ATF/CREB-like transcription factor, is required for thioredoxin induction upon oxidative stress, while Yap4p, an AP-1 like protein, is responsible for induction under nitrosative stress (Missall and Lodge 2005).

9.5.3 Skn1p

In *S. cerevisiae* Skn7p is involved in the regulation of heat and oxidative stress response genes, as well as genes relevant to cell wall biosynthesis (Coenjaerts et al. 2006). An orthologue of Skn7p has been identified in *C. neoformans* and contains a similar heat shock factor DNA-binding domain and a receiver domain to that, which is found in *S. cerevisiae* (Coenjaerts et al. 2006). In *C. neoformans* Skn7p is essential for the response to oxidative stress and notably intracellular survival in endothelium but not for survival in phagocytic cells such as neutrophils. However, the downstream targets of Skn7p are still unknown (Coenjaerts et al. 2006).

9.5.4 Ssa1p

Ssa1p a member of Hsp70 family, acts as a GC-rich, DNA-binding transcriptional co-activator and is able to form a complex with heat shock transcription factors (HSF) and TATA-binding proteins (Zhang et al. 2006). As there is no activation domain in Ssa1p, it is believed that the interaction between Ssa1p and HSF can lead to the conformational change of HSF (Zhang et al. 2006). Such conformational changes can activate HSF and thereby, activate laccase by binding to its heat shock element upon stress (Zhang et al. 2006). Moreover, the phenotype of the *SSA1* deletion strain suggested that, Ssa1p contributes to the virulence of *C. neoformans* (Zhang et al. 2006).

9.5.5 Cir1p

CIR1 encodes a transcription factor, which is required for iron regulation in *C. neoformans* (Jung et al. 2006). Cir1p contains a cysteine-zinc domain and a zinc finger motif which are found in other fungal ion-response GATA-type transcription factors (Jung et al. 2006). Previous experimental findings show that, Cir1p can control the expression of genes in iron transport, homeostasis functions, calcium signaling, cAMP signaling, cell-wall integrity and virulence (Jung et al. 2006). Moreover, a c*ir1* mutant leads to an increased sensitivity to iron and phleomycin. Additionally, the strain is avirulent in an infection model suggesting that Cir1p is important for iron regulation and pathogenesis of *C. neoformans* (Jung et al. 2006).

9.6 Conclusion

Three main virulence factors, capsule and melanin production, and the ability to grow at high temperatures protect *C. neoformans* against various stresses in host cells. In addition, *C. neoformans* is able to form a biofilm to protect itself from environmental changes. These multiple phenotypes demonstrate the importance of stress responses in relation to pathogenesis in this fungus. Genomic and proteomic approaches have paved the way forward to the discovery of the responsible signaling cascades, the genes involved ,and the transcription profiles to specific stress responses in *C. neoformans*. Some of these genes and pathways are conserved among other fungal species including, *S. cerevisiae* and *C. albicans*. However, *C. neoformans* has also evolved unique signaling mechanisms which function in response to specific stresses, which may have all contributed to the fact that this organism has become a successful pathogen.

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Chapter 10
Malassezia

H. Ruth Ashbee and Annika Scheynius

Abstract Over the last decade, there has been a big increase in our understanding of many aspects of yeasts of the genus *Malassezia*. The description of several new species and characterisation of the genetic diversity of several other species has now resulted in 13 recognised species, with others that may be defined in the future. Recent studies have also examined the metabolites that are produced by the species of *Malassezia*, and how they may be related to the disease with which *Malassezia* has been associated. Although many studies have sought to associate a particular species with a particular disease, this has been controversial and the pattern that is now emerging is that there may be geographic variations in both commensal flora and also species associated with disease. The role of *Malassezia* in pityriasis versicolor, seborrhoeic dermatitis and, especially atopic eczema, has been studied, and the complex interactions between the organism and the host are being slowly revealed. As further studies continue, we may finally begin to understand how these organisms can exist on the skin as commensals, with minimum immune stimulation, but in some people they are able to either cause or exacerbate various cutaneous conditions.

10.1 Introduction

Yeasts of the genus *Malassezia* have been the subject of intensive research over the last decade. For the first time, their taxonomy is being unravelled by detailed molecular studies; their role in human disease has been dissected, and some of

H.R. Ashbee (✉)
Mycology Reference Centre, Department of Microbiology, Leeds General Infirmary, Leeds LS1 3EX, UK
e-mail: h.r.ashbee@leeds.ac.uk

A. Scheynius
Karolinska Institutet, Department of Medicine Solna, Clinical Allergy Research Unit L2:04, Karolinska University Hospital, Solna 171 76, Stockholm, Sweden
e-mail: Annika.Scheynius@ki.se

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the mechanisms by which they cause disease are being elucidated; and the ways in which they interact with the immune system, both in health and disease are being described. At such an interesting time in the history of *Malassezia* research, it is worth examining exactly what we now know and what remains to be understood.

The aim of this chapter is to provide an overview of the most recent research in this area in the hope that it will stimulate researchers to continue to study this interesting and paradoxical organism.

10.2 Taxonomy of *Malassezia*

Until 1996, the taxonomy of *Malassezia* was very fragmented with different groups using different classifications. *Malassezia* species are dimorphic and this confused many of the early researchers. The yeast phase was classified as *Pityrosporum* and the mycelial phase as *Malassezia*, although it was thought that there was some relationship between the two forms. In 1995, Guillot and Gueho studied the large subunit sequence of rRNA and the nuclear DNA complimentarity of 104 strains and defined seven species of *Malassezia* (Guillot and Gueho 1995). These were subsequently named and widely accepted as a reliable classification, to which others have subsequently added species over the intervening years. There are now 13 species included in the genus, which are listed in Table 10.1.

Within some species (particularly *M. sympodialis* and *M. pachydermatis)*, there is considerable genetic variation, leading to speculation that in the future, further species may be separated out from those which are currently recognised (Cabanes et al. 2005; Sugita et al. 2005).

Since the first description of the "new" species in 1995, many methods have been published for the identification of the species. Guillot described a method relying on the microscopic appearance of the yeast cells, and the ability of different species to use different Tweens (Guillot et al. 1996). This was further refined by the

Table 10.1 Currently recognised species of *Malassezia*

Species	Source or occurrence	Reference
M. furfur	Human skin	Gueho et al. (1996)
M. sympodialis		Simmons and Gueho (1990)
M. restricta		Gueho et al. (1996)
M. globosa		
M. obtusa		
M. slooffiae	Pig skin	
M. dermatis	Atopic dermatitis	Sugita et al. (2002)
M. japonica	Atopic dermatitis	Sugita et al. (2003)
M. nana	Cats, cows	Hirai et al. (2004)
M. yamotoensis	Human skin	Sugita et al. (2004)
M. caprae	Goat, horse	Cabanes et al. (2007)
M. equina	Horse, cow	
M. pachydermatis	Various animals	Gueho et al. (1996)

addition of utilisation of Cremophor and splitting of esculin (Mayser et al. 1997). Subsequently, a wide range of molecular methods had been published, but no one method is able to identify all 13 species, although a PCR-RFLP method was described that could identify 11 species (Mirhendi et al. 2005)

10.3 Physiology and Biochemistry

The physiology and biochemistry, as with many areas related to *Malassezia,* have received a lot of attention over the last 5 years, and we are beginning to understand the complex physiology of this genus.

Malassezia yeasts reproduce by asexual reproduction by monopolar or sympodial budding usually from a broad base. The daughter cells often have pronounced collarettes at the site of separation from the mother cells, which may be seen during microscopic examination (Ahearn and Simmons 1998).

10.3.1 Cell Structure

Malassezia cells range from approximately 2–8 µm, varying in both size and shape with the different species. The cell wall of these yeasts are very thick in comparison to other yeasts, being about 0.12 µm (Keddie and Barajas 1972) and consisting of ~70% sugars, ~10% protein and 15–20% lipids (Thompson and Colvin 1970). The cell wall appears to be lamellar and is surrounded by a lipid-rich capsular-like structure (Mittag 1995), which is now thought to be involved in the organism's interaction with the host (Thomas et al. 2008).

10.3.2 Dimorphism

Early studies of *Malassezia* were hampered because the organism is dimorphic, able to exist both as a yeast and also as short, distorted hyphae, usually seen in the lesions of pityriasis versicolor. The yeast phase was initially thought to be a separate organism to the hyphal phase and was assigned to the genus *Pityrosporum* (Lodder and Kreger-van Rij 1952) ,whilst the hyphal phase was designated *M. furfur*. This division persisted for many years, although several authors had suggested that there might be a relationship between the yeast phase and the hyphal phase. In 1977, three separate groups succeeded in inducing the conversion of yeast forms to hyphae, using glycine (Dorn and Roehnert 1977), cholesterol and cholesterol esters (Nazzaro-Porro et al. 1977), or potassium nitrate (Salkin and Gordon 1977). This led to the acceptance that the yeast and hyphal phases were the same organism and to the acceptance of the name *M. furfur* for both phases (Cannon 1986).

10.3.3 Lipid Dependence and Culture Requirements

Apart from *M. pachydermatis*, all species of *Malassezia* require an exogenous source of lipid to grow *in vitro* due to a block in the *de novo* synthesis of myristic acid (Shifrine and Marr 1963). Various lipids have been used in culture media to fulfil this requirement, including olive oil, Tweens, milk and glycerol. Because of their requirement for lipid, routinely used media will not support the growth of *Malassezia*, meaning that in most clinical laboratories, recovery of *Malassezia* will be significantly underestimated. Specialist media such as Dixon's medium (Van Abbe 1964) and Leeming and Notman agar (Leeming and Notman 1987) have widely replaced the use of Sabouraud's agar overlaid with olive oil as the newer media incorporate the lipid into the medium and hence are much easier to work with. Recently, it has been shown that some of the essential growth requirements are ox bile, glycerol monostearate, glycerol, and Tween 60 and that if these components are added to other agars, they are sufficient to support the growth of most species of *Malassezia* (Kaneko et al. 2005).

10.3.4 Metabolite Production

Malassezia species produce a wide range of metabolites, including enzymes and pigments, some of which are related to their nutritional requirement for lipid (see Table 10.2).

One of the most active areas of research over the last ten years has been to characterise the pigments produced by *M. furfur*. When *M. furfur* is grown on minimal medium, with tryptophan as the sole nitrogen source, it produces a wide range of pigments and fluorochromes (Mayser et al. 1998). Since the initial description of this ability, the compounds produced and their biological activities have been extensively studied (see Table10.2). Many of the activities which these compounds possess appear to correlate well with several features seen in patients with pityriasis versicolor, and it is tempting to speculate that they may be the explanation for findings such as de-pigmentation and lack of inflammation seen in the lesions. However, all the data have been collected *in vitro* and there is no conclusive proof that these same compounds are produced *in vivo*.

The recent sequencing of the genome of *M. globosa* and *M. restricta* has also shed light on the metabolites and enzymes produced by these species (Xu et al. 2007). Unlike every other free-living fungus studied, neither species produce a fatty acid synthase, accounting for their lipid dependence. In *M. globosa*, the sequencing revealed genes encoding 14 lipases, 9 phospholipases and 18 aspartyl proteases, of which 8 lipases and 3 phopholipases are predicted to be secreted. This pattern of gene expression is similar to that of *C. albicans*, which though only distantly related to *Malassezia*, occupies a similar niche as an opportunistic pathogen, leading Xu et al. to suggest that the pattern of gene expression is an adaptation to their lifestyle as human pathogens.

Table 10.2 Metabolites produced by *Malassezia* species

Metabolite	Comments	Function/effect	Reference
Azelaic acid	Produced when cultures are grown in the presence of oleic acid	Inhibitor of tyrosinase – may be involved in changes in pigmentation in pityriasis versicolor Inhibits oxygen-dependent mechanisms of phagocytosis	Nazzaro-Porro and Passi (1978)
Gamma lactone	Produced when cultures are grown in the presence of lecithin, oleic acid, triolein or human sebum	Characteristic “fruity” smell associated with many species of *Malassezia*	Labows et al. (1979)
“Hydrolase”		Hydrolysed Tween 60 or Tween 80 *in vitro*	Mayser et al. (1996)
Lipase	Many different lipases described and characterised, both cellular and extracellular. Shown to occur in *M. furfur* and *M. globosa* at least	Likely to be involved in releasing lipids present in culture media to fulfil the need for lipid for growth. Presence and role in vivo not proven	Brunke and Hube (2006), Catterall et al. (1978), DeAngelis et al. (2007)
Phospholipase A_2	Releases arachidonic acid from Hep-2 cells *in vitro*	Might be involved in triggering inflammation in the skin	Plotkin et al. (1998)
Malassezin	Produced *in vitro* when *M. furfur* is grown with tryptophan as the sole nitrogen source	Agonist of the arylhydrocarbon receptor. Induces apoptosis of melanocytes.	Kramer et al. (2005b), Wille et al. (2001)
Pityriacitrin		Acts a filter against UVA, UVB and UVC	Mayser et al. (2002)
Pityrialactone		May be responsible for yellowy fluorescence of lesions of pityriasis versicolor; may act as free radical trap	Mayser et al. (2003)
Pityriarubins A, B & C		Inhibit the respiratory burst of neutrophils	Kramer et al. (2005a)
Pityriazole		Unknown	Irlinger et al. (2005)

10.4 Commensalism

Malassezia species are part of the normal human cutaneous microbial flora, occurring mainly on the trunk and head, with the highest densities associated with sebum-rich areas of skin. They are thought to use the fatty acids present in normal sebum to fulfil their requirement for lipids. The pattern of carriage differs in children and adults, with highest densities occurring between puberty and late middle age after which densities decrease, tracking the pattern of activity of the sebaceous glands throughout life.

There are many studies that have examined the carriage of *Malassezia* species in healthy children and adults, but the findings have been conflicting. If reliable sampling and culture methods are used, organisms can be recovered from newborns within a few days of birth (Leeming et al. 1995), although levels in children are often relatively low compared to adults. During early childhood about 5% of children are colonised, rising to 25% by the age of 10 and most children by the age of 15 (Faergemann and Fredriksson 1980). In adults, population densities vary at body sites, with densities of up to 10^4 yeasts.cm^{-2} on the chest and the ear and densities of up to 10^3 yeasts.cm^{-2} on the back, forehead and cheeks (Leeming et al. 1989).

Since the description of the new species of *Malassezia*, many studies have sought to define if certain species predominate at different body sites. Recently, the data have been reviewed, and although *M. globosa, M. sympodialis* and *M. restricta* are most commonly seen on human skin, there are significant differences between the various studies (Ashbee 2007), although individuals are often colonised with more than one species at a single site. Whilst differences in sampling and culture methods may partly explain the variation, there may be genuine differences between carriage rates in different ethnic and racial groups, rendering comparison of the worldwide data meaningless. It would therefore be of considerable interest to use the same sampling techniques and identification methods to look at carriage in different countries, and hence determine whether there are genuine geographic differences. A recent study has examined the genetic diversity of *M. furfur* strains from natives of Greece, Bulgaria, China and Scandinavia and it was found that they formed distinct group clusters, suggesting that there is indeed geographic diversity in the commensal *Malassezia* population (Gaitanis et al. 2009).

As members of the commensal flora, *Malassezia* species will be regularly exposed to the host immune system and even in the absence of disease, humoral and cellular responses specific to their antigens can be measured. Immunoglobulins of the classes IgG and IgM specific to *Malassezia* can be detected in adults and children, although levels are lower in children (Faergemann 1983) and appear to decrease in older people. Titres of IgA are generally low in healthy individuals, demonstrating that mucosal sensitisation is not important (Cunningham et al. 1992). Cellular immune responses can also be measured in healthy individuals and, although studies are limited, it appears that levels of cellular responses remain relatively constant in adults and only tail off in later life (Ashbee and Evans 2002).

10.5 Role in Disease

Malassezia was first described in 1846 associated with the lesions of pityriasis versicolor (Eichstedt 1846), but since then it has been isolated from many cutaneous and systemic diseases. The precise role it plays in some of these diseases is still a matter of debate – the evidence for each disease and how the organism interacts with the host immune system will be discussed in the following pages.

Although *Malassezia* species are not usually considered to be "pathogens" they do possess several interesting properties that probably help them to cause the conditions with which they have been associated. Examination of their interactions with both peripheral mononuclear cells and keratinocytes, have demonstrated that the yeasts do have immunomodulatory potential, which in certain circumstances might contribute to the initiation or exacerbation of disease.

The description in 1995, of a lipid-rich layer surrounding the yeast cells of some isolates of *Malassezia,* has paved the way for several interesting studies. The first indications that the lipid-rich layer might interact with the host immune system was in a study examining the effects of co-culturing *Malassezia* species with peripheral blood mononuclear cells (PBMNCs), which demonstrated a decrease in the release of IL-1β by PBMNC's during certain culture conditions (Walters et al. 1995). Further work with a wider range of strains and culture conditions, confirmed that *Malassezia* species could significantly modulate the release of pro-inflammatory cytokines from PBMNCs (Kesavan et al. 1998). When the cells were treated to remove the lipid-rich layer around the yeast cells, the immunosuppressive effects of the yeasts were removed and cytokine production returned to constitutive or higher levels (Kesavan et al. 2000). Similar results have just been reported with keratinocytes, looking at a range of cytokines whose production was affected by the lipid-rich layer (Thomas et al. 2008). This presents an interesting parallel with *Cryptococcus*, a distantly-related basidiomycetous yeast that causes human infections, and also possesses a capsule with immunomodulatory ability. Understanding the interaction of the lipid-rich layer with the host immune system may be a key area in elucidating how *Malassezia* species switch between their commensal and "pathogen" phenotypes.

10.5.1 Pityriasis Versicolor

Pityriasis versicolor (PV) is a chronic cutaneous condition, which can present as either hyper or hypopigmented scaling, itchy lesions, usually on the upper trunk. It occurs most commonly between puberty and middle age, paralleling the periods of maximal carriage of *Malassezia* on skin. Prevalence is higher in tropical, humid countries, reaching upto 40%. The disease is characterised by its recurrent nature and despite extensive and thorough treatment, most patients will experience relapses (Ashbee and Evans 2002).

Many factors may be involved in the alterations in skin pigmentation associated with this condition Azelaic acid, a metabolite of *Malassezia*, is an inhibitor of tyrosinase, an enzyme involved in melanogenesis, whilst several of the tryptophan-metabolites have a UV filtering action. *M. furfur* also produces several pigments, some of which are red or yellow, which may influence the colour of lesions in the hyperpigmented form of disease.

One area of intense research has been to examine the species of *Malassezia* associated with lesions of PV, to determine if one species or group of species could

be identified as the causative agent/s. As with commensal carriage, there have been marked differences in the results from different studies. The first study to look at the expanded list of species on lesions of PV was that of Crespo Erchiga in Spain (Crespo-Erchiga et al. 1999b). They studied 100 patients and 220 healthy skin samples, and it was found that *M. globosa* was isolated alone from 55% of patients and in association with another species in a further 32% of patients. The predominant isolate from healthy skin was *M. sympodialis*, however, 37% of the samples from healthy trunk skin and >90% of samples from healthy foreheads were culture negative. This is in contrast to many other studies, which document significant populations of *Malassezia* at both of these sites and is probably a reflection of the inefficiency of the sampling methods that are used to collect samples. From their results, the authors suggested that *M. globosa* should be considered the causative agent of PV. Following on from this study, several other groups have also reported *M. globosa* as the predominant species that is isolated from lesions of PV. These studies include patients studied in Greece (Gaitanis et al. 2006), Iran (Tarazooie et al. 2004), Mexico (Hernandez et al. 2003), India (Dutta et al. 2002), Japan (Nakabayashi et al. 2000),Tunisia (Ben Salah et al. 2005) and Boznia Herzegovina (Prohic and Ozegovic 2007). However, studies from other countries have reported other species as predominating – *M. sympodialis* in Canada (Gupta et al. 2001a), *M. furfur* in Panama (De Quinzada 2005), Indonesia (Krisanty et al. 2008) and Brazil (Gandra et al. 2006) and *M. globosa* with *M. restricta* in Japan (Morishita et al. 2006). Thus, the situation is still rather confused and it appears that as yet, we cannot designate one species as the causative agent of PV, but rather several species may be important, perhaps to differing extents depending on other factors.

Most of the studies on host interaction in patients with PV have examined either humoral or cellular immune responses to various preparations of *Malassezia*. The findings from the studies on humoral immunity have been largely inconclusive, with some studies showing increased levels of antibodies in patients (Silva et al. 1997; Wu and Chen 1985) and some showing no differences between patients and controls (Ashbee et al. 1994a; Faergemann 1983). These differences may be due to differences in antigenic preparations used, differences in the sensitivities of the methodology or it may be that antibody production is a multifactorial response and not correlated simply with presence or absence of disease.

Early studies on cellular immune responses in PV reported that patients appeared to have cellular immune deficiencies specific to *Malassezia* (Sohnle and Collins-Lech 1978), however, subsequent studies have not all confirmed these results. One study found a similarly decreased response in patients (Bergbrant et al. 1999), but another reported increased responses in patients compared to controls (Ashbee et al. 1994b). It has been suggested that some species of *Malassezia* are more immunostimulatory than others and that if these species were used in the early studies, this may explain the apparent lack of cellular response (Ashbee and Evans 2002).

Another approach to examine the host-pathogen interaction has been to characterise the lesional infiltrate in PV lesions. The predominant cell type in the infiltrate is Th cells (Scheynius et al. 1984), but there is also an increase in the number of

Langerhans' cells present (Brasch et al. 1993). Recently, it has been noted that despite the large amount of fungal material in the lesions, there is very little neutrophil infiltrate and it has been postulated that this may be due to the large amount of lipid around *Malassezia* reducing its antigenicity, or that the metabolites produced by *Malassezia* may suppress neutrophil infiltration (Wroblewski et al. 2005).

10.5.2 Seborrhoeic Dermatitis and Dandruff

The exact relationship between seborrhoeic dermatitis (SD) and dandruff is a matter of dispute, with some researchers considering them to be distinct clinical entities and others considering them to be a part of a continuum. For the purposes of this review, they will be considered as the opposite ends of the same spectrum of disease.

SD presents as scaly, itchy, red lesions, occurring on the face, scalp and trunk. The condition is chronic and patients often report exacerbations due to stress or dry environments, with improvements during the summer months. SD occurs in approx 2–5% of normal individuals, but has often been the presenting condition in untreated AIDS patients, occurring in 70–80% of these patients (Mathes and Douglass 1985). Although most people consider that *Malassezia* is involved in SD, the exact role that it plays is still controversial. *Malassezia* can be isolated from lesions of SD and some groups have found that the population densities are higher on lesions (Pierard-Franchimont et al. 1995) and that a decrease in population densities due to antifungal treatment produces clinical benefit (Heng et al. 1990; Pierard-Franchimont et al. 1998). However, other groups have found no differences in population densities between lesional and normal skin (Ashbee et al. 1993) and it is known that steroid treatment alone can also produce clinical improvement.

As with PV, there have been several studies that have examined the species of *Malassezia* found on lesions of SD to determine if there is any association between a particular species and this condition. Again, there is no consensus in the findings, with *M. globosa* reported as predominating in studies from Greece (Gaitd et al. 2006) and Canada (Gupta et al. 2001b); *M. restricta* from studies in Spain (Crespo-Erchiga et al. 1999a), Holland (Gemmer et al. 2002) and Japan (Tajima et al. 2008) and *M. sympodialis* from Korea (Lee et al. 2001). Mixtures of species have been reported in other studies (Nakabayashi et al. 2000; Sandstrom Falk et al. 2005).

In the absence of any obvious correlation between either overgrowth of *Malassezia* or specific species, research on the pathogenesis of SD has begun to examine more subtle possible causative factors. Although a cohesive pathway has not yet been described, several contributory facets have been reported. The structure of the stratum corneum of patients with dandruff is altered, with the corneocytes only loosely associated and with reduced numbers of desmosomes (Warner et al. 2001). The lipids present are also reduced, which leads to an increased itch response to histamine (Harding et al. 2002). This compromise in barrier function which may

allow various metabolites produced by *Malassezia* on the scalp to initiate inflammation. It has been shown that *M. furfur* strains from SD patients produce indolo-[3,2-b] carbazole and malassezin, both of which are ligands for the aryl hydrocarbon receptor (AhR) (Gaitanis et al. 2008). AhR are ligand-activated transcription factors, which are involved in a diverse range of processes, including cell proliferation, differentiation, adhesion and migration, as well as immunological homeostasis (Barouki et al. 2007). If *Malassezia* produced these metabolites on the skin of susceptible individuals, they may initiate or contribute to inflammation and hence precipitate or exacerbate SD. Another study demonstrated that when oleic acid was applied to the scalp of susceptible individuals, this caused flaking and the authors suggested that this might be another way in which *Malassezia* might contribute to dandruff and SD (DeAngelis et al. 2005). Although it has been suggested previously that fatty acids released by cutaneous commensals contribute to skin inflammation, doubt has been cast on this hypothesis as it is unlikely that the levels of fatty acids tested experimentally would be produced in vivo (Ingham et al. 1981).

The cellular infiltrate in SD lesions is mainly Th cells (Bergbrant et al. 1991), but expression of NK1and CD16 are also increased, indicating a non-immunogenic irritant reaction (Faergemann et al. 2001).

10.5.3 Atopic Eczema

Atopic eczema (AE), or atopic dermatitis, is a chronic relapsing inflammatory skin disease, with a prevalence in industrialised countries of around 15–30% in children and 2–10% in adults (Bieber 2008). The disease is characterised by severely itchy, red, dry and crusted skin where the distribution on the skin surface varies with age. Today,the pathogenesis of AE is considered to be a combination of a disturbed skin barrier which enables allergens to enter the skin (Elias and Steinhoff 2008) and inappropriate immune responses with contributions from both genetic and environmental factors (Akdis et al. 2006; Bieber 2008). A number of genes have been connected with the disease, some of which are related to the barrier function of the epidermis and others are involved in immunological responses, favouring the development of IgE-mediated sensitization with an allergen-specific Th2 polarisation (Akdis et al. 2006; Bieber 2008). cDNA microarrays have been used to characterise the global gene-signature in lesional skin, and atopy patch-tested skin from eczema patients and corresponding skin from healthy individuals. Through this genome-wide expression profiling, a distinct reciprocal expression pattern has been described of induced inflammatory genes and repressed lipid metabolism genes in skin from patients with AE (Saaf et al. 2008). Genes encoding key enzymes and structural proteins involved in assembly of the cornified layer also demonstrated altered expression in AE skin. It should be remembered that AE is a complex clinical syndrome with several subgroups; for example, elevated IgE levels are not present and allergen reactivity has not been detected in approximately 20% of adult AE patients. Another subgroup of AE patients have, in addition to IgE

reactivity to exogenous allergens, an autoimmune IgE-mediated reactivity against self antigens (Altrichter et al. 2008; Zeller et al. 2008).

Environmental triggers of AE can be different life style factors (Alfven et al. 2006), stress, allergens, and microorganisms (Bieber 2008). *Malassezia* yeasts are one such group of microorganisms. Among the *Malassezia* species, *M. sympodialis* is one of the most frequently isolated species from both AE patients and healthy individuals (Sandstrom Falk et al. 2005; Scheynius et al. 2002) but here as for other diseases associated with *Malassezia,* various patterns of species have been reported in different parts of the world (Ashbee 2007). The first report of an association between *Malassezia* (at that time denoted *Pityrosporum)* and AE was by Clemmensen and Hjort in 1983 (Clemmensen and Hjorth 1983). They described how treatment with the antifungal drug ketoconazole improved AE, especially in adult patients with a head and neck distribution of the eczema and a positive skin prick test (SPT) to *Malassezia* extract. Later studies have supported their findings (Back et al. 1995; Scheynius et al. 2002; Schmid-Grendelmeier et al. 2006). In approximately 50% of adult patients with AE, specific IgE- and /or positive SPT and atopy patch test (APT) to *Malassezia* have been found, as well as specific T-cell reactivity (Scheynius et al. 2002; Schmid-Grendelmeier et al. 2006) but rarely in other allergic diseases (Casagrande et al. 2006) indicating a specific link between AE and *Malassezia*. The global transcriptional response in positive APT reactions to *M. sympodialis* is very similar to the gene-signature identified in lesional AE skin (Saaf et al. 2008) supporting the association of *Malassezia* with AE pathogenesis. The disturbed skin barrier and elevated pH of AE skin (Elias and Steinhoff 2008) can also induce an enhanced allergen release from *M. sympodialis*, leading to increased host-microbe interactions (Selander et al. 2006)

Several IgE binding components in the 10–100 kDa molecular weight range have been identified in *Malassezia* (Crameri et al. 2001; Schmid-Grendelmeier et al. 2006). Thirteen allergens from *Malassezia* species are reported to date by the official allergen nomenclature list (www.allergen.org), ten from *M. sympodialis*, Mala s 1, and Mala s 5 – Mala s 13, and three derived from *M. furfur*, designated Mala f 2 – Mala f 4. Interestingly, four of the *M. sympodialis* allergens, Mala s 1 and Mala s 7–9, encode proteins of unknown function without sequence homology to known allergens or to other known proteins. The crystal structure of Mala s 1 shows a sixfold β-propeller structure representing a new fold among allergens (Vilhelmsson et al. 2007). The putative active site of Mala s 1 overlaps structurally to putative active sites in the potential homologues Q4P4P8 and Tri 14, from the maize parasite *Ustilago maydis* and the wheat parasite *Gibberella zeae*, respectively (Martinez-Espinoza et al. 2002; Schisler et al. 2002). Tri14 is a protein of the mycotoxin (trichothecene) synthesis gene cluster largely responsible for the pathogenicity of *G. zeae* (Dyer et al. 2005) and suggested to be involved in host-cell recognition or in cell-wall processes involved in plant cell invasion by the parasite. The conserved residues within the potential active sites might suggest similar functions for Mala s 1 and for the two related proteins of the plant fungal parasites. Interestingly, Mala s 1 is localised in the cell wall and exposed to the yeast cell surface (Zargari et al. 1997). The genomes of the *Malassezia* species *M. globosa*

and *M. restricta* were recently sequenced (Dawson 2007). The resemblance between *Malassezia* and *U. maydis* is not limited to Mala s 1 and Q4P4P8 since DNA sequence comparison indicated *U. maydis* as the fungus most closely related to *Malassezia* among all fungi with complete genome sequences (Xu et al. 2007).

The sequences of Mala s 6, 10, 11, and 13, reveals significant homology with human endogenous proteins. The crystal structure of Mala s 6, a cyclophilin (Glaser et al. 2006), and Mala s 13, a thioredoxin (Limacher et al. 2007), have been resolved at high resolution. They belong to the class of phylogenetically highly conserved proteins and are members of so called pan-allergen families (Fluckiger et al. 2002; Glaser et al. 2006; Limacher et al. 2007). These proteins, together with Mala s 11 (a manganese dependent superoxide dismutase) (Andersson et al. 2004), share a high degree of sequence identity to the corresponding human enzymes and might play an essential role in the pathogenesis of AE (Zeller et al. 2008). Mala s 10 and Mala s 12, encode a heat shock protein (Andersson et al. 2004) and a glucose-methanol-choline (GMC) oxidoreductase (Zargari et al. 2007), respectively. Heat shock proteins are well known as IgE-binding structures (Shen and Han 1998), whereas Mala s 12 homologous allergens have so far not been reported (Zargari et al. 2007). Mala f 2 and Mala f 3 represent peroxisomal proteins reported as cross-reactive allergens derived from many fungal species (Bowyer et al. 2006; Hemmann et al. 1997; Weichel et al. 2002). Mala f 4 codes for a malate dehydrogenase, a known allergen structure reported also as a latex allergen (Posch et al. 1997), whereas Mala s 5 represents an additional peroxisomal protein (Lindborg et al. 1999). The available recombinant allergens have been proven to be useful reagents for a highly specific detection of *Malassezia*-sensitised AE patients (Casagrande et al. 2006; Johansson et al. 2002; Schmid-Grendelmeier et al. 2006; Zargari et al. 2001), for the understanding of cross-reactivity at molecular level (Glaser et al. 2006; Limacher et al. 2007), and for the investigation of the pathologic background played by allergens in AE.

Sensitization to *Malassezia* is most likely to be mediated by antigen presenting dendritic cells (DCs) in the skin. It has been shown that human monocyte-derived DCs (MDDCs) can efficiently bind and rapidly internalise *M. sympodialis* as well as allergenic components from the yeast (Buentke et al. 2000). This process is associated with maturation of the MDDCs, induction of lymphocyte proliferation and of a Th2-like immune response (Buentke et al. 2001). DC can interact with NK cells in the skin and *M. sympodialis* stimulates this interaction in patients with AE (Buentke et al. 2002). Furthermore, *M. sympodialis* enhances NK cell-induced DC maturation in healthy subjects (Buentke et al. 2004). NK and/or NKT cells might selectively eliminate DCs which have picked up *Malassezia* before they activate the immune system, a function that might be impaired in AE.

Another function that has been reported to be hampered in AE patients is the innate immune response. Antimicrobial peptides, like LL-37 and human β-defensin-2 (HBD-2), are produced in the skin as a first line of defence against bacteria, fungi and some viruses (Ganz 2003). *M. furfur* induces up-regulation of HBD-2 in normal cultured human keratinocytes which thus limits the uptake of further yeast cells and reflects a regulated innate host

defence (Donnarumma et al. 2004). It has been found that patients with AE lack appropriate induction of these antimicrobial peptides when compared to patients with psoriasis, and also it suggests an explanation for the frequent infections with *Staphylococcus aureus* in the skin of AE patients (Ong et al. 2002). Another recent study shows, however, that patients with AE exhibit enhanced expression of LL-37 in lesional skin compared to non-lesional, suggesting a role of LL-37 in AE that might be associated with the process of re-epithelialization (Ballardini et al. 2009).

The dominating symptom in AE is severe itch which provokes scratching and increased inflammation. Mast cells most likely play a central role in this vicious circle. Fungal products like zymosan can activate mast cells through TLR2 (Olynych et al. 2006) and cross-linking of the high-affinity IgE receptor, FcεRI, on mast cells leading to the release of potent inflammatory mediators, such as histamine, proteases, chemotactic factors, cytokines, and arachidonic acid metabolites (Henz et al. 2001). It was recently found that *M. sympodialis* can activate IgE-sensitised mast cells (Selander et al. 2009), a novel mechanism for the contribution of *Malassezia* to the inflammation and itch in AE.

10.5.4 Other Cutaneous Diseases

In addition to the diseases detailed in previous sections, *Malassezia* has been linked with many other cutaneous diseases, including psoriasis (Rosenberg et al. 1989), acne (Aron-Brunetiere and Avram 1978), confluent and reticulate papillomatosis (Bruynzeel-Koomen and De Wit 1984), onychomycosis (Ertam et al. 2007), nodular hair infection (Lopes et al. 1994), blepharitis (Nelson et al. 1990), neonatal cephalic pustulosis (Niamba et al. 1998), canaliculitis (Romano et al. 1978), dacryocystitis (Wolter 1977), and keratitis (Suzuki et al. 2007).

Of the other conditions, most attention has been focused on psoriasis and there are now several molecular studies attempting to define species found on psoriatic lesions. The first study could not differentiate specific species, but found that the populations on healthy and psoriatic skin did not differ (Paulino et al. 2006). A subsequent study found that *M. restricta* was the predominant species detected in psoriatic scale, but as they did not study healthy control samples in this study, they could not determine whether this differed from the normal population. Using historical control data from healthy individuals and studying 22 psoriatic patients, a group in Japan found that *M. globosa, M. restricta* and *M. sympodialis* were all commonly isolated from both groups and did not differ between lesional and healthy samples (Amaya et al. 2007). Therefore, as with other diseases, there is currently no definitive evidence for an association between psoriasis and a particular species of *Malassezia*.

Further evidence for the role of *Malassezia* in psoriasis was provided by a recent study which demonstrated that *Malassezia* upregulated various molecules involved in cellular proliferation and migration, and that this effect was more pronounced in

*Malassezia*colonised psoriatic skin (Baroni et al. 2004). Thus, colonisation with *Malassezia* may contribute to the cellular hyperproliferation, which is a characteristic of psoriatic lesions.

10.5.5 Systemic Diseases

The first report of *Malassezia* causing systemic disease was its isolation from a case of "sterile" peritonitis in 1979 (Wallace et al. 1979). The patient was undergoing continuous ambulatory peritoneal dialysis (CAPD) and had experienced several episodes of peritonitis, from which no organism had previously grown. Subsequent reports confirmed the ability of various species to cause peritonitis, usually in CAPD patients (Fine et al. 1983; Gidding et al. 1989; Johnson et al. 1996).

The other form of systemic disease with which *Malassezia* yeasts are associated is catheter-related fungaemia. The first report was in a neonate born at 28 weeks gestation, receiving total parenteral nutrition (Redline and Dahms 1981). She developed cardiomegaly, oedema and cholestasis and when her respiratory function worsened, an open lung biopsy was carried out. On histology, yeasts were seen and amphotericin B and flucytosine were initiated, but the child subsequently died. The authors suggested that as intralipid contains significant amounts of C_{16}–C_{18} fatty acids, these fulfilled the requirement of *Malassezia* for lipid and hence the organism was able to grow. Since this initial report, over 100 cases have been reported and *Malassezia* is now a well-recognised cause of fungaemia, especially in premature, low-birth weight neonates (Kaufman and Fairchild 2004).

Malassezia fungaemia has also been reported in immunocompromised adults and children, usually in association with parenteral nutrition administered through central venous catheters (Curvale-Fauchet et al. 2004). Catheters may become colonised during placement and biofilms form around the organism, protecting them against antifungals and hence removal of catheter is often the only way to eradicate the infection. Unless clinicians have a high index of suspicion, diagnosis may not be made as blood culture systems have low rates of recovery for *Malassezia* (Lyon and Woods 1995; Nelson et al. 1995).

To date, their have been no studies of host-pathogen interaction in systemic *Malassezia* infections.

10.6 Summary and Conclusions

Malassezia species are commensals of normal human skin, but also cause or contribute to disease in a significant number of people. Our understanding of the interactions between these yeasts and the immune system is enabling us to define how they cause disease in some individuals, but remains as commensals in others. The range of metabolites and antigens that they produce is diverse and these have

been shown to have interact with different cell types within the skin and the host immune system. The activities of some of these metabolites may partly explain some of the characteristics of PV, SD and AE. As we continue to map these interactions, the hope is that we will understand the diseases better and in the future perhaps modulate the effects which give rise to them.

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Chapter 11
Emerging Systemic Yeast Pathogens

Mary S. Mathews and H. Ruth Ashbee

Abstract The range of yeasts reported as the cause of infections has increased over the last decade. Patients who would once have died of their underlying disease are now surviving longer, but often in an immunocompromised state. Many yeasts have now been recognised as having potential to cause disease in these patients, including less common species of *Candida, Blastoschizomyces capitatus* and yeasts from the genera *Trichosporon, Malassezia, Saccharomyces* and *Pichia*. This chapter will review the microbiology, epidemiology, clinical presentations, diagnosis, antifungal susceptibilities and clinical outcomes for infections caused by these emerging yeast pathogens.

11.1 Introduction

There are many yeasts which are well-known pathogens of humans, including *Candida* and *Cryptococcus*. However, over the last decade, as patients have survived diseases which would once have been fatal and become more immunocompromised due both to treatments and diseases, many unusual yeasts have been reported to cause systemic disease. Many of these yeasts are lacking in virulence factors and are usually associated with environmental sources or superficial disease. As patients in the future survive evermore radical therapies and debilitating diseases, the range of yeasts that are reported as pathogens is likely to continue increasing.

M.S. Mathews
Department of Microbiology, Christian Medical College, Vellore 632004, Tamil Nadu, India

H. Ruth Ashbee (✉)
Mycology Reference Centre, Department of Microbiology, Leeds General Infirmary, Leeds LS1 3EX, UK
e-mail: h.r.ashbee@leeds.ac.uk

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11.2 Non-*albicans Candida*

11.2.1 Microbiology

The genus *Candida* includes over 200 species of yeasts, occupying a diverse range of ecological niches, but relatively few species cause disease in humans. *C. albicans* is the species most commonly isolated from clinical samples. Most clinically relevant species will grow readily on Sabouraud's agar and also in blood culture systems. As well as causing disease in humans and animals, several species are commensals of humans, occurring on the skin and mucosal membranes and in the gastrointestinal tract. Over the last decade, molecular studies have defined many new species of *Candida* and started to unravel the complex taxonomy of this genus.

11.2.2 Epidemiology

Several species of *Candida* are well-known pathogens of human and animals and in the USA they are the fourth most common cause of bloodstream infections (Wisplinghoff et al. 2004). Four species (*C. albicans*, *C. glabrata*, *C. parapsilosis* and *C. tropicalis*) account for 95% of all episodes of candidaemia (Pfaller and Diekema 2004), but in recent years there have been changes in the epidemiology. Contributing to this change is the widespread use of fluconazole, leading to the emergence of species that are less susceptible to fluconazole. In addition, molecular analysis has lead to the creation of several new species, which are associated with infections and can be considered emerging causes of candidosis.

C. parapsilosis is a commensal of human skin, but is also well-known as a pathogen of neonates, often causing catheter-related fungaemia, related to total parenteral nutrition and its ability to form biofilms on catheters (Trofa et al. 2008). Three groups of *C. parapsilosis* were recognised, but in 2005 these were formally proposed as three separate species: *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis* (Tavanti et al. 2005). Most infections are caused by *C. parapsilosis*, but several of the isolates of *C. orthopsilosis* were clinical isolates from blood or catheters and so this species should be considered an emerging pathogen.

Candida guillermondii is generally considered an unusual cause of disease in humans, but recent data suggests that it may be more significant than previously thought, constituting 1% of clinical isolates (Pfaller et al. 2006b). Although its frequency of isolation did not change over the 9-year study period, it was more frequently isolated from certain parts of the world, including Latin America, where it represented 3.7% of yeast isolates from that region.

Candida rugosa was previously considered to be non-pathogenic, until two cases of fungaemia were reported in 1985 (Reinhardt et al. 1985; Sugar and Stevens 1985). Subsequent to those initial reports, there have been several case reports of candidaemia with this unusual species (Colombo et al. 2003; Dube et al. 1994).

A recent international study reported that *C. rugosa* represented 0.4% of the *Candida* isolates collected from 127 centres over 6 years and that it was more common in Latin America (Pfaller et al. 2006a).

Candida nivariensis was first reported as the causative agent of human disease after isolation from three patients in Spain, in whom it was isolated from broncho-alveolar lavage fluid, urine and blood (Alcoba-Florez et al. 2005). Subsequent isolates have been reported from Japan (Fujita et al. 2007), Indonesia (Wahyuningsih et al. 2008) and the UK (Borman et al. 2008).

Other *Candida* species that have been reported from clinical isolates include *C. norvegensis* (Sandven et al. 1997), *C. membranaefaciens* (Fanci and Pecile 2005), *C. lipolytica* (Agarwal et al. 2008), *C. subhashii* (Adam et al. 2009) and *C. bracarensis* (Bishop et al. 2008).

11.2.3 Clinical Manifestations

The clinical manifestations associated with systemic non-*albicans Candida* species are varied, but most species have been associated with fungaemia. *C. rugosa* and *C. guillermondii* were also reported to be commonly isolated from urine, in addition to blood. Peritonitis has also been caused by some of the other non-*albicans Candida* species.

In addition to particular clinical manifestations, some of the non-*albicans Candida* species appear to be associated with specific groups of patients. Both *C. rugosa* and *C. guillermondii* have been mostly isolated from either medical or intensive care unit patients (Pfaller et al. 2006b; Pfaller et al. 2006a) and *C. parapsilosis* is known to be associated with newborns, particularly those in neonatal intensive care units (Trofa et al. 2008).

11.2.4 Diagnosis

Because most species of *Candida* will grow readily on routinely used culture media, diagnosis of infections is relatively straightforward. Samples plated onto Sabouraud's agar or into blood culture bottles and incubated at 35–37°C are likely to yield positive cultures. It is the identification of these unusual species which is likely to present the diagnostic challenge, in addition to ascribing a pathogenic role to them.

11.2.5 Antifungal Susceptibility, Therapy and Outcome

One common feature of several of the non-*albicans Candida* species is their relative resistance to various antifungal agents.

C. guillermondii susceptibilities were recently reported from a multinational study which found that 15.7% of the isolates tested from urine (n = 51) were resistant to voriconazole, whilst 15.4% of the 78 tested were resistant to fluconazole. Resistance to fluconazole was also seen in 12.5% of the respiratory isolates tested (n = 136) and many of the isolates from sterile body fluids, urine and skin or soft tissue were susceptible – dose dependent to fluconazole (Pfaller et al. 2006b).

C. rugosa has also been reported to be resistant *in vitro* to amphotericin B (Dube et al. 1994; Sugar and Stevens 1985), and although a recent study found the isolates tested to be susceptible *in vitro*, all four patients who received amphotericin therapy died (Colombo et al. 2003). Recent data has also documented widespread resistance to fluconazole in isolates from blood, urine and respiratory samples (64.9%, 54.5% and 41.5% resistant in 74, 99 and 41 isolates tested, respectively) and resistance in 44% of the 59 isolates from blood tested against voriconazole (Pfaller et al. 2006a).

Susceptibility testing for *C. membranaefaciens*, *C. nivariensis* and *C. norvegensis* have also shown decreased activity of the azoles *in vitro*, but in most cases it is not known whether this correlates with clinical treatment failure for these species.

11.3 *Trichosporon* Species

11.3.1 Microbiology

Trichosporon species produce yeast-like colonies, which become more wrinkled with age. The colonies are usually white to cream in colour. Production of barrel-shaped arthroconidia is characteristic of the genus, although hyphae, pseudohyphae and blastoconidia are also produced by some species.

The first *Trichosporon* species, *T. beigelii*, was described in 1890, associated with a case of white piedra. Since that time, the taxonomy of the genus has undergone considerable expansion and revision and by the mid-1990s, there were 17 species recognised (Gueho et al. 1994). More recently, newer species have been proposed and there are now 38 species within the genus (Fuentefria et al. 2008; Middelhoven et al. 2004; Molnar et al. 2004), although not all experts accept this revised and expanded taxonomy.

11.3.2 Epidemiology

Trichosporon species are commonly found in the environment, particularly in soil and decomposing wood, but may also be transient commensals of human skin and the respiratory tract.

Based on the latest taxonomy, the species now considered to be associated with white piedra are *T. ovoides* and *T. inkin*, with *T. cutaneum* and *T. asteroides*

associated with other forms of superficial disease. The two species most commonly associated with systemic disease are *T. asahii* and *T. mucoides*.

Systemic infections with *Trichosporon* species have mainly been reported in immunocompromised patients, most commonly those with leukaemia (Bayramoglu et al. 2008; Fournier et al. 2002; Girmenia et al. 2005; Rieger et al. 2007), but also in solid organ transplant (Nettles et al. 2003) and haematopoietic stem cell recipients (Moretti-Branchini et al. 2001) and rarely in diabetic patients (Ebright et al. 2001).

Risk factors for infection include neutropenia, receipt of corticosteroids, surgery, presence of vascular catheters and cytotoxic therapy.

11.3.3 Clinical Manifestations

White piedra presents as white or light brown nodules on the hair shaft and affects the hair of the beard, moustache and pubic area more than that of the scalp. Other forms of superficial infection, including onychomycosis and tinea pedis have also been reported (Archer-Dubon et al. 2003; Ruiz-Esmenjaud et al. 2003).

Systemic infections with *Trichosporon* species may present in many different ways. Fungaemia is seen in a significant proportion of patients (Girmenia et al. 2005), and cutaneous lesions and lung involvement are also relatively common (Fournier et al. 2002). Less common manifestations include peritonitis (Jian et al. 2008), septic shock (Kim et al. 2007), endocarditis (Mooty et al. 2001) and meningitis (Mathews and Prabhakar 1995).

11.3.4 Diagnosis

Diagnosis of white piedra is usually based on clinical presentation, with the light coloured nodules adherent to the hair shaft visible to the naked eye. Growth of a yeast from the nodule and its subsequent identification as a species of *Trichosporon* is confirmatory.

For systemic infections, the diagnosis may be more difficult as growth of a cream-coloured yeast colony may initially be confused with some of the more unusual species of *Candida*. Rapid identification of the organism is important and use of PCR may be helpful in some cases (Sano et al. 2007).

11.3.5 Antifungal Susceptibility, Therapy and Outcome

Several susceptibility studies have been carried out on *Trichosporon* species, but most use the previous taxonomic nomenclature, so it is difficult to interpret old data

in light of the currently recognised species. A recent study examined isolates that had been identified either on the basis of conventional or molecular methods and determined susceptibility to amphotericin B, flucytosine, fluconazole, itraconazole and voriconazole (Rodriguez-Tudela et al. 2005). Resistance to amphotericin B has previously been reported and this was confirmed for *T. asahii*, with all 15 isolates being resistant. MICs varied for fluconazole, but itraconazole and voriconazole were the most active drugs *in vitro*. For other species of *Trichosporon*, there was more resistance to the azoles and generally greater susceptibility to amphotericin B. The outcome of therapy in cases of systemic *Trichosporon* infection parallels these findings *in vivo*, with a mortality rate of approximately 80% in patients treated with amphotericin B (Walsh et al. 2004). Echinocandins have limited activity against *Trichosporon* species and breakthrough infections in patients receiving caspofungin and micafungin have been seen (Bayramoglu et al. 2008; Matsue et al. 2006). Whilst voriconazole has been used to successfully treat some cases (Fournier et al. 2002), breakthrough infections have occurred in patients treated with posaconazole (Rieger et al. 2007). Because of the variable patterns of antifungal susceptibility in different species, susceptibility testing of isolates is probably pertinent to tailor treatment in individual cases.

11.4 *Rhodotorula*

11.4.1 Microbiology

Rhodotorula species are predominantly found in the environment, often associated with water, food and plants. The species which have been isolated from clinical samples, unusually for yeasts, have pigmented colonies on Sabouraud's agar, having an orange-pink colour due to the presence of carotenoid pigments. They are nutritionally non-fastidious and grow easily on most media.

11.4.2 Epidemiology

The three species of *Rhodotorula* which have been associated with human infections are *R. mucilaginosa* (previously called *R. rubra*), *R. glutinis* and *R. minuta*. *R. mucilaginosa* is the most commonly isolated species from fungaemia, causing over 70% of cases (Tuon et al. 2007; Tuon and Costa 2008), with *R. glutinis* responsible for only 7% (Tuon and Costa 2008).

Fungaemia due to *Rhodotorula* species is associated with central venous catheters (Duboc de Almeida et al. 2008; Zaas et al. 2003) in patients with underlying disease, most commonly cancer (Tuon et al. 2007). Some studies have reported a preponderance of disease in males (Duboc de Almeida et al. 2008; Tuon and Costa 2008).

11.4.3 Clinical Manifestations

Rhodotorula was first isolated from a human infection when it was associated with a case of endocarditis (Louria et al. 1960). After that initial case, many different forms of disease have been reported, but the most common is fungaemia. Signs and symptoms are non-specific in patients, in common with most other causes of fungaemia, but often include fever.

Rhodotorula species have also caused cases of endocarditis (Naveh et al. 1975), meningitis (Gyaurgieva et al. 1996) and peritonitis (Eisenberg et al. 1983).

11.4.4 Diagnosis

Diagnosis of infections due to *Rhodotorula* can be made on the basis of growth of the typically pigmented colonies from clinical samples. The organism grows in several blood culture systems and so is likely to be recovered in patients with fungaemia. Identification can be made using the API32C system.

11.4.5 Antifungal Susceptibility, Therapy and Outcome

There have been few studies of the susceptibility of *Rhodotorula* species to antifungals. However, from the data available, they appear to be largely susceptible to amphotericin B and flucytosine, have variable susceptibility to itraconazole and ravuconazole and mainly resistant to fluconazole and the echinocandins (Galan-Sanchez et al. 1999; Zaas et al. 2003).

Although there is no widely accepted therapeutic regimen for systemic *Rhodotorula* infections, use of amphotericin B appears to be the most advisable course, with use of fluconazole and the echinocandins contra-indicated (Tuon and Costa 2008; Zaas et al. 2003).

The overall mortality associated with systemic *Rhodotorula* infection has been reported as 12.6%, with fungaemia in the absence of a central line having a higher mortality of 20% (Tuon and Costa 2008).

11.5 *Blastoschizomyces capitatus*

11.5.1 Microbiology

Blastoschizomyces capitatus is the sole member of the genus and is the accepted synonym of the now obsolete *Geotrichum capitatum* and *Trichosporon capitatum*.

It is an environmental yeast, found in soil, sand and wood, but also as a commensal in the human respiratory and digestive tracts.

On Sabouraud's agar, it grows as white-cream colonies that develop aerial hyphae with age.

11.5.2 Epidemiology

Infections due to *B. capitatus* have been reported mainly from patients in Europe, with approximately 75% of cases occurring in European patients and over 90% of the European cases originating from Italy, Spain and France (Girmenia et al. 2005). Cases have also been reported from outside Europe, including India (Mathews and Sen 1998) and USA (Wills et al. 2004).

The main risk factor is an underlying haematological malignancy, most commonly acute myeloid leukaemia (Girmenia et al. 2005; Martino et al. 2004) with associated extended periods of profound neutropenia; central venous catheters were present in 88% of patients in one large series in leukaemic patients (Martino et al. 2004). Men are more commonly affected than women (Girmenia et al. 2005; Martino et al. 2004).

There have been a few cases of systemic disease reported in immunocompetent patients, but these are the exception (Mathews and Sen 1998; Wills et al. 2004).

11.5.3 Clinical Manifestations

The clinical manifestations of systemic infection with *B. capitatus* include fungaemia (Girmenia et al. 2005; Martino et al. 2004), pneumonia (Wills et al. 2004), spondylodiscitis (Mejdoubi et al. 2009), meningitis (Girmenia et al. 2005), endocarditis (Polacheck et al. 1992) and hepatosplenic disease, similar to that seen in candidosis (Amft et al. 1996). Fungaemia occurs in approximately 80% of cases (Girmenia et al. 2005) and this can lead to spread to many organs of the body. The lungs are affected in two-thirds of patients; liver, spleen, bone and joints, gut, kidneys and the oesophagus are other common sites of dissemination (Girmenia et al. 2005).

11.5.4 Diagnosis

Diagnosis is made in most cases by positive blood culture, with *B. capitatus* growing easily in blood culture media (Martino et al. 1990) and on Sabouraud's agar. The organism can grow upto 45°C and identification of isolates can be made using the API 32C system.

11.5.5 Antifungal Susceptibility, Therapy and Outcome

Few studies have examined the susceptibility of *B. capitatus*, but the limited data has shown that isolates may have reduced susceptibility or be resistant to fluconazole and amphotericin B (Buchta et al. 2001; Perez Sanchez et al. 2000), although other studies have shown good activity for fluconazole (Girmenia et al. 2003; Pfaller et al. 2009).

Despite apparent *in vitro* reduced susceptibility to amphotericin B, many patients have been successfully treated with this drug, either in combination with other antifungals or alone (Martino et al. 2004). Voriconazole also appears to be a useful drug for these infections with both *in vitro* (Pfaller et al. 2009) and *in vivo* efficacy (Etienne et al. 2008; Mejdoubi et al. 2009).

The outcome associated with *B. capitatus* infections is dismal, with mortality ranging from 40% to 75% (Martino et al. 2004; Perez Sanchez et al. 2000). Admittedly these infections occur in patients with serious underlying disease but *B. capitatus* infection was considered to be the main cause of death in 50% of patients in one series (Martino et al. 2004).

11.6 *Saccharomyces*

11.6.1 Microbiology

Yeasts of the genus *Saccharomyces* are widespread in nature, but are less commonly found associated with humans. They may occasionally be found as commensals of the gastrointestinal and genitourinary tract. *Saccharomyces* species are used in the production of food, wine and beer; and more recently has been used as a component of health foods and also as a probiotic in the treatment of antibiotic-associated diarrhoea (Surawicz 2008). Species include *S. cerevisiae*, *S. carlsbergensis* and *S. fragilis*, with the former most often seen in humans. On Sabouraud's agar, *S. cerevisiae* grows as white-cream coloured colonies, often with a typical central point to the colony when observed from the side. This species will grow at 35–37°C and can be identified using the API 32C identification system.

11.6.2 Epidemiology

S. cerevisiae is the species that in the past was most commonly associated with human infections. It has been reported as the aetiological agent of many conditions, including fungaemia, endocarditis, abscesses and osteomyelitis (Enache-Angoulvant and Hennequin 2005) However, recently, the use of *S. boulardii* in the treatment of antibiotic-associated diarrhoea has been shown to be linked to disease in patients receiving these preparations (Fredenucci et al. 1998; Perapoch et al. 2000),

although genetic analysis indicates that *S. boulardii* is an invalid taxon and is either a subtype of variety of *S. cerevisiae* (Cassone et al. 2003).

Risk factors for disease include the presence of an intravenous catheter, prior antibiotic therapy and, where relevant, receipt of a preparation containing *S. cerevisiae* (Munoz et al. 2005).

11.6.3 Clinical Manifestations

Systemic infections with *Saccharomyces* have usually been associated with fungaemia and have mainly occurred in patients with some form of immunocompromise or surgical intervention (Munoz et al. 2005). Signs and symptoms are often non-specific.

Other diseases caused by *Saccharomyces* include peritonitis (Aucott et al. 1990) and endocarditis (Stein et al. 1970).

11.6.4 Diagnosis

Diagnosis of systemic infections due to *Saccharomyces* is relatively simple as it can be readily grown on Sabouraud's agar and will grow in most blood culture media. Difficulty may occur in ascribing a pathogenic role when it is isolated either from a site which it may colonise or if it is found in conjunction with another organism.

11.6.5 Antifungal Susceptibility, Therapy and Outcome

Susceptibility testing of *S. cerevisiae* has demonstrated in most studies that isolates are susceptible to amphotericin B and 5-flucytosine, with MIC_{50} and MIC_{90} for all isolates in the susceptible range as defined for *Candida* (Barchiesi et al. 1998; Pfaller et al. 1997; Zerva et al. 1996). Most of the azoles are active against *S. cerevisiae* with MIC_{90} for fluconazole in several studies below 8 mg/L (Barchiesi et al. 1998; Zerva et al. 1996), although Pfaller reported a higher MIC_{90} of 16 mg/L for 22 isolates. Isolates from a surveillance study in haematology patients receiving chemotherapy were found to be highly resistant to fluconazole with an MIC_{90} of 128 mg/L (n = 160); (Salonen et al. 2000). MIC_{90} for itraconazole have been reported from 0.5 – 1 mg/L (Barchiesi et al. 1998; Pfaller et al. 1997; Zerva et al. 1996) and for voriconazole 0.064 mg/L (Swinne et al. 2004). A recent study has shown that 89.9 and 95.8% of *S. cerevisiae* isolates were sensitive to fluconazole (n = 1,022) and voriconazole (n = 1,010), respectively (Pfaller et al. 2009).

The optimum therapy for infections due to *S. cerevisiae* is not yet established, but cessation of probiotic administration, removal of central venous catheters if possible and use of antifungal therapy, probably amphotericin B are all warranted (Munoz et al. 2005), although fluconazole may also be effective (Cassone et al. 2003).

Mortality rates for systemic *Saccharomyces* infections in the two large published series were 29.5% (Munoz et al. 2005) and 37% (Enache-Angoulvant and Hennequin 2005), although this condition occurs mainly in patients with serious underlying disease, which will also contribute to mortality.

11.7 Malassezia

11.7.1 Microbiology

Yeasts of the genus *Malassezia*, except *M. pachydermatis*, are unable to grow on standard mycological agar due to an inability to synthesise certain fatty acids, so they require lipid supplementation. Various media have been formulated for their growth and incorporate a range of lipids, including olive oil, cow's milk, glycerol and Tweens.

Colonial morphology varies with the species, but most grow as creamy-yellow-coloured colonies, often with clearing or precipitation in the medium. Colonies may be flat, umbonate or wrinkled. Microscopically, the yeast morphology varies and micromorphology may be used as part of the identification process.

The taxonomy of the genus has undergone considerable revision over the last decade and there are now 13 recognised species, details of which can be found in Chap. 10.

11.7.2 Epidemiology

Although *Malassezia* species are most frequently considered to be agents of superficial disease, causing pityriasis versicolor and seborrhoeic dermatitis amongst other things, they have also been reported as the causative agent of systemic disease. Most infections have occurred in premature newborns and the use of central venous catheters and parenteral nutrition are known risk factors (Shek et al. 1989). The lipids within the parenteral nutrition fulfil the organism's requirement for lipid (Powell et al. 1986).

The species associated with systemic infections were mainly reported as *M. furfur*, but as many cases were reported before the taxonomic revision of the genus it may be that they are not *M. furfur* as currently defined.

11.7.3 Clinical Manifestations

The first report of *Malassezia* associated with a deep-seated infection was its isolation from the peritoneal dialysis fluid of a patient undergoing continuous ambulatory peritoneal dialysis (Wallace et al. 1979). The patient had previously

had several episodes of apparently "sterile" peritonitis and *Malassezia* was only grown after the addition of olive oil to the cultures. Subsequent to this first report, peritonitis due to *Malassezia* in CAPD patients has been reported in a further three patients (Fine et al. 1983; Gidding et al. 1989; Johnson et al. 1996). Although *Malassezia* is unlikely to be a major cause of CAPD peritonitis, the fastidious growth requirements of *Malassezia* also mean that it may have been present but simply not isolated in many more cases.

The main form of systemic disease with which *Malassezia* has been associated is fungaemia in premature newborns. Before the first case was reported, it was known that use of parenteral nutrition lead to the deposition of lipid droplets in the pulmonary vasculature of newborns and that the lipid affected the functioning of the reticulo-endothelial system in these children (Friedman et al. 1978). However, it was only with the report of a case of pulmonary vasculitis that it was realised that this combination of factors was ideal for *Malassezia* and that they would be able to take advantage of these circumstances to initiate infections (Redline and Dahms 1981). Since this first report, more than 100 cases of fungaemia or disseminated infection with *Malassezia* have been reported, in both newborns and also adults receiving parenteral nutrition through central venous catheters (Alpert et al. 1987; Brooks and Brown 1987; Chryssanthou et al. 2001; Richet et al. 1989; Welbel et al. 1994). *Malassezia* can grow in parenteral nutrition emulsions (Powell et al. 1986) and so carryover of the emulsion during blood collection may provide enough lipids to allow the organism to grow. In the absence of lipid supplementation, most blood culture systems are relatively poor at supporting growth of these yeasts (Lyon and Woods 1995; Nelson et al. 1995).

Less common manifestations of *Malassezia* infections include meningitis (Rosales et al. 2004), cardiac mass (Schleman et al. 2000) and septic arthritis (Wurtz and Knospe 1988).

11.7.4 Diagnosis

Whilst diagnosis of pityriasis versicolor is easily made on the basis of the characteristic finding of yeasts and short hyphae – the so called "spaghetti and meatballs" appearance, diagnosis of systemic *Malassezia* infections presents a greater challenge. In blood culture systems, the organism may not be able to multiply due to insufficient lipid in the medium, but it may be seen on microscopy as budding yeasts, leading to the mistaken assumption that the organism is a species of *Candida*. A high index of suspicion in "at-risk" patients is important and in these patients, supplementation of blood cultures with exogenous lipid might be useful to increase the chances of a positive culture (Nelson et al. 1995).

Where it is possible to obtain samples other than blood, these should be plated onto an appropriate culture medium, such as that described by Leeming and Notman (Leeming and Notman 1987), which will support the primary isolation of *Malassezia* species.

Identification of the species of *Malassezia* causing systemic infection, whilst of academic interest, is not strictly required for the management of the infections (see Sect. 16.6.5).

11.7.5 Antifungal Susceptibility, Therapy and Outcome

Susceptibility to antifungal agents varies with the species of *Malassezia*, but there are several general trends. Of the different species, *M. furfur* shows the widest range of MIC's for most agents, with many isolates showing *in vitro* resistance. All species are resistant to flucytosine and most are susceptible to amphotericin B and itraconazole. A summary of antifungal susceptibility data has recently been published (Ashbee 2007).

Systemic infections with *Malassezia* do have a significant associated mortality (Athar and Stafford 1993; Garcia et al. 1987; Redline and Dahms 1981). Therapy and management of these infections should include the removal of any venous catheters and cessation of parenteral nutrition, which may be curative (Morrison and Weisdorf 2000). However, in cases where this is not feasible, therapy with amphotericin B or fluconazole has been successful (Barber et al. 1993; Chryssanthou et al. 2001). A novel approach, locking a high concentration of amphotericin B into the catheter, without catheter removal or systemic administration was also successful in one patient (Arnow and Kushner 1991).

11.8 *Pichia* Species

11.8.1 Microbiology

Pichia are ascomycetous yeasts and many of the species are teleomorphs of *Candida* species. Species that have been documented to cause infection in humans (and their respective *Candida* anamorph) include *Pichia anomala* (*C. pelliculosa*), *P. fabianii* (*C. fabianii*), *P. farinosa* (*C. cacaoi*) and *P. ohmeri* (*C. guillermondii var membranaefaciens;* synonym *Kodamaea ohmeri*).

Many *Pichia* species are able to grow on Sabouraud's agar and in blood culture systems, although identification can be problematic. Some species of *Pichia* are included in the API 32C database, but others require molecular identification (Bhally et al. 2006).

11.8.2 Epidemiology

Pichia infections have occurred in a range of patient groups, including neonates and children (Bhally et al. 2006; Chakrabarti et al. 2001; Pasqualotto et al. 2005),

immunocompromised patients (Bakir et al. 2004; Ostronoff et al. 2006) and patients with a range of chronic health problems (Lee et al. 2007). The main risk factor for development of infection is the presence of a central venous catheter (Pasqualotto et al. 2005), although parenteral nutrition and treatment with broad spectrum antibiotics are common factors preceding the onset of infection (Lee et al. 2007).

11.8.3 Clinical Manifestations

Most *Pichia* infections have usually manifested as fungaemia, although cases of peritonitis (Choy et al. 2000), endocarditis (Reina et al. 2002) and phlebitis (Shin et al. 2003) have also been reported. Signs and symptoms are not specific, but have included fever in association with neutropenia (Bakir et al. 2004; Ostronoff et al. 2006).

11.8.4 Diagnosis

Diagnosis of infection has usually been based on the recovery of *Pichia* from blood cultures. Species of *Pichia* that have been recovered from blood cultures include *P. ohmeri* (Ostronoff et al. 2006), *P. anomala* (Bakir et al. 2004), *P. fabianii* (Bhally et al. 2006) and *P. farinosa* (Adler et al. 2007). Other samples from which species have been recovered include urine (Puerto et al. 2002), skin at the site of phlebitis (Lee et al. 2007) and peritoneal fluid (Choy et al. 2000).

11.8.5 Antifungal Susceptibility, Therapy and Outcome

There is only one large scale study on the antifungal susceptibility of *Pichia* species, which included 58 isolates of *P. anomala* (da Matta et al. 2007). All isolates were susceptible to voriconazole, amphotericin B and caspofungin, with only 36% of isolates susceptible to itraconazole and 97% susceptible to fluconazole.

There is a paucity of susceptibility data on other species, but resistance to fluconazole developing during therapy has been reported for *P. fabianii* (Hamal et al. 2008) and resistance *in vitro* has also been reported for *P. ohmeri* (Lee et al. 2007).

Although there is no consensus on how best to approach treatment of these infections, the most commonly used approach has been administration of amphotericin B, usually with concomitant removal of the central venous catheter (Ostronoff et al. 2006). Outcomes from infection vary, but mortality rates of 20–41.2% have been reported (Bakir et al. 2004; Pasqualotto et al. 2005).

11.9 Conclusions

In addition to the species already described, there has been a miscellany of other yeasts reported as the aetiological agents of systemic infections over the last decade. Unusual species, including *Lodderomyces elongisporus*, *Issatchenkia terricola*, *Metschnikowia pulcherrima* and *Blastobotrys prolificans* have all been reported from deep sites causing disease (Linton et al. 2007; Lockhart et al. 2008; Quirin et al. 2007); although, in most cases they were not previously known as human pathogens. As patients survive evermore invasive and debilitating diseases and their associated therapies, we are likely to see the range of yeasts causing disease expand considerably. Whereas the distinction between "pathogens" and "non-pathogens" was once clear, those boundaries are now blurring and vigilance is essential in interpreting the significance of isolates isolated or detected in sterile body sites, especially in immunocompromised patients.

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Chapter 12
Yeast Pathogens of Domestic Animals

F.J. Cabañes

Abstract Mycoses of domestic animals caused by yeasts have been recorded for approximately 150 years. The majority of these infections are cutaneous and superficial and are of minor clinical significance but fatal systemic infections are also reported. Currently, most common pathogenic yeasts of domestic animals are included in the genera *Candida*, *Cryptococcus* and *Malassezia* and they are reviewed in depth in this chapter. *Candida* and *Cryptococcus* species continue to cause sporadic mycoses in animals. However, in comparison to the high number of cases of candidiasis and cryptococcosis reported in humans, they are uncommon mycoses reported in veterinary medicine. However, during the last two decades, interest in the genus *Malassezia* amongst mycologists and veterinary dermatologists has increased considerably. A controversial pathogen as *Malassezia pachydermatis* is now recognized as an important cause of dermatitis and otitis externa in dogs. However, very little is known about the pathogenic role of the lipid-dependent *Malassezia* species in animal skin.

12.1 Introduction

Mycoses of domestic animals caused by yeasts have been recorded for approximately 150 years, since Eberth described candidiasis in poultry in 1858 (cited in Jungerman and Schwartzman 1972). Numerous yeasts are potential agents of animal mycoses. The majority of these infections are cutaneous and superficial and are of minor clinical significance but fatal systemic infections are also reported. Currently, most common pathogenic yeasts of domestic animals are included in the genera *Candida*, *Cryptococcus* and *Malassezia* and they are reviewed in depth in

F.J. Cabañes
Veterinary Mycology Group, Department of Animal Health and Anatomy, Faculty of Veterinary, Universitat Autònoma de Barcelona, Bellaterra 08193, Barcelona, Spain
e-mail: javier.cabanes@uab.es

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this chapter. Yeasts that occur in other genera (e.g. *Rhodotorula*, *Sporobolomyces*, *Trichosporon*) have been infrequently reported from veterinary patients (Greene and Chandler 2006; Jungerman and Schwartzman 1972; Smith 1989). A comprehensive review of opportunistic mycoses of animals (humans included) was published by Smith (1989) in the late eighties. This latter book reviews important aspects of the pathogenesis of these mycoses and includes a detailed list of opportunistic fungal species affecting a wide variety of animal species. In this chapter, emphasis has been placed on literature published within the last two decades, but prior noteworthy reviews and case reports are included.

The names used throughout this chapter are those appearing in the original publications. In some reports, mainly due to the opportunistic nature of the pathogens dealt with in this review, it was difficult to assess the significance of the fungal species reported (e.g. retrospective studies) or the validity of the identifications (e.g. identification technique not cited). In these cases an uncritical compilation of the yeast species as reported is included. On the other hand, when old nomenclature was detected in the original publication the most proper synonyms are also indicated.

12.2 *Candida*

Candidiasis has been recognized in domestic animals since at least 1858. However, other than in poultry, the early data regarding animal infections is scanty and often unsatisfactory (Jungerman and Schwartzman 1972). More than 160 *Candida* species are currently accepted (Meyer et al. 1998). A few of these species commonly reside as commensals in the mucosal tissues of various warm-blooded domestic animals. However, they have been also isolated from the gastrointestinal tract of different reptile species (Kostka et al. 1997).

When the balance of the normal microbiota is disrupted or the immune defences are compromised, some of these species can act as opportunistic pathogens in susceptible animals. Antibacterial therapy, immunosuppressive drug therapy, immunosuppressive or debilitating systemic disease, malnutrition, poor management, unsanitary conditions and the stress of captivity are important predisposing factors for this disease in domestic animals. However, in comparison to the high number of cases of candidiasis reported in humans, it is a rare mycosis reported in veterinary medicine.

Candida albicans is the more important species involved in this mycosis, but it is not always the most frequently isolated species in some clinical forms. The occurrence of *C. albicans* in domestic animals is not as well known as it is in humans. More than 20 *Candida* species have been implicated in different animal infections (Smith 1989). In Table 12.1, some *Candida* species cited as opportunistic pathogens in domestic animals are detailed.

Analyzing by DNA fingerprinting *C. albicans* isolates from different human and animal individuals, Edelmann et al. (2005) obtained no evidence for host-specific

Table 12.1 Some *Candida* species cited as opportunistic pathogens in domestic animals[a]

Candida spp.	Animal species	Clinical forms, diseases, infections
C. albicans	Various	Cutaneous and urinary tract infection in cat and dog. Abortion and mastitis in cattle. Systemic, genital tract and alimentary tract infections in horses. Cutaneous and alimentary tract infections in poultry and swine.
C. cariosilignicola	Horse	Keratomycosis in horses.
C. catenulata	Poultry	Alimentary tract infections.
C. famata	Horse	Arthritis.
C. glabrata	Various	Urinary tract infection in cat and dog. Abortion in cattle. Keratomycosis in horses. Alimentary tract infections in swine.
C. guilliermondii	Various	Cutaneous and urinary tract infection in cat and dog. Abortion and mastitis in cattle. Keratomycosis in horses. Alimentary tract infections in poultry.
C. hellenica	Cattle	Mastitis in cattle.
C. kefyr	Cattle	Abortion and mastitis in cattle.
C. krusei	Various	Urinary tract infection in cat and dog. Abortion and mastitis in cattle. Alimentary tract infections in horses and poultry.
C. lusitaniae	Cattle and horses	Abortion and mastitis in cattle. Keratomycosis in horses.
C. parapsilosis	Various	Cutaneous and urinary tract infection in cat and dog. Abortion and mastitis in cattle. Arthritis, endocarditis and keratomycosis in horses. Alimentary tract infections in poultry.
C. pelliculosa	Cattle	Mastitis.
C. pintolopesii	Swine	Alimentary tract infections.
C. rugosa	Various	Urinary tract infection in cat and dog. Mastitis in cattle. Genital tract infection in horses.
C. sake	Poultry	Alimentary tract infections.
C. slooffiae	Swine	Alimentary tract infections.
C. tropicalis	Various	Urinary tract infection in cat and dog. Abortion and mastitis in cattle. Arthritis and keratomycosis in horses. Alimentary tract infections in poultry.
C. zeylanoides	Swine	Keratomycosis in horses.

[a]This table is intended to provide some examples of infections caused by *Candida* species in domestic animals. It is not a complete list

genotypes and for the existence of species-specific lineages, even though a certain degree of separation between human and animal isolates was found. These authors also indicated that animals have to be considered as potential sources of *Candida* infections of human individuals especially when humans are immunodeficient. More recently, Wrobel et al. (2008) explored whether wildlife species serve as the reservoir for human *C. albicans* strains in a given geographic area by multilocus sequence typing (MLST) analysis. Clade distributions between human and wildlife isolates were significantly different, demonstrating population isolation between the groups. These authors suggested a greater likelihood of *C. albicans* transfer from humans to animals than from animals to humans. Jacobsen et al. (2008)

carried also MLST out of *C. albicans* strain types from animals, including mammals and avian species from diverse global sources and compared them with strain types for human isolates. Their results showed a strong statistical trend towards genetic selection of different *C. albicans* strain types adapted to non-human animal hosts, but without complete genetic separation.

12.2.1 Cat and Dog

As inhabitants of the alimentary tract, genital and upper respiratory mucosae, some *Candida* species may produce both localized mycoses and disseminated mycoses in immunocompromised and/or debilitated animals (Greene and Chandler 2006). Candidiasis is rare in dogs and cats (Jungerman and Schwartzman 1972) but prolonged immunosuppression, cytotoxic chemotherapy causing neutropenia, diabetes mellitus, long-term glucocorticoid therapy and prolonged antimicrobial therapy have resulted in an increased incidence in the last few years of both localized and disseminated candidiasis.

Cutaneous candidiasis is an uncommon fungal skin disease reported in cats and dogs. Usually is seen as an opportunistic infection secondary to underlying immunosuppressive or debilitating systemic disease and immunosuppressive drug therapy. The oral mucosa, muco-cutaneous junctions and distal extremities are the most frequently involved sites (Gross et al. 2005). There are few reports of cutaneous candidiasis in these pets in the veterinary literature. The most frequent pathogen is *C. albicans* (Král and Uscavage 1960; Guillot et al. 1996; Moretti et al. 2004). However other species, such as *C. parapsilosis* (Dale 1972), *C. guilliermondii* (Mueller et al. 2002) have been identified from canine diseased skin.

Recently, other clinical forms of candidiasis have been mentioned in the veterinary literature. In a retrospective study of more than 8,000 microbial isolates from canine urinary tract infection, *Candida* spp. were the predominant fungal species which accounted for less than 0.2% of the total isolates (Ling et al. 2001). Urinary tract infections with *Candida* spp. are often associated with some predisposing factors such as diabetes mellitus, indwelling urinary catheters or prolonged antimicrobial or glucocorticoid administration (Greene and Chandler 2006). Recently, in another retrospective study about fungal urinary tract infection, *C. albicans* was the most common species isolated, found in 48% of dogs and 42% of cats. *Candida tropicalis, C. krusei, C. glabrata* and *C. guilliermondii* were also recovered. Lower urinary tract diseases, diabetes mellitus, neoplasia, and renal failure were the most common concurrent or preceding diseases identified (Jin and Lin 2005).

In another study, *C. albicans* was also the most common isolate from urinary tract infection, found in 62% of dogs and 43% of cats (Pressler et al. 2003). *Candida rugosa* and *C. krusei* were isolated only from dogs and *C. glabrata* and *C. parapsilosis* were isolated only from cats. Concurrent diseases or non-antifungal drugs administered included antibiotics, corticosteroids, diabetes mellitus, non-urogenital neoplasia, and non-candidal urogenital disease.

In the last few years, systemic candidiasis has often been reported in dogs (Heseltine et al. 2003; Kuwamura et al. 2006; Linek 2004) and in a case affecting an apparently immunocompetent animal (Brown et al. 2005). In these cases, species level identification of clinical isolates was not performed.

On the other hand, the occurrence of *Candida* species in the skin and mucous membranes of cats and dogs is not very well known. In a recent study of the mycobiota on cutaneous and mucosal surfaces of cats infected with feline immunodeficiency virus or feline leukaemia virus, *C. albicans* was rarely isolated (from approximately less than 10% of cats) from both infected and noninfected animals (Sierra et al. 2000). More recently, Wrobel et al. (2008) cited that *C. albicans* isolation from canine and feline oral and anal swabs was also very infrequent (approximately from less than 2% of pets). No other *Candida* species were mentioned in this survey.

Very limited information is available about the prevalence of fungal organisms in the gastrointestinal tract of dogs and their role as part of the intestinal microbiota. Recently, Suchodolski et al. (2008) evaluated the prevalence of fungal DNA in the small intestine of healthy dogs and dogs with chronic enteropathies. Fungal DNA was detected in 60.9% of healthy dogs and in 76.1% of dogs with chronic enteropathies. The most commonly observed sequences were classified as *Pichia* spp., *Cryptococcus* spp., *Candida* spp., and *Trichosporon* spp. Eight different *Candida* spp. were detected in 14 dogs. Sequences showing 100% similarity with *C. albicans*, *C. tropicalis*, *C. parapsilosis* were detected mainly in diseased dogs.

12.2.2 Cattle

12.2.2.1 Bovine Abortion

Aspergillus spp. and some species of the Zygomycetes are the most frequent fungi isolated from mycotic placentitis, a major cause of abortion in cattle. *Candida* spp. are associated with only a low percentage of the mycotic cases (Jensen et al. 1991; Knudtson and Kirkbride 1992). *Candida albicans, C. glabrata, C. krusei, C. lusitaniae, C. parapsilosis, C. pseudotropicalis* (synonym of *C. kefir; Kluyveromyces marxianus* (Lachance 1998)) and *C. tropicalis* have been associated with bovine abortion (Foley and Schlafer 1987; Knudtson and Kirkbride 1992; Sarma et al. 1979; Smith 1967). In a retrospective study (Foley and Schlafer 1987), *C. parapsilosis* was the only pathogenic agent demonstrated in the four cases of yeast-associated abortion found among 1,323 bovine abortions. *Candida guilliermondii* has been also reported (Smith 1989).

12.2.2.2 Bovine Mastitis

Mastitis is one of the major causes of serious economic losses to the dairy industry and an important factor influencing the health of dairy cows. The great majority of

the cases are caused by bacteria, but since the advent of antibiotics there has been an increasing number of reports of mycotic mastitis, almost invariably associated with prior antibiotic treatment of bacterial mastitis.

Plastridge (Plastridge 1959) reviewed the few first cases of yeast-like fungi associated with bovine mastitis. A *Trichosporon* sp. and *C. neoformans* were reported as etiological agents of this disease, but *Candida* species were not involved in these first cases. Today, yeast mastitis is generally a sporadic condition of low incidence, where *Candida* species are the predominant cause of sporadic cases and outbreaks. The most common organisms causing mycotic mastitis reported in U.S. dairy herds belonged to the *Candida*, *Cryptococcus* and *Trichosporon* genera, with *Pichia* and *Torulopsis* also being important in other countries (Kirk and Bartlett 1986). Sources responsible for more widespread outbreaks seems to be linked to homemade antibacterial infusion products given with reused syringes or teat cannulas.

Interestingly, non-*albicans* species such as *C. krusei* or *C. rugosa* are predominant in some surveys on milk from clinical and subclinical mastitis or responsible for mastitis outbreaks in intensive dairy farms (Crawshaw et al. 2005; Santos and Marin 2005; Spanamberg et al. 2008). However, a steady increase in clinical *C. albicans* mastitis has been recently described in smallholder dairy herds of Tanzania (Kivaria and Noordhuizen 2007). The prevalence of *C. albicans* increased from <1% in 1971 to >17% in 2002. Other species isolated in this study were *C. guillermondii*, *C. tropicalis* and *C. pelliculosa* (anamorph of *Pichia anomala* (Kurtzman 1998)). Other species such as *C. steatolytica* (*C. hellenica* anamorph of *Zigoascus hellenicus* (Smith 1998)), *C. kefyr*, *C. lusitaniae* and *C. parapsilosis* have been associated with bovine mastitis (Smith 1989).

12.2.3 Horse

Candidiasis is rarely reported in horses. Only a few case reports mainly concerning reproductive tract infections of mares have been cited in classic reference books (Jungerman and Schwartzman 1972; Smith 1989). With the exception of a case of pyometra caused by *C. rugosa* (Abou-Gabal et al. 1977), only non-identified species of *Candida* associated with endometrial, cervical and uterine infections were mentioned in these reports. They were considered to be secondary pathogens and related to antibiotic therapy. More recent literature indicates that besides *C. albicans*, many other fungal species including various *Candida* spp and other yeasts (e.g. *Rhodotorula* spp., *Hansenula* spp.) may be isolated from the reproductive tract of mares (Daschanio et al. 2001). In foals, oral candidiasis (McClure et al. 1985) and gastroesophageal candidiasis associated with *C. albicans* and *C. krusei* (Gross and Mayhew 1983) have also been reported. A case of endocarditis with massive amounts of *C. parapsilosis* in the valvular vegetations of a horse has been also described (Buergelt et al. 1985).

A few cases of keratomycosis caused by non-*albicans* species (e.g. *C. cariosilignicola* (synonym of *Pichia methylivora* (Kurtzman 1998)), *C. glabrata, C. guilliermondii, C. parapsilosis, C. tropicalis, C. lusitaniae, C. zeylanoides*) and other yeasts (e.g. *Trichosporum* spp., *Pichia* spp.) have been reported (Ball et al. 1997; Barton 1992; Ledbetter et al. 2007; McLaughlin et al. 1983; Moore et al. 1983). Equine fungal keratitis is a relatively common ocular disease in horses, compared with other domestic animal species. It is caused mainly by filamentous fungi, with *Aspergillus* and *Fusarium* spp. the most common (Ball 2000). Yeast infections tend to be more discrete, focal and suppurative than filamentous fungal keratomycosis. The most commonly reported events that predispose horses to keratomycosis are corneal trauma or indiscriminate use of topical corticosteroids (Barton 1992).

Cases of arthritis associated with fungal organisms are rare. However, infections produced by *C. famata* (Riley et al. 1992b), *C. parapsilosis* and *C. tropicalis,* respectively (Madison et al. 1995), affecting inflamed joints have been described in three horses. The fungal arthritis were probably developed after an accidental laceration of the fetlock of a mare (Riley et al. 1992b), an intra-articular injection in another horse and through an haematogenous spread of the yeast from the gastrointestinal tract or a surgical wound infection in the third animal (Madison et al. 1995).

Systemic *Candida* spp. infections are also rarely found in the veterinary literature. A unique report (Reilly and Palmer 1994) described the isolation of *C. albicans* from four neonatal foals with complex problems. Some possible risk factors for contracting candidiasis such as the use of intravenous catheters, urinary catheters, or endotracheal tube, and prior treatment with multiple antibiotics were identified in these immunologically immature newborns.

12.2.4 Poultry

Candidiasis is the principal fungal infection of digestive tract of poultry. This mycosis was first reported in chickens by Eberth in 1858 (cited in Jungerman and Schwartzman 1972). Major outbreaks with severe losses were recorded in North America in the early 1930s. Poultry pathologists and researchers are not in complete agreement regarding the significance of this mycosis and some of them minimize its importance. However, these cases are no doubt much more frequent than reports indicate (Smith 1989). Avian candidiasis is most frequently caused by *C. albicans* and is usually manifested as a crop mycosis. In these cases, the crop wall is thickened and the mucosal surface is usually covered by whitish, pseudomembranous exudate and ulceration, also seen in the mouth and oesophagus (Kunkle and Richard 1998). This yeast is often present in the upper gastrointestinal tract of normal birds. A mycological survey of the crops of approximately 100 healthy broilers from each of 6 grow-out operations revealed that the incidence of *Candida* colonization ranged from 17.4% to 51.5% (Wyatt and Hamilton 1975). The occurrence of avian candidiasis is sporadic, but outbreaks can be costly

(Kunkle 2003). In the Wyatt and Hamilton (1975) survey, of the 573 birds examined, less than 1% exhibited lesions attributable to *Candida*, with *C. albicans* being the most frequently isolated species (approx. 95%). Other species identified in this study were *C. brumptii* (synonym of *C. catenulata* (Meyer et al. 1998)), *C. catenulata, C. guilliermondii, C. parapsilosis, C. ravautii* (synonym of *C. catenulata* (Meyer et al. 1998)) and *C. salmonicola* (synonym of *C. sake* (Meyer et al. 1998)) which were also isolated from the crops. Other species such as *C. krusei* and *C. tropicalis* have been also reported (Smith 1989).

Thrush has been observed also in turkeys, pigeons, geese, guinea fowl, pheasants, ruffed grouse, quail, peacocks, lories, lovebirds, finches, parrots and parakeets. Usually it is an opportunistic infection. Factors predisposing to candidiasis include the presence of another primary disease, immunosuppression, prolonged administration of antibiotics which suppresses normal bacterial microbiota, malnutrition and poor husbandry (Kunkle 2003).

Cutaneous candidiasis has been also reported in birds. *Candida albicans* has been involved in chicken dermatitis and loss of feathers (Kuttin et al. 1976) and in a recent cutaneous infection affecting the comb, wattles, facial skin and neck of healthy broiler breeder roosters (Osorio et al. 2007).

Recently, the fungal contamination in a turkey confinement house was evaluated (Fulleringer et al. 2006). Occurrence of *C. albicans* in many environmental samples (68.7%) including air samples was detected. Densities of this yeast were very high in litter samples. However, no case of candidiasis was detected in the facility.

12.2.5 Swine

In pigs, *C. albicans* appears to colonize debilitated skin surfaces and lesions on other mucous surfaces. The predisposing factors appear to include the effects of artificial rearing of piglets and chronic enteritis often associated with treatment with broad-spectrum antimicrobials. Piglets with candidiasis are often in poor condition with chronic diarrhoea. Systemic invasion is rare (Straw et al. 2006).

A unique report of cutaneous candidiasis affecting approximately 180 garbage-fed swine was caused by *C. albicans* (Reynolds et al. 1968). The distribution of lesions in these animals correlated well with exposure of pigs to wet conditions, from lying either in damp bedding or cooked garbage. Correction of management conditions with the maintenance of dry housing controlled the infection. Specific epithelial lipids can modulate adherence of *C. albicans* to keratinized epithelial surfaces. Removal of lipid from the porcine stratum corneum lead to a doubling of the number of adherent organisms, whereas additional skin lipid inhibited adherence (Law et al. 1997).

Some species, such *C. albicans, C. glabrata, C. slooffiae* and *C. pintolopesii* have been associated with alimentary tract infections and gastric ulcers (Kadel et al. 1969; McGillivery 1989; Smith 1967; Smith 1989; Tannock and Smith 1970). Some of these yeasts (e.g. *C. albicans* and *C. slooffiae*) have been found in

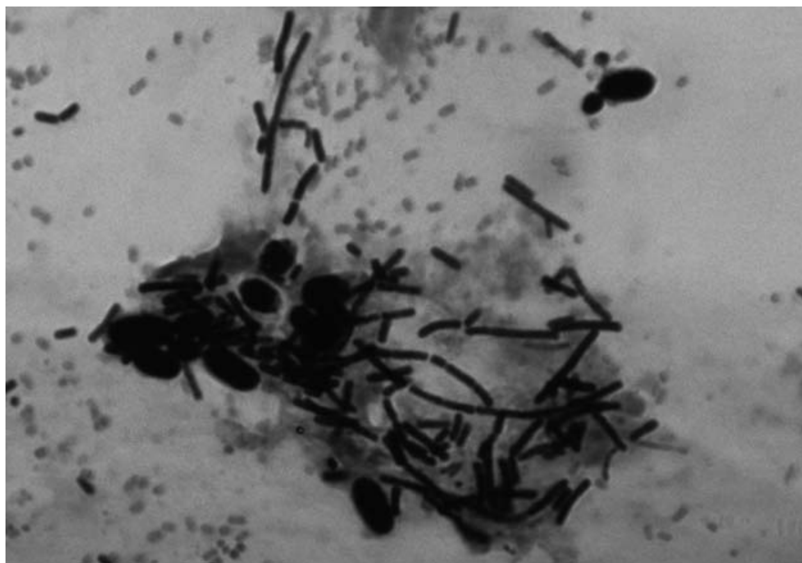

Fig. 12.1 Microbiota of a gastric ulcer (pars oesophagea) from a pig. Note the presence of yeast-like cells of *Candida* spp. Gram stain

association with the mucosa as well as in the stomach contents and can be regarded as members of the stomach microbiota (Fig. 12.1). They seem to be capable of proliferating on abnormal epithelial areas of the pars oesophagea (Tannock and Smith 1970). *Candida slooffiae* and *C. pintolopesii* have been recently assigned to the teleomorphic genus *Kazachstania* (Kurtzman et al. 2005).

Andrutis et al. (2000) demonstrated that immunosuppressed gnotobiotic (IGB) piglets orally inoculated with wild-type *C. albicans* developed extensive intestinal lesions and disseminated infection. Severe ulceration of the ileal mucosa was observed overlying regions of colonization and necrosis of the gut-associated lymphoid tissue. Despite the high susceptibility of IGB piglets to many microbial pathogens, an avirulent mutant strain of *C. albicans* failed to produce intestinal lesions and exhibited poor dissemination, demonstrating that these effects required virulent organisms.

Recently, Zlotowski et al. (2006) described two cases of muco-cutaneous candidiasis produced by *C. albicans* in postweaning multisystemic wasting syndrome (PMWS) affected pigs. PMWS is a recently described disease caused by porcine circovirus type 2 which affects the immune response of the animals. The presence of oral, oesophageal and gastric lesions associated with yeasts and pseudo-hyphae in these lesions in growing pigs suggested a failure in the immune response of the hosts. An increase in the incidence of opportunistic agents, such as *C. albicans* in swine herds may be expected with the worldwide spread of PMWS (Zlotowski et al. 2006).

Table 12.2 Main *Cryptococcus* species cited as pathogens in domestic animals

Cryptococcus spp.	Serotypes (genotypes[a])	Main diseases and remarks
More common:		
C. neoformans (*Filobasidiella neoformans*)	A, D, AD	Cutaneous, subcutaneous and systemic mycoses affecting CNS in various animal species. Bovine mastitis. Immnunocompromised patients. Worldwide distribution. Associated with avian guano (especially from pigeons); zoonotic transmission via aerosol.
C. neoformans var. *grubii*	A (VNI, VNII)	Worldwide. Predominant in domestic animals.
C. neoformans var. neoformans	D (VNIV)	More common in Europe.
Hybrid form	AD (VNIII)	Rare. More commonly found in Europe.
C. gattii (*Filobasidiella bacillispora*)	B, C (VGI, VGII, VGIII, VGIV)	Same diseases as *C. neoformans* but less frequently isolated. Immunocompetent and immnunocompromised patients. Tropical and sub-tropical regions. Setotype B common in domestic animals in Australia. Serotype C is rarely found in animals. Associated with trees (*Eucalyptus* spp and other tree species).
Rare:		
C. albidus	–	Cited causing systemic infection in a cat and in a dog. Genital tract infection and keratomycosis in horses. Bovine mastitis.
C. laurentii	–	Subclinical bovine mastitis. Genital tract infection in horses.
C. curvatus	–	Subclinical bovine mastitis.

[a]Molecular genotypes by PCR fingerprinting (Meyer et al. 1999, 2003). Other molecular types and subtypes have been defined (e.g. AFLP type)

12.3 *Cryptococcus*

This anamorphic basidiomicetous genus is polyphyletic and currently contains more than 30 accepted species. However, only a few of these species are involved in animal mycoses (Table 12.2). Some species are anamorphic states of *Filobasidium, Filobasidiella* and other teleomorphic genera (Fell and Statzelll-Tallman 1998).

Cryptococcosis is an uncommon mycosis in domestic animals and its occurrence is sporadic. Apparently, the first record of infection by these yeasts in a domestic animal was described by Sanfelice in 1895 when he isolated these microorganisms from the lymph node of an ox. He had also isolated these yeasts from peach juice a year before, which he named *Saccharomyces neoformans*. A few years later, Vuillemin found cryptococci in a pulmonary lesion in a pig and transferred it to the genus *Cryptococcus* (cited in Jungerman and Schwartzman 1972).

In animals, this mycosis is caused mainly by *Cryptococcus neoformans*, although *Cryptococcus gattii* has also been frequently reported. Cats appear to be particularly affected by this disease in comparison to other domestic animals

(Malik et al. 2006). Since studies on these yeasts started in 1894, their speciation has been a controversial issue, due mainly to their diversity and to the different opinions among taxonomists on the definition of species. Currently, these two species form the *C. neoformans–C. gattii* species complex which includes species, microspecies, hybrids, serotypes and genotypes. Two anamorphic species are recognized in this complex, *C. neoformans* (Sanfelice) Vuillemin, with the teleomorph *Filobasidiella neoformans* Kwon-Chung and *C. gattii* (Vanbreuseghem and Takashio) Kwon-Chung et al. with *Filobasidiella bacillispora* Kwon-Chung as teleomorph (Bovers et al. 2008a).

These species have distinctly different environmental niches and geographic-climatic distribution (Table 12.2). *Cryptococcus neoformans* is noted for its association with accumulations of avian guano, especially pigeon excreta and has a worldwide distribution. For this organism, the disease is frequently related to the exposure of immunocompromised patients to avian droppings (Sorrell and Ellis 1997; Rosario et al. 2008). Infections with *C. gattii* occur almost only in immunocompetent individuals, and have an apparent restriction to tropical and sub-tropical regions. It has been ecologically associated with several tree species, mainly with eucalyptus trees (Bovers et al. 2008a; Sorrell and Ellis 1997). However, both species have been found sharing the same natural biotope on hollows of living trees (Lazera et al. 2000). The rapidly expanding spectrum of host tree species for *C. gattii* and also for *C. neoformans* have recently been documented (Randhawa et al. 2008, Refojo et al. 2009). In a recent study (Nielsen et al. 2008), pigeon guano supported the growth of both species. However, *C. neoformans* exhibited prolific mating, whereas *C. gattii* did not. Because *C. gattii* grows on pigeon guano but cannot sexually reproduce, this substrate represents a fundamental but not a realized niche for this species. This is an interesting issue because animals are thought to be exposed by inhalation of basidiospores. In humans, a pulmonary infection with *C. neoformans* or *C. gattii* most likely starts with inhalation of infectious particles, which have not been identified with certainty, although basidiospores which are smaller than yeast cells seem to be the most likely candidate to reach the alveoli (Bovers et al. 2008a).

Very few other species of this genus have been cited as pathogens in domestic animals. In recent years, systemic infections produced by *Cryptococcus albidus* in a dog (Labrecque et al. 2005) and in a cat (Kano et al. 2008) have been reported. This species has been also recovered from nodular lesions on the prepuce of a stallion, the cornea of a horse and bovine mastitis (Smith 1989) and otitis externa of dogs (Bernardo et al. 1998). *Cryptococcus laurentii* has been also recovered from the genital tract of horses (Smith 1989). This latter species and *Cryptococcus curvatus* have been detected in milk from subclinical bovine mastitis (Spanamberg et al. 2008).

12.4 *Cryptococcus neoformans*

This species contains two recognized varieties corresponding to the two major serotypes: *C. neoformans* var. *grubii* (serotype A) and *C. neoformans* var. *neoformans*

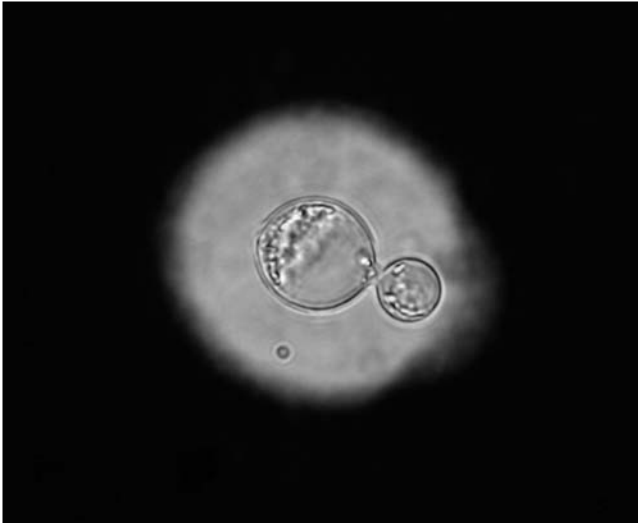

Fig. 12.2 A thick-capsuled budding yeast cell of *Cryptococcus neoformans* var. *neoformans* from a fine-needle aspirate of a lymph node of a cat seropositive for feline immunodeficiency virus with cryptococcosis. India ink

(serotype D) (Franzot et al. 1999). PCR fingerprinting distinguish four genotypic groups within *C. neoformans* (VNI and VNII = serotype A); VNIII = serotype AD; VNIV = serotype D (Meyer et al. 1999). Using several molecular techniques, two monophyletic lineages corresponding to *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* have been consistently found within this species (Bovers et al. 2008b). The epidemiological distribution pattern of these varieties in animal cryptococcosis is not well known. *Cryptococcus* spp. detected in animals is rarely taxonomically identified to the serotype or variety level in diagnostic cases. In some veterinary reports, the special micromorphology of these yeasts (capsulated round cells with narrow-based budding) (Fig. 12.2) and/or their response to various assimilation tests are usually the only characters used for identification. Both varieties can cause disease in animals but they differ in their geographic distribution; *C. neoformans* var. *grubii* (serotype A) is by far the most common cause of human cryptococcal infection worldwide, and it seems to be also predominant in domestic animals; *C. neoformans* var. *neoformans* (serotype D) occurs worldwide as well, but seems to occur more commonly in Europe (Bovers et al. 2008a; Kwon-Chung and Bennett 1984; Malik et al. 2006). In some areas of Europe, a moderately high percentage of the clinical isolates were found to be AD hybrids (serotype AD) (Bovers et al. 2008a). On the contrary, in Australia *C. neoformans* var. *neoformans* (serotype D) and the hybrid forms (serotype AD) are extremely rare (Pfeiffer and Ellis 1993; Bui et al. 2008).

Cryptococcosis is the most common of the systemic mycoses of cats. In these animals, upper respiratory tract signs are most common, and in some cases a polyp-like mass is evident in the nostril. Major organ systems affected in the dog include

the nasal cavity, the central nervous system and the eyes. It appears that most cases of canine and feline cryptococcosis begin as mycotic rhinitis. Unlike in humans, in cat and dogs, among other animals, the nasal cavity is usually the primary site of infection. This may probably be due to the more developed nasal passages which more efficiently filter small particles (Malik et al. 2006). Asymptomatic carriage of blastoconidia and/or basidiospores of *C. neoformans* in the nasal cavity of dogs and cats has been demonstrated (Malik et al. 1997). Interestingly, only this species was isolated from nasal washings in this study, even though the animals used were drawn from a wide Australian geographical area in which *C. gattii* accounts for 25% or more of clinical isolates in cats, dogs and HIV-negative humans.

Infection with *C. neoformans* has been reported in a variety of other domesticated species, including cattle, goats, horses and sheep, among others. The infection in cattle and goats usually involves the mammary gland, causing subsequent severe mastitis; horses and sheep can manifest nasal discharges and respiratory distress and also generalized disease affecting the CNS (Jungerman and Schwartzman 1972; Smith 1989).

The definitive environmental niche for *C. neoformans* has not been determined, although a strong historical association exists between weathered bird (especially pigeon) guano and growth in decaying plant matter in hollows of certain trees. However, a consistent association between pigeons, their guano and cryptococcosis has never been convincingly documented for any animal species (Malik et al. 2006; Rosario et al. 2008). The common suspicion that birds may be the source for the infection is now becoming a demonstrable fact thanks to the use of molecular typing methods. So far, zoonotic transmission of *C. neoformans* from pet bird's excreta to an immunocompromised human patient (Nosanchuk et al. 2000) and an immunocompetent human patient (Lagrou et al. 2005) has been demonstrated using these techniques. The pet birds involved in these cases were a cockatoo and a magpie, respectively. Cryptococcosis produced by *C. neoformans* is included in the list of zoonotic diseases of importance in USA and it is classified as not nationally notifiable for human or animal cases (Elchos et al. 2008). Few mycoses are usually considered as zoonoses. The role that animals play in the epidemiology of the main human mycoses is still not well known and definitive environmental niches for their fungal agents have not been completely determined (Cabañes 2008). Pigeon and other birds are cited as the most common animal species associated with *C. neoformans* transmission to humans via aerosol.

12.4.1 Cryptococcus gattii

Although several molecular methods distinguish four genotypic groups within *C. gattii* (VGI, VGII, VGIII and VGIV), these groups have not yet been interpreted as distinct taxa. Interestingly, *C. gattii* serotypes B and C do not correspond to a specific group, but appear to occur in all genotypic groups (Bovers et al. 2008a; Meyer et al. 2003).

There is not much epidemiological information concerning this species in veterinary patients. Most of the published reports are from Australia. Malik et al. (2006) emphasize that *C. gattii* is important in certain geographic regions such as Australia, Papua New Guinea, Southeast Asia and Central Africa. Kwon-Chung and Bennett (1984) also reported an unusually high prevalence of this species in Brazil, Hawaii, southern California, Mexico and Paraguay. In Australia, *C. gattii* (serotype B) exists in highest concentration within the dead plant material in eucalyptus tree hollows and approximately 20% of cat and dog cryptococcosis cases are caused by this species (Malik et al. 1992, 1995, 2006). Interestingly, in a retrospective study of cryptoccosis in dogs (Malik et al. 1995) all *C. gattii* isolates were obtained from dogs normally residing in rural or suburban, rather than urban environments, supporting the idea that increased exposure to eucalyptus is an important epidemiological factor in these infections. Animals in some rural areas of Australia tend to be infected by this species, and also for this reason all cases recorded in koalas have been attributable to *C. gattii* (Malik et al. 2006; Sorrell et al. 1996). In cases of equine cryptococcosis reported in Western Australia, isolates of *C. gattii* were recovered from two of the five horses in which cultures were attempted. Most horses in this report had been in areas in which *Eucalyptus camaldulensis*, or the closely related *Eucalyptus rudis*, were growing (Riley et al. 1992a). This species has also been recovered from nasal cavity and brain of sheep (Sorrell et al. 1996), localized invasive disease of the upper respiratory tract of captive parrots and from captive brown kiwis with severe diffuse cryptococcal pneumonia or widely disseminated disease (Malik et al. 2003), and ferrets with different clinical forms of cryptococcosis (Malik et al. 2002). All these animals were also from Australia and New Zealand. Molecular types VGI and VGII are frequently isolated from these animals in these areas (Sorrell et al. 1996).

Few case reports caused by *C. gattii* are published outside these regions. Recently, several case reports and outbreaks suggest that *C. gattii* is becoming more prevalent in temperate climates. In Spain, *C. gattii* (serotype B) was isolated from goats with predominantly severe pulmonary disease, during several outbreaks (1990–1994 years). Between 2.5 and 12% of the goats were affected (Baró et al. 1998). These isolates were molecular typed as VGI (Meyer et al. 2003). A *C. gattii* (serotype B) strain molecular type VGII from a sick goat from Aruba is also cited in molecular studies (Bovers et al. 2008b). Since 1999, an exceptional emergence of *C. gattii* serotype B in humans and various animal species occurred on Vancouver Island in British Columbia, Canada (Stephen et al. 2002) and is now considered endemic in the environment. All outbreak and environmental isolates belonged to this species. The vast majority of clinical and veterinary infections were caused by isolates of the molecular type VGII/AFLP6 and all environmental isolates belonged also to this type (Kidd et al. 2004). Some studies indicated that asymptomatically infected cats and dogs in this area may clear *C. gattii*, remain sub-clinically infected or progress to clinical disease (Duncan et al. 2005).

Serotype C is rarely found in animals. The only cited isolate in the literature is the reference strain of the molecular type VGIV (WM779). It was isolated from a cheetah from South Africa (Bovers et al. 2008b; Meyer et al. 2003). Cryptococcosis

is the most common systemic mycoses in the cheetah (Millward and Williams 2005). In a report of the worldwide distribution of *Cryptococcus* species (Kwon-Chung and Bennett 1984), among the two serotypes of *C. gattii*, serotype B was cited 4.5 times more prevalent than serotype C. In this report, the majority of type C isolates were from southern California.

12.5 Malassezia

This anamorphic genus belongs to the basidiomicetous yeasts and is classified in the Malasseziales (Ustilagomycetes) (Batra et al. 2005). However, the sexual form (teleomorph) is still unknown. *Malassezia* species are lipophilic yeasts that are usually members of the normal mycobiota of the skin and mucosal sites of a variety of homeoterm animals. The natural habitat of these lipophilic yeasts is the stratum corneum of human and animal skin where they grow readily owing to the presence of skin surface lipids.

The genus *Malassezia* was created by Baillon in 1889 and remained limited to only two species for a century: *M. furfur* (Robin) Baillon 1889 and *M. pachydermatis* (Weidman) Dodge 1935 (Batra et al. 2005). Traditionally, the lipid-dependent species *M. furfur* (*sensu lato*) was thought to occur only on human skin, being the causal agent of different skin disorders, while the lipophilic, but non lipid-dependent, species *M. pachydermatis* was restricted to animal skin.

At the moment, few studies have been published in order to know the distribution and pathogenicity of the different *Malassezia* spp. on animals following the last taxonomic revision of this genus (Guého et al. 1996). In this revision, on the basis of molecular data and lipid requirements, this genus was enlarged to seven species. They include three former taxa *M. furfur*, *M. pachydermatis* and *M. sympodialis* (Simmons and Guého 1990) and four new taxa *M. globosa*, *M. obtusa*, *M. restricta* and *M. slooffiae*. Furthermore, in most of these surveys, the isolates have been identified only on the basis of phenotypic characteristics without molecular analysis confirmation. Difficulties remain in obtaining a high level of certainty in the identification of some lipid-dependent strains by means of physiological tests (e.g. Tween assimilation) without molecular characterization and comparisons of recent work to older data are difficult due to changes in nomenclature (Table 12.3).

More recently, six new lipid-dependent species, *M. caprae* (Cabañes et al. 2007), *M. dermatis* (Sugita et al. 2002), *M. equina* (Cabañes et al. 2007), *M. japonica* (Sugita et al. 2003), *M. nana* (Hirai et al. 2004) and *M. yamatoensis* (Sugita et al. 2004) have been described. Of these last species, *M. caprae*, *M. equina* and *M. nana* were isolated from domestic animals.

12.5.1 *Malassezia pachydermatis*

Malassezia pachydermatis, the only species in the genus that does not require lipid supplementation for development in culture medium, is considered to be zoophilic,

Table 12.3 Current described *Malassezia* species and their main hosts[a]

Malassezia spp.	Main host	Main diseases and remarks
M. caprae	Goat	Unknown. Member of the normal skin microbiota of goats. Cited also on a horse. Lipid-dependent.
M. dermatis	Man	Associated with atopic dermatitis. Lipid-dependent.
M. equina	Horse	Unknown. Member of the normal skin microbiota of horses. Cited also on a cow. Lipid-dependent.
M. furfur	Man	Deep infection (e.g. blood, urine). Member of the normal human skin microbiota. Not detected or detected in moderate or low % in pityriasis versicolor, seborrheic dermatitis, atopic dermatitis. Cited also on: cow, elephant, ostrich, pelican, pig, monkey. Lipid-dependent.
M. globosa	Man	Detected in high % in pityriasis versicolor, seborrheic dermatitis, dandruff and atopic dermatitis. Member of the normal human skin microbiota. Cited also on: cheetah, cow. Lipid-dependent.
M. japonica	Man	Associated with atopic dermatitis. Lipid-dependent.
M. nana	Cat. Cow.	Otitis externa. Member of the normal skin microbiota of cat and cow. Cited also on: dog. Lipid-dependent.
M. obtusa	Man	Unknown. Member of the normal human skin microbiota. Lipid-dependent.
M. pachydermatis	Dog, cat.	Otitis externa. Dermatitis. Zoonotic transfer has been documented from dogs to neonates by healthcare workers who own dogs. It is not a member of the normal human skin microbiota. Cited also on a large variety of warm-blooded animals (mainly carnivores) and birds. Non lipid-dependent.
M. restricta	Man	Detected in high % in dandruff, seborrheic dermatitis and atopic dermatitis. Member of the normal human skin microbiota. Lipid-dependent.
M. slooffiae	Man. Pig.	Member of the normal pig and human skin microbiota. Not detected or detected in moderate or low % in pityriasis versicolor, seborrheic dermatitis, atopic dermatitis. Cited also on: goat, pig, sheep. Lipid-dependent.
M. sympodialis	Man	Detected in high % in pityriasis versicolor, seborrheic dermatitis, dandruff and atopic dermatitis. Member of the normal human skin microbiota. Cited also on: horse, pig, sheep. Lipid-dependent.
M. yamatoensis	Man	Not detected or detected in moderate or low % in seborrheic dermatitis and atopic dermatitis. Member of the normal human skin microbiota. Lipid-dependent.

[a]Cited only those (cases, isolates) confirmed by rDNA sequencing analysis

and is frequently found on wild and domestic carnivores (Guillot and Bond 1999). This yeast has been also implicated as a zoonotic agent. Zoonotic transfer of *M. pachydermatis* has been documented from dogs to immunocompromised patients by healthcare workers who own dogs, mainly in neonates (Chang et al. 1998).

The first association of this yeast with skin disease and the first description of this species (as *Pityrosporum pachydermatis*) were reported by Weidman (Weidman 1925) in a case of an exfoliative dermatitis in an Indian rhinoceros. However, *M. pachydermatis* is usually associated with otitis externa and different kind of

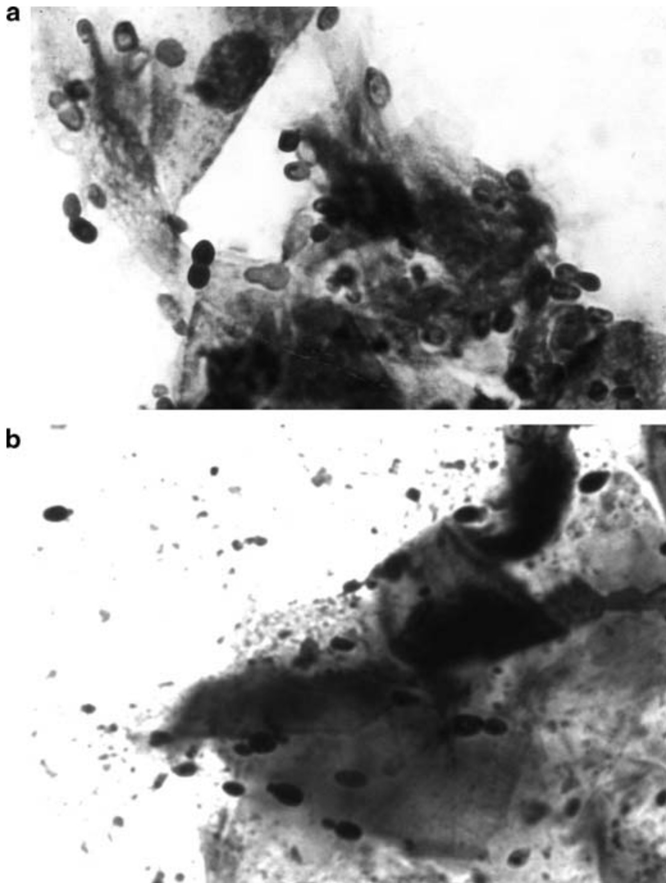

Fig. 12.3 Diff-Quick stains of smears from otic swabs of two cats with otitis externa associated with *Malassezia* species. (**a**) Presence of numerous *Malassezia pachydermatis* cells. Note the typical monopolar budding on a broad base. (**b**) Presence of numerous *Malassezia nana* cells. Note that buds are formed on a narrow base

dermatitis in domestic animals, but especially in dogs. This species is more frequently isolated from dogs than cats and appears to be a relatively infrequent pathogen in other animals (Guillot and Bond 1999; Crespo et al. 2002a). Different authors have mentioned the high incidence of this yeast in canine otitis externa and dermatitis and it seems to be a less frequent pathogen in cats (Fig. 12.3a). However, the pathogenic role of *M. pachydermatis* in the otitis externa and dermatitis has been a matter of controversy. This yeast seems to have an opportunistic nature and it may become pathogenic with any alteration in the skin surface microclimate or in the host defence. In some canine breeds, hypersensitivity conditions such as flea allergy dermatitis, food hypersensitivity or atopy and antimicrobial or corticosteroid therapy may be factors favouring proliferation of these yeasts (Guillot and Bond 1999; Pier et al. 2000).

The factors, which favour proliferation of *M. pachydermatis* from a commensal organism to an opportunistic pathogen of the skin are poorly understood. Various molecular methods have been used to correlate genetic types of this yeast with pathogenesis, skin disease or a particular animal species. Guillot et al. (1997) described seven genetic groups by 26S rRNA sequence analysis and some of them were host-specific (e.g. type Ic for rhinoceros; type Ig for ferrets). None of the sequence types correlated with skin disease. Aizawa et al. (2001) found four genetic types based on RAPD and chitin synthase gene sequencing data but they could not correlate them with pathogenicity. However, isolates from cats showed only a single genetic type. Castellá et al. (2005) showed that an animal could be colonized by more than one genetic type, detecting different genetic types in the same body site. Some types were only isolated from diseased skin. Cafarchia and Otranto (2004) reported that a high percentage of isolates obtained from lesion sites produced phospholipase, compared to the strains obtained from healthy skin of the same dog with localized lesions and healthy dogs. Isolates derived from skin lesions also showed a significantly higher phospholipase activity than those from healthy skin (Cafarchia et al. 2008). Cannizzo et al. (2007) demonstrated the ability of *M. pachydermatis* to adhere to and form biofilms on the surfaces of different biomaterials. Biofilm formation could be related to the pathogenesis.

In addition to dogs and cats, otitis externa associated with *M. pachydermatis* has been also cited in fennecs (Guillot et al. 1994), ferrets (Dinsdale and Rest 1995, Guillot et al. 1994), in pigs and in a camel (Kuttin and Glas 1985; Pinter et al. 2002). This yeast has been also associated with different skin disorders in a black bear (Salkin et al. 1978) in red foxes, porcupines and coyotes (Salkin et al. 1980), sea lions (Guillot et al. 1998; Nakagaki et al. 2000) and a goat (Pin 2004).

12.5.2 Lipid-dependent Species

Recently, lipid-dependent species have been isolated from the healthy skin of different animals. However, recent changes in nomenclature of these yeasts make it difficult to keep track of lipid-dependent species identities cited in papers when these species were identified only by phenotypic methods. In Table 12.3 those lipid-dependent species isolated from animals confirmed by rDNA sequencing analysis are cited. Lipid-dependent species seem to be more frequent in cats (Bond et al. 1996; Bond et al. 1997; Cafarchia et al. 2005; Coutinho et al. 2006; Crespo et al. 1999; Crespo et al. 2002a; Dizotti and Coutinho 2007; Guillot et al. 1994; Nardoni et al. 2005) than in dogs (Crespo et al. 2002a; Duarte et al. 2002). On the other hand, very little is known about their pathogenic role in animal skin. There is one report about the presumptive implication of a lipid-dependent species in a skin disorder in milking goats similar to those seen in pityriasis versicolor in humans (Bliss 1984). However, the lipid dependence of this yeast could not be demonstrated because the isolation was unsuccessful. Lipid-dependent species were first isolated from cats and dogs with otitis externa by Crespo et al. (2000a,b) (Fig. 12.3b).

Malassezia sympodialis (Crespo et al. 2000a) and *M. furfur* and *M. obtusa,* (Crespo et al. 2000b) were phenotypically identified, respectively. More recently, lipid-dependent strains from a cat and cows with otitis externa were included in the description of *M. nana* (Hirai et al. 2004). Several isolates from cats phylogenetically related to *M. sympodialis* were studied using DNA sequence analysis and grouped together by the *M. nana* D1/D2 rDNA sequence (Cabañes et al. 2005). This species seems to be the most common lipid-dependent species isolated from feline skin. This species and *M. slooffiae* have been recently isolated from cats with different disorders (Perrins et al. 2007) as well as *M. slooffiae* from the claw folds of healthy and seborrhoeic Devon Rex cats (Ahman et al. 2007)

Lipid-dependent species constitute the major population of the lipophilic mycobiota in herbivores such as horses, goats, sheep and cows. During a study of the occurrence of *Malassezia* spp. in 112 animals (50 horses, 25 goats, 25 sheep and 12 cows), *Malassezia* spp. were isolated from 60% of horses, 28% of sheep, 44% of goats and 58% of cows. In these animals, the occurrence of lipid-dependent species (42%) was much greater than the occurrence observed for *M. pachydermatis* (3%; Crespo et al. 2002b).

Several lipid-dependent species have been associated with otitis externa in bovines (Duarte et al. 1999, 2002, 2003). Different species such as *M. furfur, M. sympodialis, M. slooffiae, M. globosa* were identified from cerumen or secretions of the external ear of these animals.

More recently, a case of generalized dermatitis in an adult Pygmy goat associated with *M. slooffiae* has been reported (Uzal et al. 2007). Yeast cells were visualized in the skin and this species was identified in the skin scrapings by DNA sequencing. However, *Malassezia* spp. were not recovered in culture media. *Malassezia caprae*, a new lipid-dependent species isolated mainly from healthy goats has been recently described (Cabañes et al. 2007).

On the other hand, *M. sympodialis* related to skin lesions of a horse has also been described (Senczek et al. 1999). This species has been also reported in horses without skin disease together with other lipid-dependent species and non-identified isolates using phenotypic techniques (Crespo et al. 2002b). Several isolates from horses phylogenetically related to *M. sympodialis* grouped together by D1/D2 and ITS sequence analysis and were considered conspecific strains (Cabañes et al. 2005). These yeasts have been recently included in the new described species *M. equina* (Cabañes et al. 2007).

As far as birds are concerned, the most frequently isolated yeast from clinically altered combs of adult chickens was also *M. sympodialis* (Gründer et al. 2005). In this study, yeasts were phenotypically identified and no other *Malassezia* species was isolated.

It is possible that lipid-dependent species are more common in the skin and mucosae of animals and play a similar role as the non lipid-dependent *M. pachydermatis*. However, the isolation and identification of these strains continues to be difficult due, among other problems, to the low viability associated with some isolate types and lack of suitable methods for its isolation and preservation (Crespo et al. 2000c). In addition, the identification of *Malassezia* spp. only on the

basis of morphology and assimilation profiles of Tweens may produce misidentification (e.g. *M. sympodialis* versus *M. nana*). Although various molecular techniques have been proposed to identify *Malassezia* spp. (e.g. PCR-REA, AFLP), analysis of the rDNA regions, particularly D1/D2 sequences of the 26S rRNA gene, are especially useful because they clearly discriminate between the current described species (Cabañes et al. 2007). Sequence analysis of the D1/D2 regions is the gold standard technique to confirm the identification to species level of lipid-dependent isolates from animals.

12.6 Concluding Remarks

Candida and *Cryptococcus* species continue to cause sporadic mycoses in animals. However, in comparison to the high number of cases of candidiasis and cryptococcosis reported in humans, they are uncommon mycoses reported in veterinary medicine. *Candida albicans* is usually the more important species involved in this mycosis, but it is not always the most frequently isolated species in some clinical forms and animal species. It is still not clear whether a wildlife reservoir for this species exists. It has been suggested a greater likelihood of *C. albicans* transfer from humans to animals than from animals to humans. As far as cryptococcosis is concerned, this mycosis is caused mainly by *Cryptococcus neoformans*, although *Cryptococcus gattii* has also been frequently reported. Cats appear to be particularly affected by this disease in comparison to other domestic animals.

However, during the last two decades, interest in the genus *Malassezia* amongst mycologists and veterinary dermatologists has increased considerably. This fact is reflected by the high number of recently published papers in this field. A controversial pathogen *M. pachydermatis* is now recognized as an important cause of dermatitis and otitis externa in dogs. On the other hand, the lipid-dependent species which was thought to occur only on human skin are now isolated from various animal species and are the major population of the lipohilic mycobiota in some herbivores. However, very little is known about the pathogenic role of this lipid-dependent species in animal skin.

However, other emerging yeast pathogens have been reported to produce mycoses in a variety of wild and domestic animal species but unfortunately they are not within the scope of this review and I shall cite here only a couple of recent and interesting examples.

An organism commonly referred to as "megabacterium" that colonizes the stomach of many species of birds was recently described as an anamorphic ascomycetous yeast and named *Macrorhabdus ornithogaster* (Tomaszewski et al. 2003). The prevalence of *M. ornithogaster* infection in budgerigar, parrotlet, canary and finch aviaries seems to be high and it can be found in these birds throughout the world. An acute and chronic form of *M. ornithogaster*-associated disease has been described (Phalen 2005). In vitro efforts to grow this organism on traditional bacterial and fungal media have been largely unsuccessful. Very recently, this

species has been repeatedly passaged without loss of viability in a special culture medium under microaerophilic conditions at 42ºC (Hannafusa et al. 2007).

Only a small number of fungal species are pathogenic to fish and among them, yeasts are very infrequently reported as pathogens. *Metschnikowia bicuspidata* var. *bicuspidata* has been reported the causative agent of unexplained mortality in Chinook salmon fed live adult brine shrimp. Necropsy examinations revealed systemic fungal yeast infections and the yeast was cultured from the kidneys. This yeast was also observed within the live brine shrimp used as food for the salmon (Moore and Strom 2003). A pathogenic yeast which could cause milky disease in cultured crabs (*Portunus trituberculatus*) in China was recently identified to be also *M. bicuspidata* (Wang et al. 2007). This explosive epidemic disease affected cultured crabs causing high mortality and great economic losses in this sector.

Nevertheless, yeasts that occur in other common genera such as *Rhodotorula*, *Sporobolomyces* or *Trichosporon* continue to be rarely reported from veterinary patients.

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Chapter 13
Laboratory Diagnosis of Yeast Infections

Richard Barton

Abstract The diagnosis of yeast infections has been dominated by culture-based methods and this continues in the modern medical microbiology laboratory. The germ tube test to distinguish *Candida albicans* and *Candida dubliniensis* from other yeasts remains one of the few useful morphological identification tests; otherwise, identification of yeasts depends largely on panels of sugar assimilation assays. In recent years, chromogenic agars have become popular for mixture or full identifications. Automated biochemical systems for yeast identification are gaining popularity in larger laboratories. Molecular identification has become an important approach, with sequences in rRNA genes and spacers being used in DNA sequence analysis, microarrays or peptide nucleic acid probe based fluorescent hybridisation assays. Non-culture based approaches to the diagnosis of yeast infections have been suggested as ways to improve the sensitivity and speed of a diagnosis, though currently few have been proved as sufficient and reliable alternatives to culture.

13.1 Introduction

Yeasts cause a variety of infections, and the medical microbiology laboratory plays an important part in the diagnosis of these infections. The cultivation of yeasts on agar from clinical specimens is likely to be one of the earliest laboratory methods for the diagnosis of yeast infections. This may be directly from swabs taken from superficial sites as in oral and vaginal candidosis or indirectly from automated blood culture systems when yeast cells are seen in gram-stained samples taken from culture bottles flagging up as positive. Growth of yeasts on plates allows approximate quantification, identification of potential causal agents and, often nowadays,

R. Barton
Department of Microbiology, Mycology Reference Centre, Leeds General Infirmary, Leeds, LS1 3EX, UK
e-mail: Richard.Barton@leedsth.nhs.uk

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examination for sensitivity to antifungal agents. Non-culture-based methods of laboratory diagnosis of yeast infections including antibody detection, antigen detection and DNA detection are, with some notable exceptions, usually less useful but may be of considerable importance in some circumstances.

13.2 Direct Microscopy

13.2.1 Direct Microscopy of Unfixed Specimens

The visualisation of yeasts, pseudohyphae and hyphae typically in vaginal smears or oral swabs is an indication of the presence of yeasts within the vaginal and buccal mucosae (Fig. 13.1a). Microscopic examination can be enhanced by the use of fluorescent stains such as calcofluor that bind chitin in the yeast cell walls and fluoresce under certain wavelengths of light (Fig. 13.1b). However, microscopic detection of yeasts is known to be less sensitive than culture (Milne 1989) and will not provide definitive information on the identity of the organism or organisms involved. The view that the hyphal form of *Candida albicans* is pathogenic and the yeast form commensal has largely been debunked (Odds 1988). However, many authors persist in attributing a greater significance in terms of mucosal infection to the presence of the hyphal form of *C. albicans* in terms of infection, though this appears to be based on anecdotal evidence (Segal and Elad 2005) or experiments involving the inoculation of animal models with non-germ tube forming strains (Sobel et al. 1984). It is important to note that certain agents of candidosis such as *Candida glabrata* do not produce hyphae or pseudohyphe in vitro or in vivo.

Microscopic examination of other clinical specimens may be more revealing and may give an early indication of a yeast infection particularly where this was previously unexpected. The observation of capsulated *Cryptococcus neoformans* and *Cr. gatti* yeasts in cerebrospinal fluid (CSF) with India ink will usually provide a firm diagnosis of cryptococcal meningitis. The capsule around yeast cells can be readily observed in the clinical specimen (usually cerebrospinal fluid) when mixed with India ink, which the capsule excludes (Fig. 13.1c) – an approach first noted in 1924 (Weidman and Freeman 1924). A modification of this method involves the addition of 2% chromium mercury which improves the visualisation of the yeast cell structure and reduces confusion with artefacts (Zerpa et al. 1996). Histoplasmosis may be diagnosed by the presence of the characteristic small *Histoplasma capsulatum* yeast cells in phagocytic cells in a blood smear (Fig. 13.1d). Yeast cells and the hyphae derived from yeasts are frequently seen in skin and nail preparations. The subglobose yeast cells and hyphae of *C. albicans* from a skin scraping may be present in conditions such as Candida interigo. However, the presence of round yeast cells together with short sections of hyphae, the so-called “grapes and bananas” seen in skin scrapings, is diagnostic of pityriasis versicolor (Fig. 13.1e). This is a solely microscopic diagnosis – almost unique in mycology – since the *Malassezia* species that cause pityriasis versicolor are difficult to culture and will be universally present on the skin in any case.

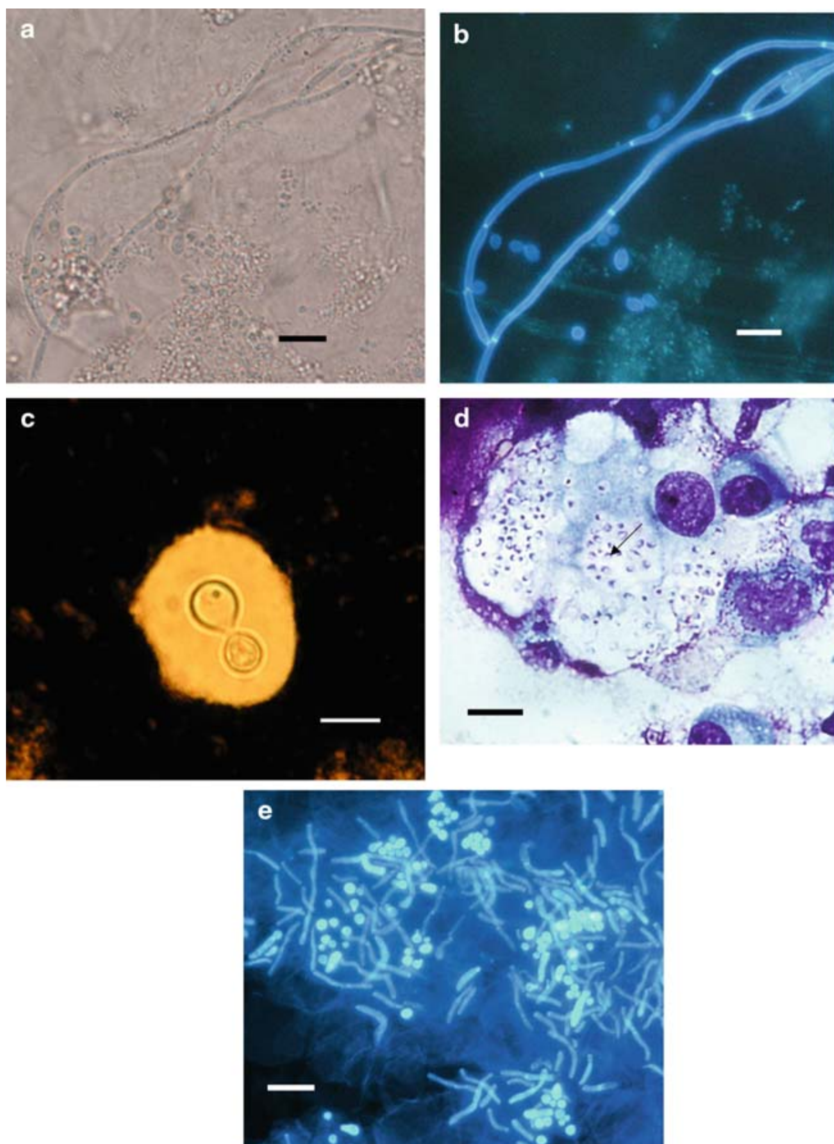

Fig. 13.1 (**a**) Yeast cells and hyphae of *Candida albicans* in an oral rinse specimen seen by light microscopy (*Bar*=10 μm). (**b**) Yeasts and hyphae (from 1a) stained with calcofluor and viewed by fluorescence microscopy (*Bar* = 10 μm). (**c**) *Cryptococcus neoformans* cell in a cerebrospinal fluid specimen, mounted in India ink (*Bar*=5 μm). (**d**) Yeast cells of *Histoplasma capsulatum* (*arrowed*) within blood monocytes in blood smear stained with Geimsa (*Bar*=10 μm). (**e**) Yeast and hyphal cells of *Malassezia* sp. stained with calcofluor by fluorescence microscopy photo M. Copland (*Bar* = 10 μm)

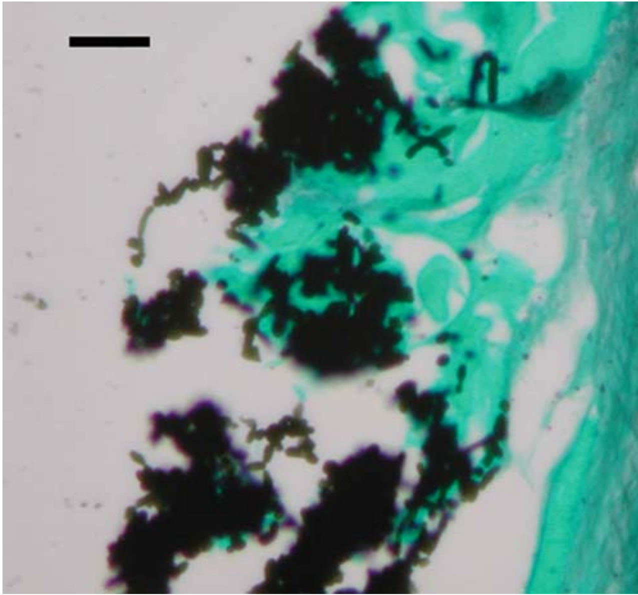

Fig. 13.2 Tissue section of a corneal scrape from a patient with keratitis caused by *Candida parapsilosis*, stained with Grocott's silver and counterstained with *light green* (Bar = 10 μm)

13.2.2 Histopathological Examination

Histopathological examination of tissue may provide the only firm evidence of a yeast infection. Yeasts and in some cases hyphae and pseudohyphae may be noted by histopathologists from routine haematoxylin- and eosin-stained sections but are often easier to see by periodic acid Schiff, or Grocott's silver staining (Elenitsas et al. 2009) (Fig. 13.2). In most cases, yeasts seen in fixed sections are impossible to identify with any degree of certainty; however, the asteroid body formed around *Sporothrix schenkii* yeasts cells in infected tissue is reasonably diagnostic (Daniel de Rosa et al. 2009). Species-specific monoclonal antibody methods for the identification of yeasts in fixed tissues have been reported (Monteagudo et al. 1995) but problems of specificity have prevented their widespread use.

13.3 Blood Culture Systems

For many systemic yeast infections, the isolation of the organism from a blood specimen is the first indication of an infection. Blood culture methods have developed rapidly in the last few decades and many automated blood culture systems are very sophisticated. Most detect the presence of microbial growth through increases

in carbon dioxide by colorimetric, fluorimetric or infrared spectrophotometric means (Richardson and Carlson 2009). Some systems provide blood culture bottles optimised for the detection of yeasts and other fungi such as the BD Systems Mycosis IC/F bottle on the Bactec 9240 machine, and these have been shown to give a higher yield compared to a bacteriological bottle (Meyer et al. 2004). The use of such bottles for patients at risk of systemic yeast infections has been proposed to avoid concomitant bacterial infections that may otherwise reduce the rate of yeasts isolation (Chiarini et al. 2008).

While the culture of blood for the detection of systemic yeast infections, particularly for candidosis, is an easy and convenient approach to diagnosis, the sensitivity of blood culture is regularly questioned with a maximum figure of 50% often quoted (e.g. Kullberg and Filler 2002). However, the accurate determination of the sensitivity of blood culture to detect systemic yeast infections is an extremely difficult exercise. The invasive sampling of blood culture-negative patients or a series of autopsies (which are themselves inherently biased) is required in order to determine if the blood culture result was a true or false negative result. There are relatively few reports of this nature in the literature, and those that are present are often relatively old (Pizzo and Walsh 1990) and/or make use of methods such as lysis centrifugation that are not currently widely used (Berenguer et al. 1993). More recently, in an autopsy series of 720 haematology patients the sensitivity of blood culture for the diagnosis of systemic candidosis was estimated to be 21.3% (Kami et al. 2002). While this was a careful analysis of this issue a significant proportion of the blood culture testing was carried out using a manual system. Recent developments in blood culture methodology and the use of automated blood culture systems may make the estimates of sensitivity inaccurate. Furthermore, from the list of the organs involved in patients with invasive candidosis, which were listed independently, the lung was listed as the site of infection in 67% of cases. It was not possible to identify cases where the lung was the only site of infection. Distinguishing between local pulmonary invasion shortly after death and a genuine infection is likely to be difficult.

A different approach to examine the sensitivity of blood culture for systemic yeast infections is to look at the results of other specific tests such as PCR-based tests to detect, for example, *Candida* DNA from blood samples. For example, it has been shown in one study that all twelve candidaemic patients were PCR-positive for *Candida* spp., but nine of 16 blood culture-negative patients suspected of having a systemic candidosis were positive by PCR (Ahmad et al. 2002). However, it remains to be proven whether this DNAemia is indicative of a systemic infection or perhaps also sometimes a reflection of extensive superficial yeast growth. Recently, a similar study found that PCR in serum extracts was exactly as sensitive and specific as culture for the detection of candidaemia (Metwally et al. 2008).

The analytical sensitivity of the most widely used blood culture systems has been well defined, and in simulated candidaemia as few as 10 colony-forming units per bottle could be readily detected for most yeast species tested (George et al. 2005), with little difference in the detection rate between blood culture systems (Horvath et al. 2004). Most yeasts grow best aerobically but interestingly

C. glabrata appears to grow better in anaerobic blood culture bottles (George et al. 2005; Horvath et al. 2004). However, this appears to relate more to the composition of the media in the bottles than to the presence of anaerobic atmosphere (Foster et al. 2007).

13.4 Culture Media

Most yeasts grow on a wide variety of media including those not designed for fungal culture. Sabouraud's agar is used routinely in most clinical laboratories (Odds 1991). Growth of *C. albicans* on chocolate (heated blood) agar produces a characteristic filamentous colony in an atmosphere of 5–6% CO_2 (Sheth et al. 2009); (Barnes and Vale 2005). The identity of some other species can be guessed from colony morphology on Sabouraud's agar: *Cryptococcus* spp. are frequently somewhat mucoid, whilst those of *Candida krusei* are flat and dry.

Chromogenic and fluorogenic agars are frequently used to detect mixtures of yeasts, and to make presumptive identifications, they incorporate compounds which when hydrolysed by certain enzymes produce coloured or fluorescent products respectively (Freydiere et al. 2001). The enzyme β-galactosaminidase is produced by *C. albicans*, and substrates consisting of coloured and fluorescent moieties linked to galactosamine have been used in agars to differentiate *C. albicans* from other yeast species. Fluorogenic agars require a UV lamp to observe the fluorescence and the fluorescence also tends to diffuse into the agar and make the determination of mixed cultures difficult (Freydiere et al. 2001). Chromogenic agars range from those designed to differentiate *C. albicans* from other yeasts (Lipperheide et al. 2009) to media designed to presumptively identify a small number of clinically important species (Odds and Bernaerts 1994; Eraso et al. 2006). The emphasis on a presumptive identification is important as the range of species-specific colony colours is typically small. Furthermore, there are often atypical reactions such as colourless isolates of *C. glabrata*, and white colonies are also seen in the closely related *C. nivariensis* and *C. bracarensis* (Bishop et al. 2008a). Four cases of *C. albicans* growing as a pink colony rather than the typical blue-green on Chromagar have also been reported (Saunte et al. 2005). Assessment of colony colour is inherently subjective and also time- and temperature-dependent (Odds and Davidson 2000). Ultimately other methods for the definitive identification of yeasts are required (see below).

The role of chromogenic agars for the primary isolation of yeasts in the clinical mycology laboratory is unclear. It has been suggested that Chromagar and Sabouraud's agar have similar rates for the efficiency of *Candida* spp. isolation (Willinger and Manafi 1999). However, in a larger study of 3,296 specimens (Murray et al. 2005), 164 specimens yielding yeasts on routine media (Sabouraud's or brain heart infusion agar) did not result in yeast growth on CHROMagar Candida agar, including 13 blood culture specimens. This was comparable to 91 specimens that grew yeasts on the chromogenic agar and not on routine media. This suggests that

the improvement in time to presumptive identification is balanced by a reduction in culture yield on at least some chomogenic agars, particularly in specimens where the numbers of yeasts tends to be low. Some authors have proposed an algorithm for the most cost-effective use of chromogenic agars (Ainscough and Kibbler 1998).

Another use of chromogenic agars is in the detection of mixtures of yeasts. Many yeasts species are indistinguishable on routine mycology media (Murray et al. 2005; Eraso et al. 2006) though the extent to which mixtures can be detected depends on the agar used (Letscher-Bru et al. 2002); (Eraso et al. 2006).

13.5 Methods for Identification of Yeasts

13.5.1 The Germ Tube Test

In contrast to moulds, many yeast species are morphologically indistinguishable at the macroscopic and microscopic level under routine growth conditions. Thus, identification of yeasts requires additional approaches. Since *C. albicans* still remains the most commonly isolated yeast in the clinical microbiology laboratory, tests to screen out *C. albicans* from other yeasts are popular. The best known of these tests is the germ tube test first described in 1960 in order to identify *C. albicans* (Taschdjian et al. 1960). The inoculation of mammalian serum (the precise source appears to be relatively unimportant; (Hilmioglu et al. 2007)) with yeast cells and incubation for 1.5–2 h at 37°C will result in the yeast cells of the vast majority of isolates of *C. albicans* producing the characteristic hyphal outgrowth, the germ tube (Fig. 13.3a). Detailed analysis of this reaction has determined that D-glucose plays an important part in the induction of germ tubes in *C. albicans* (confirming much earlier observations) but that there is also a non-dialysable component of serum that is involved in induction (Holmes and Shepherd 1988; Hudson et al. 2004). Since the first report of this method, a variety of methods have been described to modify the germ tube test including modified Sabouraud broth (Odds and Evans 1975), the use of N-acetyl glucosoamine (Cassone et al. 1985), proline (Holmes and Shepherd 1987) or other defined media to replace serum (Lee et al. 1975) and the use of 39°C to enhance specificity (Kim et al. 2002). The continued popularity of the germ tube test relates to its low cost, speed and relative ease of use, although it has been calculated that where laboratory staff time is expensive chromogenic agars may provide cost savings (Freydiere et al. 2001). A novel microtitre plate format has been proposed to improve throughput (Peltroche-Llacsahuanga et al. 1999). The germ tube test is traditionally applied to yeasts isolated on agar plates but it has been shown that the test can usefully be applied directly to samples from blood culture bottles where a yeast has been seen in a gram stain (Sheppard et al. 2008). The interpretation of the microscopy does require a certain level of technical skill, though an audit of the germ tube test in Taiwanese hospitals suggested a sensitivity and specificity of 95 and 98.6% respectively (Lo et al. 2001). False positives can occur with *C. tropicalis* (Martin 1979)

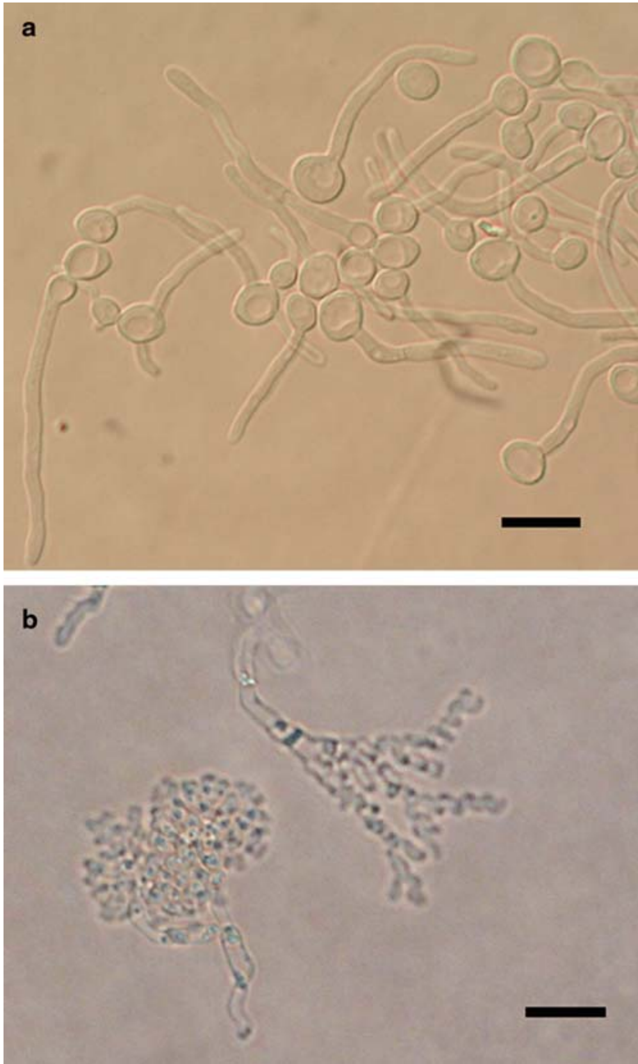

Fig. 13.3 (**a**) Germ tubes produced by *Candida albicans* (**b**) Appressoria formed by *Trichosporon inkin*. (Bar = 10 μm)

though in practise this tends to happen very rarely and only after long incubation periods (Hilmioglu et al. 2007).

There were reports in the 1980s of *C. albicans* isolates that were germ tube-negative. Notably some reports showed that some non-germinative strains produced larger numbers of chlamydospores, with round, thick-walled spore forms seen only in *Candida albicans* growing on certain media (see below) compared to typical *C. albicans* isolates (Torosantucci and Cassone 1983). *Candida dubliniensis* is a yeast closely related to *C. albicans* found initially in the human oral mucosa that

was described by Sullivan *et al*. (Sullivan et al. 1995). In this first report, all isolates of *C. dubliniensis* were germ tube-positive, but later studies showed that this species has a reduced capacity to produce germ tubes (Davis et al. 2005). As *C. dubliniensis* is notable for increased chlamydospore production, the non-germinative variants of *C. albicans* seen earlier may have been *C. dubliniensis*. A study of the quantitative ability of *C. albicans* yeast cells to form germ tubes from oral isolates suggested that while all isolates were positive for this reaction, germ tube production was reduced in isolates from patients with denture-related stomatitis (Pinto et al. 2008).

13.5.2 Chlamydospores, Hyphae and Pseudohyphae

Growth on agar using a combination of a defined media such as Czapek Dox, or a complex media such as Cornmeal, together with the detergent Tween 80 is used as a standard inducer of a variety of morphological forms for the identification of clinically important yeasts (Odds 1988). Using such agars and inoculating yeasts by the Dalmau technique (under a sterile glass coverslip to create a gradient of oxygen tension), chlamydospores: refractile, thick-walled round cells at the end of short hyphae or pseudohyphae, are produced by *C. albicans* and *C. dubliniensis* (reviewed by Staib and Morschhauser 2007). A simple method of differentiation of *C. albicans* and *C. dubliniensis* can be achieved by growth on Staib or Nigerseed (*Guizotia abyssinica*) agar (Staib and Morschhauser 1999) which stimulates the production of hyphae and chlamydospores in *C. dubliniensis* but not *C. albicans*. A number of reports have described alternatives to Nigerseed agar often including reagents that are locally available such as rosemary and oregano extract (de Loreto et al. 2008), tobacco (Khan et al. 2004) and tomato juice (Alves et al. 2006). A combination of Pal's medium (sunflower seed agar) with Chromagar created a medium with the advantages of Chromagar that could also differentiate *C. albicans* and *C. dubliniensis* (Sahand et al. 2005).

Agars such as Czapek Dox or Cornmeal with Tween 80 are used to induce hyphal and pseudohyphal production in other yeasts and are also often used in the identification process. The distinction between pseudohyphae and hyphae is a function of the extent to which parallel-sided hyphae and septa without constrictions in the hyphal compartment are observed (Merson-Davies and Odds 1989). In practise, it is often only necessary to score for the presence of pseudohyphae or hyphae and not differentiate between the two in many tests (Milan et al. 1997). Species of *Candida* such as *C. albicans, C. tropicalis* and *C. lipolytica* will often produce true hyphae on Czapek Dox or Cornmeal agar whilst many other species such as *C. parapsilsosis* and *C. krusei* produce pseudohyphae (Campbell et al. 1996). Notably some species do not produce any filamentous forms and this is an important aspect of their identification. The most important example of this is *C. glabrata*, which does not produce hyphae or pseudohyphae agars such as Czapek Dox-Tween 20 (Odds et al. 1997). Although, pseudohyphal growth can be induced in *C. glabrata* by promoting colony morphology switching on copper

sulphate-containing medium (Lachke et al. 2002), a lack of filamentation can be reliably used on media used in the clinical laboratory to aid the identification of this species. Another species characterised by a lack of filamentation is *Candida famata* (*Debaryomyces hansenii)*, though unfortunately it is not a useful method to differentiate this species from the phenotypically similar *C. guilliermondii,* as the latter may or may not have the ability to form pseudohyphae (Desnos-Ollivier et al. 2008). Rice agar Tween (RAT) medium was shown to be unreliable for inducing pseudohyphal production isolates of *C. inconspicua*, thus allowing these yeasts to be easily confused with *C. norvegenesis* (Majoros et al. 2003).

Many species of *Cryptococcus* including *Cr. laurentii* and *albidus* are unable to produce hyphal or pseudohyphal forms under most conditions, although *Cr. laurentii* can be induced to form hyphae in vitro (Kurtzman 1973). Similarly, *C. neoformans* grows as a yeast under most conditions but, at mating, dikaryotic hyphae form in the telmorphic state *Filobasidiella neoformans* (Erke 1976). Yeasts in the genus *Malassezia* grow almost exclusively as yeasts in vitro and it is difficult to convert them to the hyphal forms seen in vivo in cases of pityriasis versicolor (Saadatzadeh et al. 2001).

Some yeasts tend to grow in vitro as mixtures of hyphae and yeasts, including the arthrosporic yeasts in the genera *Trichosporon* and *Geotrichum*. Identification of *Trichosporon* species is aided by scoring for the presence of arthroconidia and appressoria (an irregularly branched attachment structure, Fig. 13.3b) (Gueho et al. 1994).

13.5.3 Capsule Formation

A capsule is produced by *Cr. neoformans* (and the recently separated *C. gatti*) during growth in vivo during an infection, particularly meningitis (Fig. 13.1c). In culture, *C. neoformans* loses the capsule rapidly in many isolates associated with the loss of the mucoid appearance of the colony. Capsule production in vitro is regulated in part by osmolarity, with high osmotic concentrations in the media inhibiting capsule production (Dykstra et al. 1977) and by growing *C. neoformans* under conditions of physiological carbon dioxide and iron limitation (Vartivarian et al. 1993). In the diagnostic laboratory it may be difficult to detect the presence of a capsule in many *C. neoformans* isolates. It is also important to note that non- or poorly capsulated strains of *C. neoformans* have been reported (Sugiura et al. 2005). Capsules are formed by other *Cryptococcus* species including *Cr. laurentii* and *Cr. albidus*, and by *Rhodotorula* species.

13.5.4 Structures Associated with Sexual Reproduction in Yeasts

Many clinically important yeasts are classified as Ascomycota and some diploid strains may produce asci under certain conditions. The ascospores of many yeasts

are round and unremarkable as in *Saccharomyces*. However, the ascospores of *Debaryomyces hansennii* (the perfect state of *Candida famata*) are ornamented (Kurtzman et al. 1975). The ascospores of several *Pichia* species including the clinically relevant *P. anomala* are hat-shaped (Yamada et al. 1994).

13.5.5 Assimilation and Fermentation in Yeasts

While morphological approaches to the identification of yeasts are often useful, the recording of patterns of assimilation and sometimes fermentation in panels of different carbon compounds has formed the mainstay of yeast identification for many years. Fermentation assays require the observation of gas production seen in a Durham tube. Because of their high technical complexity and also the general lack of fermentation activity of many yeasts compared to assimilation activity (Yarrow 1998), fermentation tests are infrequently used in diagnostic laboratories. Assimilation of carbon compounds is much more widely used and varies in terms of the number of different compounds tested and the format. The original format for testing the assimilation of carbon compounds was the inoculation of tubes of liquid media and assaying of growth visually described by (Wickerham and Burton 1948) and this approach often bears the former scientist's name (Wickerham tubes). Using a yeast nitrogen base broth supplemented with 0.5% of the carbon compound to be tested, tubes are inoculated with yeasts and incubated with or without shaking for 3–4 weeks (Yarrow 1998). Alternative approaches are to incorporate the carbon compounds into an agar plate and inoculate the surface with the yeast to be tested and score for growth (Shifrine et al. 1954) or to inoculate the agar plate with the yeast and place solid sugars (Beijerinck 1889) or paper discs soaked in sugar solutions on the agar surface and to score for growth around the carbon source. Nowadays, this kind of testing is done almost exclusively using commercially prepared manual or automated products and systems (see Sect 19.5.9). The assimilation of nitrogen compounds can be similarly assessed in liquid or solid media using a carbon base (Yarrow 1998). Differences in the assimilation of nitrate and nitrite are often important in the differentiation of the yeast *Exophiala dermatitidis* from other *Exophiala* spp. (de Hoog and Haase 1993).

13.5.6 Other Physiological Tests

The normal human body temperature of 37°C is often used for routine incubations in the diagnostic laboratory with the view that only pathogenic yeasts will grow at this temperature. However, most *Malassezia* species grow optimally at 33°C relating to their natural habitat on the skin surface (Leeming and Notman 1987). For some species such as *Candida zeylanoides* weak or no growth at 37°C is characteristic for the species (Meyer et al. 1998). Conversely, it has been suggested

that *C. dubliniensis* can be differentiated from *C. albicans* by the inability of *C. dubliniensis* to grow at 45°C at least as a screening test, though some *C. albicans* isolates are also unable to grow at 45°C (Gales et al. 1999). Another phyisiological test which has been used to distinguish *C. albicans* and *C. dubliniensis* is the inability of the latter species to grow in Sabouraud's broth supplemented with 6.5% sodium chloride (Alves et al. 2002). The hydrolysis of urea is a useful test that typically divides yeasts in the subphylum Ascomycota from those in the subphylum Basidiomycota. The few exceptions to this are not significant pathogens (Hagler and Ahearn 1981). Urea hydrolysis can be conveniently tested for using the bacteriological Christensen agar where the production of ammonia causes an indicator to change colour. Differences in the ability of some yeasts to break down fats are used in the identification of *Malassezia* species by observing growth on media where Tween 20, 40, 60 and 80 are the sole lipid source (Gueho et al. 1996). The diazonium blue B colour reaction with yeast cells can also be used to distinguish between yeasts from the Ascomycota (negative) and Basiodiomycota (positive) (Van Der Walt and Hopsu-Havu 1976) but this is rarely used in the routine identification of yeasts. Yeast species differ in resistance to cycloheximide, an observation first made by Whiffen (Whiffen 1948). Growth in the presence of 0.01% cycloheximide varies greatly between members of the genus *Candida*, for example (Meyer et al. 1998). The importance of cycloheximide resistance for the identification of clinically important yeasts is indicated by the inclusion of a cycloheximide plate in an in-house multipoint inoculation approach to identification (Tambosis et al. 2003). Some growth-based approaches to the identification of yeasts involve assaying a combination of substrate utilisation and resistance to an antimicrobial. The best known example of this is the use of canavanine-glycine-bromothymol blue (CGB) agar. This was originally used to differentiate the varieties of *C. neoformans*. *C. neoformans* var *neoformans* are generally sensitive to canavanine and/or do not hydrolyse glycine; thus the media stays green. *C. neoformans* var *gattii* is resistant to canvanine and on hydrolysing glycine generates ammonia that turns the indicator blue (Kwon-Chung et al. 1982). Glycine, cycloheximide phenol red agar works in a similar way to differentiate the two varieties of *C. neoformans* (Salkin and Hurd 1982). More recently, *C. neoformans* var *gattii* isolates were considered sufficiently distinct to form a separate species, *C. gattii* (Kwon-Chung and Varma 2006), and *C. gattii* has been identified as an important emerging pathogen in Northwest USA and Canada (Hoang et al. 2004). It has been suggested that compounds to inhibit growth are not necessary and it is sufficient to use media containing tryptophan and proline as sole nitrogen sources to differentiate *C. gattii*, which can utilise this nitrogen source, and *C. neoformans*, which cannot (Chaskes et al. 2008). Both *C. neoformans* and *C. gattii* can produce melanin when grown on Niger seed agar and certain other media due to the production of phenol oxidase, and form brown colonies, which is a useful approach to screening for these two species (Staib et al. 1989). The Microring YT system makes use of differences in the sensitivity of different species of yeasts to distinguish six different compounds, though this has been shown to have a relatively poor accuracy (52.8% in Shankland et al. 1990).

13.5.7 Enzyme-Based Yeast Identification Systems

The detection of the presence of different enzymes characteristic of a yeast species can be carried out on yeasts from an isolated colony for a rapid identification system. A number of different methods have been described for the detection of *C. albicans*, specifically through the detection of β-galactosaminidase and L-proline aminopeptidase activity (Freydiere et al. 2001). Typically, cells of the yeast to be tested are mixed with substrates, and where the relevant enzyme activity is present fluorescent and/or coloured products are produced. *C. tropicalis* isolates may have β-galactosaminidase but will be negative for L-proline aminopeptidase (Freydiere et al. 2001). Many of these methods compare well with a germ tube test with sensitivities of typically 99% and specificities in some cases of 100% for the identification of *C. albicans* (Crist et al. 1996; Carillo-Munoz et al. 2009), often leaving cost as a deciding factor. Others have targeted *C. glabrata* as the species next most prevalent in the diagnostic laboratory and make use of its ability to rapidly assimilate and/or ferment trehalose in the presence of cycloheximide within a short time period to identify this species (Land et al. 1996). Using a commercial assimilation broth (Remel Rapid Trehalose Assimilation Broth) a sensitivity of 91% and specificity of 96% at a cost significantly less than the standard API20C have been obtained by some authors (Fenn et al. 1999). A recently developed method makes use of a glucose oxidase reaction to detect assimilation of trehalose by *C. glabrata* in 20 min using maltose as a negative control (GLABRATA RTT, Fumouze Diagnostics); this method achieved increased sensitivity though it was somewhat dependent on the media from which the yeast cells were taken (Freydiere et al. 2003).

Others have developed the enzymic approach to identify multiple species. The Fongiscreen test (Sanofi Diagnostics Pasteur) used seven biochemical tests in order to detect four species (*C. albicans*, *C. tropicalis, C. glabrata* and *C. neoformans*). However, some authors found significant proportions of *C. glabrata* and *C. tropicalis* isolates were misidentified (Hoppe and Frey 1999) and the product is now no longer available. The RapID Yeast Plus system (Innovative Diagnostic Systems) uses 18 different reactions with a combination of assimilation and chromogenic reactions scored after 4–5 h and was first evaluated in 1996. The RapID test correctly identified 94% of 304 yeast isolates while others were either recognised as being identified with a low probability or were not on the systems database (Kitch et al. 1996). However, others found that while common *Candida* species were accurately identified (95.7%), the test performed less reliably with rarer *Candida* species and other yeast genera with accuracies of 75–79% (Espinel-Ingroff et al. 1998). This system continues to be used and more recent assessments of accuracy continue to be high (95.7% in (Sanguinetti et al. 2007)). An automated 4 h yeast identification system using 27 chromogenic substrates (Rapid Yeast Identification Panel, Dade Microscan) showed early promise with identification accuracy of 85% in one study (Land et al. 1991), but later studies were only able to identify 67% of yeasts without additional tests (Riddle et al. 1994) and there is little evidence of more recent evaluations.

13.5.8 *Commercial Yeast Assimilation Panels*

The mainstay of yeast identification in most clinical microbiology laboratories are commercial assimilation-based panels requiring at least 24-h incubations with either manual or automated systems of inoculation and reading. These vary in terms of the individual tests, the number of species in the database, incubation temperature, the need for additional tests and the identification of a positive reaction (turbidity or colour change; Freydiere et al. 2001). The API series of panels, and specifically the API20C (Biormerieux) with 19 tests, are often regarded as a reference standard for yeast identification (Fenn et al. 1994) and typically have accuracies of 85–95% (Freydiere et al. 2001). Biomerieux also supply a 31 test panel API32C with a larger database and the capacity for automated reading. Both API 20 and 32C are read by visual inspection of turbidity. A panel using just 10 reactions (API Candida) but with a colorimetric reading proved more accurate than both the API 20 and 32C (Verweij et al. 1999). Interestingly, the other colorimetric panel, the Auxacolor (Sanofi-Pasteur/Biorad), provided an even higher level of accuracy. Others noted that not only was Auxacolor more accurate, but that there was a higher degree of inter-observer variability in scoring results with API20C compared to Auxacolor, attributing this to the difference between assessing turbidity and a colour reaction (Sheppard et al. 1998). However, recent reports of comparisons of yeast identification methods indicate that the API 20C performs well compared to other phenotypic identification systems (Liguori et al. 2009) but not with molecular methods (Putignani et al. 2008).

Many laboratories are turning to automation in order to improve efficiency and make cost savings. The Vitek YBC and Vitek 2 systems (Biomerieux Vitek) are fully automated identification sytems with specific cards for yeasts and databases of 16 (Vitek YBC) or 51 species (Vitek 2) (Freydiere et al. 2001). Early comparisons of the Vitek YBC system using the API 20C as a reference method gave slightly disappointing results with an accuracy of 83% (Pfaller et al. 1988). Following improvements to the database and extending the incubation, a subsequent study was able to correctly identify 89.7% of 406 yeasts using the Vitek YBC (Fenn et al. 1994). The first evaluation of the Vitek 2 system using the ID-YST card for the identification of yeasts with API 32C as a reference method was reported in 2000 (Graf et al. 2000) and found that 87% of all yeasts were correctly identified by the Vitek 2, 8.7% were identified to low discrimination, 1.7% misidentified and 2.1% could not be identified. It was suggested that the Vitek 2 was a valid approach to identification in the diagnostic laboratory, with improved time to result from 48 to 15 h compared to API 32C (Graf et al. 2000). However, in a later study only 41 of 61 yeasts (67%) were fully identified, with particular problems in identifying *C. glabrata* (Massonet et al. 2004). A study of a large number of yeasts (750) typically found in a diagnostic laboratory and using rRNA sequence as a reference standard found that 98.2% were fully and correctly identified including many isolates of *C. glabrata* (Sanguinetti et al. 2007). There is still some disagreement as to whether the new colorimetric technology in the

Vitek 2 yeast cards is an improvement over the older fluorimetric cards (Aubertine et al. 2006; Loiez et al. 2006).

13.5.9 Immunological Identification of Yeast Isolates

Sera that agglutinate the cells of different species of yeasts have been described as a method of yeast identification (Shinoda et al. 1981) though this approach suffers from a lack of specificity and limited range of species that can be identified. Latex particles coated with monoclonal antibodies specific for *C. albicans* and *C. krusei* that agglutinate in the presence of the relevant species represent a very rapid technique, and this approach has been shown to have high sensitivity and specificity on colonies (Freydiere et al. 1997; Quindos et al. 1997). Furthermore, the Bichro-latex Albicans (Fumouze Diagnostics) method for the identification of *C. albicans* can also be applied to blood culture bottles prior to subculture (Laurent et al. 1996). The Krusei Color (Fumouze Diagnostics) test is not widely used due to the relatively small numbers of *C. krusei* isolates seen in the clinical laboratory.

13.5.10 Molecular Identification of Yeast Isolates

Within approximately the last 10 years it has become clear that DNA-based or molecular methods for the identification of fungi are, or should be considered, the gold standard against which all phenotypic methods should be compared (Borman et al. 2008). DNA sequence differences are typically more stable than phenotypic differences and can vary between isolates and depend on environmental conditions including growth medium (Odds 1991; Freydiere et al. 2003). The fine level of detail that can be obtained by molecular methods has unearthed new species amongst previously apparently homogeneous species. For example, *C. bracariensis* was previously often identified as *C. glabrata* and these two species cannot be distinguished reliably by the API 20C ((Bishop et al. 2008b)). *Candida dubliniensis* was originally identified as a group of *C. albicans* isolates with atypical patterns of hybridisation to a DNA probe (Sullivan et al. 1995). Conversely, *C. africana* (Tietz et al. 2001) and *C. claussenii* (Mahrous et al. 1992) are two taxa formerly thought to be separate species but closely related to *C. albicans* which tend to produce germ tubes to a lesser extent. However, recent multi-locus sequence typing analysis has indicated that they are in fact variants of *C. albicans* (Jacobsen et al. 2008).

Various differences in the DNA sequence of yeasts beween species have been exploited to effect identification. These range from the large-scale chromosomal size differences (Monod et al. 1990), relatively crude random amplified polymorphic DNA analyses (Lehmann et al. 1992) and restriction fragment length polymorphisms (RFLPs) in genomic digests (Magee et al. 1987), size differences in

polymerase chain reaction (PCR) products (Turenne et al. 1999), RFLP analysis of PCR products (Williams et al. 1995), probe hybridisation to PCR products in real-time PCR (Guiver et al. 2001), species-specific PCR (Mannarelli and Kurtzman 1998), reverse line hybridisation (Xiang et al. 2007) and microarray identification of PCR products (Leinberger et al. 2005).

However, many studies make use of the sequence analysis of PCR-amplified sections of DNA, particularly the rRNA genes (Linton et al. 2007) intervening transcribed spacers (ITS) (Leaw et al. 2007) or occasionally intergenic spacers (IGS) (Sugita et al. 2002) (Table 13.1). DNA is relatively easy to extract from yeasts (e.g Borman et al. 2006) and sequencing relatively inexpensive. There are large numbers of depositions of rRNA gene and spacer sequences in the DNA sequence databases such as Genbank that make searching by basic local alignment search tool (BLAST) (Altschul et al. 1990) easy particularly for the D1/D region of the large subunit rRNA gene 26S(Linton et al. 2007). BLAST output compares the sequence used for the search against other DNA sequences in the database and produces a number of parameters including a bit score, a probability that similarity occurs by chance (E value) and % similarity (Altschul et al. 1990). The key question is how different do two sequences need to be before two isolates should be considered different species. A study of a large number of yeasts showed that strains of the same species have two or fewer nucleotide differences in the c. 600 bp D1/D2 region of the 26S rRNA gene of the yeasts examined (Kurtzman and Robnett 1997). Different species tended to have $>1\%$ nucleotide differences in this region (Fig. 13.4). Similarly, Sugita et al. (1999) found strains of the same species of *Trichosporon* that had $>98.9\%$ sequence similarity in the ITS1/2 region for four different yeast species. Analysis of the phenotypically similar and problematic species *D. hansenii (C. famata)* and *Pichia* (*Candida*) *guilliermondii* tended to corroborate this finding (Desnos-Ollivier et al. 2008). A further study compared the RapID phenotypic method and the 25S rRNA D2 sequence using a cut-off of 2% and found discrepancies in 11 of 53 of the yeasts, with particular problems in isolates phenotypically identified as *C. krusei* and *C. glabrata* (Putignani et al. 2008). It is an indication of the currently acknowledged superiority of sequence-based identification that all discrepancies were considered errors in the phenotypic method. Increasingly, molecular sequence analysis is used to define new species as in the splitting of the *C. parapsilosis* to *C. parapsilosis* sensu stricto and *C. orthopsilosis* and *C. metapsilosis* (Tavanti et al. 2005). Publications in many journals mandate the submission of sequence data to a public database such as Genbank. However, these databases are not checked for the accuracy of the identity of the organisms from which the sequence was derived. Studies of the accuracy of the species assignments of deposited sequences in Genbank have suggested that 14–20% of fungal entries were from incorrectly identified fungi (de Hoog and Horre 2002; Nilsson et al. 2009). Interpretation of results is also complicated by the persistence in the database of obsolete names and the presence of fungi with sexual states with multiple names (Balajee et al. 2007).

Some fungi resist molecular identification by sequencing and there is a need to clone the PCR product and occasionally no PCR product can be obtained

Table 13.1 Comparisons of yeast identification by phenotypic and DNA sequence analysis

Reference	No. of yeasts	Target	Phenotypic method	Phenotypic accuracy (%)	Comments
Putignani et al. (2008)	13 reference 40 clinical	25S rRNA D2	RapID	72.5 (amongst clinical isolates)	
Sanguinetti et al. (2007)	750 clinical isolates	ITS1/2	RapID	95.5	
Page et al. (2006)	88 clinical isolates	25S rRNA D1/D2	GT, fermentation assays, urea, cornmeal	92	A flow cytometric method identified 79% of isolates and 100% of all isolates for probes that were available
Leaw et al. (2006)	299 reference 74 clinical	ITS1/2	Various traditional methods	81	Discrepancies were due to phenotypic methods as confirmed by 25S rRNA D1/D2 sequence analysis
Chen et al. (2000)	33 reference 401 Clinical	ITS1/2	API 20C/Vitek	98.5	ITS2 Product length also analysed
Chen et al. (2001)	27 unusual clinical isolates	ITS1/2 25S rRNA	API/Vitek	74	ITS1 Product length also analysed

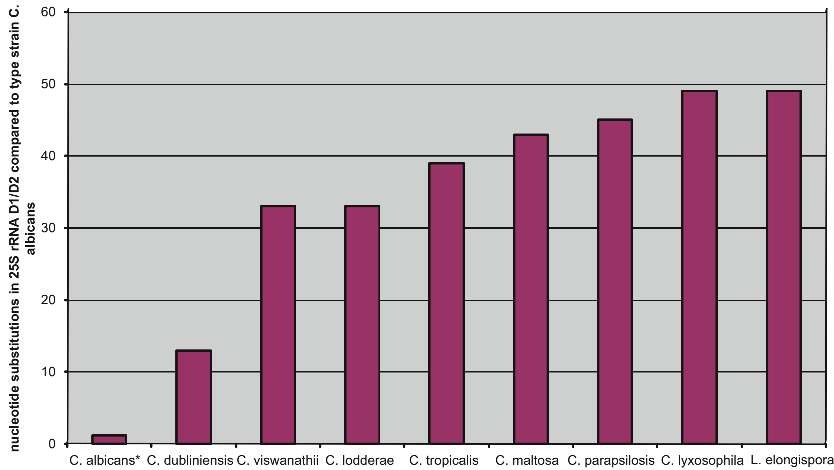

Fig. 13.4 Nucleotide substitutions in eight different species of *Candida* or *Lodderomyces* in the 25S rRNA D1/D2 region compared to type strain of *Candida albicans*.* Six different isolates of *C. albicans* were compared (from Kurtzman and Robnett 1997)

(Summerbell et al. 2005). The reasons for these difficulties in many cases remain unclear though the inhibitory effects of fungal pigment chemicals or other compounds is suspected though not proven.

Other genes and sequences such as the actin gene (Daniel et al. 2001) or analysis of multiple genes have been reported to be required for comprehensive taxonomic analysis (Taylor et al. 2000; Tsui et al. 2008), but this level of analysis will be beyond routine identification in the diagnostic laboratory.

13.5.11 Peptide Nucleic Acids

Peptide nucleic acids (PNA) are molecules with a peptide backbone but with bases attached which hybridise to nucleic acids (Egholm et al. 1993). Their hydrophobic nature allows PNA molecules to diffuse into undisrupted cells. Using a fluorescent tag, PNA fluorescent in situ hybridisation (FISH) is a method that has been used to differentiate between *C. albicans* and *C. dubliniensis* (Oliveira et al. 2001) and to determine that 2% of a collection of *C. glabrata* isolates were in fact *C. bracarensis* (Bishop et al. 2008b). Using dual labelled PNA FISH probes for *C. albicans* and *C. glabrata* has enabled some authors to rapidly identify the two most important yeasts from blood culture bottles in less than 3 h (Shepard et al. 2008). Others using five different PNA FISH probes showed that specificity was excellent; for example, a *C. albicans* probe correctly hybridised with 32 of 33 isolates of *C. albicans* and in 28 other species only one isolate (*C. parapsilosis*) was positive (Reller et al. 2007).

13.6 Algorithms for Identification

The approaches taken for the identification of an unknown yeast isolated in a medical laboratory generally take two forms. PCR array-based systems or automated phenotypic systems such as the Vitek 2 or RapID systems can be used to identify almost all yeast isolates. The small number that cannot be identified by the system chosen would require a molecular identification approach. While the disadvantage of this is its high cost, it enables all isolates to be subject to a standardised identification regime. Alternatively, one or more "screening" steps can be incorporated such as the use of germ tube testing to identify *C. albicans* and *C. dubliniensis* or chromogenic agar to identify a small number of common species. This may be followed by phenotypic methods such as Auxacolor or API20C while a small number of yeasts may require further examination, which currently will almost always be some form of DNA sequence analysis. It has been suggested that to balance cost-effectiveness and accuracy an algorithm combining the best of phenotypic and molecular methods is optimal (Ciardo et al. 2006).

13.7 Non-Culture-Based Methods of Laboratory Diagnosis

The isolation of a yeast from a clinical specimen in order to make a diagnosis of infection has many advantages including ease of further analysis such as antifungal sensitivity testing and detailed molecular analysis for validation of identification or sub-species strain typing. However, non-culture-based methods of laboratory diagnosis may provide valuable adjunctive or in some cases alternative approaches. While it is beyond the scope of this chapter to comprehensively survey this subject, this section will outline some of the current thinking in this area.

It has been recognised for many years that the pathological consequences of infection are often a function of the host's response to the pathogen. Hepatosplenic or chronic candidiasis is now recognised as an immune reconstitution syndrome reflecting an immunological response to *Candida* cells that are probably no longer viable (Dupont 2008) and where culture-based methods for diagnosis will be unproductive. For many years antibody responses to candidosis were pursued as a key diagnostic target, but after much effort they were largely abandoned due to the problems of a lack of specificity. However, in recent years there has been a resurgence of interest in antibody tests and a recent study has shown that using selected recombinant purified *C. albicans* antigens, IgG responses may provide a sensitive and timely indication of systemic *Candida* infection including non-*albicans* species (Clancy et al. 2008). In cryptococcosis, antigen detection provides the main diagnostic approach (see below), but antibody detection has been shown to be of value in identifying patients with subclinical infections that may reactivate on solid organ transplantation (Saha et al. 2007). Detection of the *C. neoformans* capsule antigen is a highly effective test using either a latex agglutination or ELISA methodology. Recent data from a large French study reported that in HIV-positive patients sensitivities of the cryptococcal

antigen test in serum were 95.2% (compared to culture 88.6%) and even higher in serum and cerebrospinal fluid in patients with meningoencephalitis (Dromer et al. 2007). There are no similarly useful *Candida* antigen tests available, though examination of sera for the presence of mannan (Platelia Mannan, Biorad) has been found to be a potential biomarker for infection, particularly if combined with detection of an anti-mannan antibody (Sendid et al. 2002). A comparison of the Biorad mannan test with a different mannan assay (Candida Monotest, Unikita) found the latter to be superior with a per patient sensitivity of 82% (Fujita et al. 2006). There is growing interest in 1-3-β-D-glucan (BDG) as a biomarker for invasive fungal infections including yeast infections. Detection is based on the *Limulus* coagulation cascade and has a high analytical sensitivity. A recent study found that 13 out of 15 patients who grew yeasts in blood culture were positive for BDG (Pickering et al. 2005) and others found this test more sensitive than two different mannan detection methods (Fujita et al. 2006). Recent data show the BDG test to achieve similar sensitivities compared to *Candida* blood culture, but interestingly also show that elevated levels of BDG can be found in patients with disseminated cryptococcosis – a somewhat unexpected result as there are much lower levels of BDG in the *C. neoformans* cell (Obayashi et al. 2008). One disadvantage of the above approaches is the limited information results yield on the precise aetiology of a yeast infection. The molecular diagnosis of yeast infections can provide both a sensitive diagnostic tool, with the potential to provide detailed information on the identity of the causal agent. However, the utility of blood culture for candidosis and antigen detection for Cryptococcosis has meant that comparatively less effort has been made to develop molecular diagnostic methods for yeast infections compared to invasive aspergillosis, for example. A real-time PCR method with probes to detect products from six different *Candida* species detected 20 of 23 cases of candidaemia (McMullan et al. 2008). A case of *C. famata* candidaemia was one of the patients who was PCR-negative, and this species was known not to be detectable by the primers and probes used, suggesting increasing sensitivity should be possible by designing further reagents. Furthermore, six patients were placed in a category of probable candidosis, and amongst these only one was PCR-positive. This patient went on to develop candidaemia subsequently. In another study with a method using a gel-based method of detecting PCR products, all candidamic patients were positive. Furthermore, 56% of patients categorised as having suspected candidosis were positive (Ahmad et al. 2002). Interestingly, some authors have suggested that serum is the optimal specimen compared to whole blood for the detection of *Candida* DNA, suggesting that free DNA may be present in the serum (Metwally et al. 2008). While these and other results are promising there remain questions over the interpretation of positive PCR results in patients whose blood cultures are negative.

13.8 The Future of the Diagnosis of Yeast Infections

What tests will the medical microbiology laboratories of the future offer for the diagnosis of yeast infections? This will depend to a large extent on the volume of work and the case mix of patients the specimens derive from relating to changes in

clinical problems and associated changes in fungal aetiologies and antifungal drug sensitivity. But it is increasingly likely that pragmatism and economics will feature in decisions on diagnostic test algorithms. Some authors have suggested that the rapid PNA FISH identification of *C. albicans* alone can save over 1,500 US dollars per patient in terms of the resultant change in antifungal prescribing (Forrest et al. 2006). Conversely, others have questioned the need to use expensive PCR-based tests to rapidly identify species such as *C. glabrata* associated with reduced susceptibility to azoles when in fact in many cases patients with *C. glabrata* respond well to fluconazole (Bennett 2008). While smaller laboratories are likely to continue to depend on culture-based methods, larger laboratories are likely to move increasingly to serological and non-culture-based techniques including BDG and PCR. Another parameter which is likely to come into play is the need for the laboratory scientist to be able to provide evidence for the clinical utility of a test (Deeks 2007). It is relatively straightforward to assess the sensitivity of a test but a clinician really wants to know the likelihood of a patient with a positive result, responding to a standard therapy. Dromer et al. (2007), for example, have determined using multivariate analysis of the treatment of cryptococcosis in which serum cryptococcal antigen titres > 1:512 are significantly associated with treatment failure ($p = 0.008$). The development of outcome-linked tests is likely to become increasingly important in medical microbiology generally and yeast infections are no exception.

In summary, the challenge to the laboratory to offer rapid, accurate, inexpensive and meaningful tests in the face of changes in pathogenic yeasts and the patient population will continue.

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Chapter 14
Antifungal Susceptibility Testing and Therapy

Manuel Cuenca-Estrella and Juan Luis Rodriguez-Tudela

Abstract The development of new therapies to treat fatal diseases is creating an increasing number of patients who have predisposing factors for infections by opportunistic yeasts. The rise in the prevalence of fungal infections has been the drive to develop and license several new antifungal agents such as new formulations of polyenes, new triazole agents and echinocandins. The availability of distinct antifungal agents with different activity profiles and the advances made in the diagnosis of mycoses and fungal taxonomy have led medical mycologists to recommend antifungal susceptibility testing techniques as routine methods for use in clinical practice. Commercial techniques are very reliable and useful to detect resistance in vitro to azole agents. Epidemiological surveys help to choose the best therapeutic alternative to treat yeast infections. However, these steps forward in the field of mycology have still not led to significant reductions in the mortality of infected patients. There is a need to develop new diagnostic tools that allow early treatment of the mycoses and to design therapeutic strategies to control these infections based on reliable epidemiological studies and sound clinical trials.

14.1 Antifungal Susceptibility Testing

14.1.1 An Update on the AST Standardization Process

Antifungal susceptibility testing (AST) procedures have become readily accessible techniques to clinical practitioners. In the last ten years, the availability of distinct antifungal agents with different activity profiles and the advances made in the

M. Cuenca-Estrella (✉) and J. Luis Rodriguez-Tudela
Mycology Department, Spanish National Center for Microbiology, Instituto de Salud Carlos III, Ctra Majadahonda-Pozuelo Km 2, 28220 Majadahonda (Madrid), Spain
e-mail: mcuenca-estrella@isciii.es

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Table 14.1 Antifungal breakpoints of EUCAST and CLSI in mg/L

Antifungal	CLSI			EUCAST		
	Susceptible	S-DD[a]	Resistant	Susceptible	Intermediate	Resistant
Flucytosine				NA	NA	NA
Fluconazole	<8	16–32	≥ 64	≤ 2	4	>4
Voriconazole	<1	2	≥ 4	≤ 0.125	–	>0.125[b]
Caspofungin	≤ 1	2	≥ 4	NA[c]	NA	NA
Micafungin	≤ 1	2	≥ 4	NA	NA	NA
Anidulafungin	≤ 1	2	≥ 4	NA	NA	NA

[a]S-DD = susceptible dependent on dose

[b]Isolates with MIC values above the 0.125 mg/L breakpoint are rare or not yet reported. This fact precludes the definition of other categories than susceptible. The identification and antimicrobial susceptibility tests on any such isolate must be repeated and if the result is confirmed, the isolate is sent to a reference laboratory. Until there is evidence regarding clinical response for confirmed isolates with MIC above the current resistant breakpoint (in italics) they should be reported as resistant

[c]EUCAST is doing several multicenter exercises to set echinacandin breakpoints according to EUCAST procedures

diagnosis of mycosis and fungal taxonomy have led medical mycologists to recommend AST techniques as methods for use in clinical practice.

AST underwent a complex standardization process in the 1990s driven by the US Clinical Laboratory Standard Institute (CLSI), former National Committee for Clinical Laboratory Standards (NCCLS). Reference methods for testing yeasts and molds were approved, and breakpoints to interpret AST of yeasts were established (Table 14.1) (National Committee for Clinical Laboratory Standards 2002; 2005). Other countries such as France and Germany also developed reference techniques. More recently, the European Committee on Antimicrobial Susceptibility Testing (EUCAST) has approved reference methodologies to test *Candida* species and filamentous fungi, and breakpoints for some antifungal compounds have been set (Subcommittee on Antifungal Susceptibility Testing, AFST of the ESCMID European Committee for Antimicrobial Susceptibility Testing, EUCAST 2008a, b, c, d). EUCAST is a standing committee jointly organized by ESCMID (European Society of Clinical Microbiology and Infectious Diseases), ECDC (European Centre for Disease Prevention and Control) and European national breakpoint committees. It was set up to standardize antimicrobial breakpoints and susceptibility testing in Europe so that reproducible methods, comparable results, and interpretations are produced. Although the reference methodologies of EUCAST and CLSI are different (Table 14.2), the results are equivalent (Cuenca-Estrella et al. 2002; Rodriguez-Tudela et al. 2007).

Although the reference techniques must be taken as one of the most significant milestones in the history of mycology, it is a well-known fact that some problems still exist. One of the most important pitfalls is the lack of reliable detection of resistance to amphotericin B. RPMI medium, the assay medium recommended in dilution reference techniques, yields a range of MIC values that spans only three or four twofold serial dilutions. Many experts believe that this short range precludes

Table 14.2 Differences between EUCAST EDEF 7.1 and CLSI M27A3 methodologies

	EUCAST EDEF 7.1	CLSI M27A3
Concentration of glucose in RPMI 1640	2%	0.2%
Shape of the well in the microplate	Flat bottom	Round bottom
Inoculum size	$0.5–2.5 \times 10^5$	$0.5–2.5 \times 10^3$
Incubation time	24 hours	48 hours
Reading	Spectrophotometric	Visual
End point		
Amphotericin B	Lowest concentration that inhibits growth by at least 90%	Optically clear and prominent decrease
Azole drugs and echinocandins	Lowest concentration that inhibits growth by at least 50%	Prominent decrease

reliable discrimination between susceptible and resistant isolates to amphotericin B (Cuenca-Estrella and Rodriguez-Tudela 2001).

Other limitations of the AST reference procedures are poor growth of non-fermentative yeasts including *Cryptococcus neoformans*, lack of reproducibility when echinocandins are tested, and the need for clinicians to establish breakpoints to interpret the susceptibility results of filamentous fungi (Espinel-Ingroff 2008; Espinel-Ingroff et al. 2008; Odds et al. 2004).

In addition, the standard reference procedures are impractical for clinical laboratories since they recommend rather complex methods for susceptibility testing. Many microbiologists prefer to use other systems which have particular advantages such as ease of performance, economy, or more rapid results (Arikan 2007).

In this sense, several modifications of the standardized dilution procedures have been developed. The CLSI has approved agar diffusion techniques to test the susceptibility in vitro of *Candida* species against both fluconazole and voriconazole, and multicenter exercises are being undertaken to standardize diffusion techniques with echinocandin disks (Arikan 2007). Other reference techniques such as methods to test dermatophytes, disk diffusion for molds, techniques for *Malassezia* species, and guidelines for testing echinocandins against molds have been developed or are under development by the CLSI.

The EUCAST is developing a reference method to test *C. neoformans* and other non-fermentative species of yeasts to overcome the limitations described above.

One of the most important tasks of EUCAST is establishing antifungal breakpoints, and it has developed a standard procedure (European Committee on Antimicrobial Susceptibility Testing 2000; Kahlmeter et al. 2006). EUCAST distinguish between clinical breakpoints and epidemiological cut-off values. Clinical breakpoints define the organism as susceptible, intermediate, and resistant to antifungal drugs. Susceptible and resistant categories are related to a high likelihood of clinical success or clinical failure respectively. The clinical outcome associated with an intermediate category is uncertain, implying that an infection caused by a microorganism classified in this category may be appropriately treated in body sites where the drugs are physically concentrated or when a high dosage of

drug can be used. Additionally, the intermediate category is defined as a buffer zone to prevent small, uncontrolled, technical factors from causing major discrepancies in interpretation. Clinical breakpoints should be used in everyday clinical laboratory work to advise the patient on therapy.

Epidemiological cut-off values are based on the wild-type MIC distribution of each microorganism. Wild-type microorganisms are defined by the absence of acquired and mutational resistance mechanisms to the antifungal. Having determined the wild-type distribution and its highest MIC value, organisms with acquired or mutational resistance mechanisms can be identified readily as organisms with reduced susceptibility compared with the highest MIC value of the wild type. These are the non-wild-type population. EUCAST has defined the MIC value encompassing the wild-type population as the *epidemiological cut-off value*. The epidemiological cut-off value is expressed as $WT \leq X$ mg/L. Epidemiological cut-off values are used as the most sensitive measure of resistance development in hospitals and the community. Also, they are useful for measuring the effect of interventions and for developing strategies to counteract further resistance development. Finally, definition of wild-type population cut-off values should ensure that clinical breakpoints do not divide wild-type populations (European Committee on Antimicrobial Susceptibility Testing 2000; Kahlmeter et al 2006).

In addition to epidemiological cut-offs, the process of setting clinical breakpoints analyzes the pharmacokinetic and pharmacodynamic characteristics of the antifungal including Monte Carlo simulations as well as data-mining analysis of patient outcome versus MIC of the microorganisms. Currently, AFST EUCAST has developed breakpoints for fluconazole and voriconazole (Table 14.1) (Subcommittee on Antifungal Susceptibility Testing, AFST of the ESCMID European Committee for Antimicrobial Susceptibility Testing, EUCAST 2008d, b).

14.1.2 Commercial Methods for AST of Yeasts

As stated above, the reference procedures are not the most convenient procedures for routine use in clinical laboratories. Several more convenient techniques have been developed which are based on agar diffusion procedures or on the use of a colorimetric oxidation-reduction indicator. Some of these techniques are commercially available and are thus faster and easier alternatives to the procedures developed by either CLSI or EUCAST (Cuenca-Estrella et al. 2005, 2008).

One of the most significant points of reference procedures is to provide a standard basis from which other methods can be developed and compared. Many works have analyzed agreements and correlations between standard and commercial procedures and quite a few commercial systems are suitable alternatives for testing susceptibility of *Candida* spp.

Techniques such as Etest® (AB Biodisk), Sensititre YeastOne® (Trek Diagnostic Systems Ltd), Neo-Sensitabs® (A/S Rosco), ASTY®, Fungitest® (Bio-Rad), Wideryst® (Soria Melguizo SA), Vitek® (bioMerieux) and others can be used to

detect the resistance in vitro to fluconazole and other azoles in *Candida* isolates. Substantial discrepancies between reference MIC values of azole agents and results obtained by commercial systems stand at less than 5%. In addition, azole-resistant isolates in vitro misclassified as susceptible by commercial systems are very uncommon (Cuenca-Estrella et al 2005, 2008).

Some of those commercial techniques are also very reliable to detect resistance to echinocandins. The identification of amphotericin B resistant organisms seems to be more difficult, however, as reference methods fail to detect it and trustworthy comparisons have not been done.

14.2 Epidemiology of Resistance of Yeasts In Vitro

Neither CLSI nor EUCAST have produced breakpoints for amphotericin B. Isolates with MICs $\geq$2 mg/L are extremely infrequent when using EUCAST or CLSI methodologies. Etest has proven to be the most reliable method to detect isolates with high MICs to amphotericin B but the number of such isolates is very limited. In summary, no breakpoints exist for amphotericin B but any strain with MIC $\geq$2 mg/L should be considered as potentially resistant. Table 14.3 shows the percentage of isolates with MIC values above 1 mg/L in a collection of strains isolated from blood cultures.

On the other hand, both CLSI and EUCAST organizations have produced breakpoints for fluconazole and voriconazole (Table 14.1). However, the breakpoints are different, with those produced by EUCAST much lower than those of CLSI. In this chapter, EUCAST criteria have been followed to interpret the rates of resistance to fluconazole and voriconazole. Tables 14.4 and 14.5 show the percentage of resistant strains for fluconazole and voriconazole according to EUCAST breakpoints for a collection of strains isolated from blood cultures in Spain. The results are completely extrapolated to other countries for *Candida albicans, Candida parapsilosis* and *Candida tropicalis*. For instance, the rates of resistance for fluconazole in the ARTEMIS DISK surveillance system from 1997 to 2003 were 1.16% for *C. albicans*, 3.1% for *C. parapsilosis* and 4.2% for *C. tropicalis* (Pfaller and Diekema 2007), similar values to those showed in Table 14.5, meaning that

Table 14.3 MIC_{50}, MIC_{90}, range and percentage of *Candida* spp. isolated from blood cultures with MICs $\geq$2.0 mg/L to amphotericin B according to EUCAST methodology (Unpublished results from Mycology Reference Laboratory of Spain)

Species	N	MIC_{50}	MIC_{90}	Range	% of Resistant $\geq$2 mg/L
C. albicans	804	0.06	0.5	0.03–1.0	0
C. glabrata	253	0.12	0.5	0.03–2.0	0.4
C. krusei	102	0.5	1.0	0.06–2.0	3.9
C. parapsilosis	660	0.25	1.0	0.03–2.0	1.8
C. tropicalis	253	0.125	0.5	0.03–2.0	0.8

Table 14.4 Percentage of resistance of *Candida* spp. isolated from blood cultures according to EUCAST fluconazole breakpoints (Unpublished results from Mycology Reference Laboratory of Spain)

Species	N	% of Susceptible ≤ 2 mg/L	% of Intermediate 4 mg/L	% of Resistant $>$ 4 mg/L
C. albicans	804	99.0	0	0.8
C. glabrata	253	15.8	38.3	45.9
C. krusei	102	0	0	100
C. parapsilosis	660	96.5	2.4	1.2
C. tropicalis	253	95.7	0.4	3.9

Table 14.5 Percentage of resistance of *Candida* spp. isolated from blood cultures according to EUCAST voriconazole breakpoints (Unpublished results from Mycology Reference Laboratory of Spain)

Species	N	% of Susceptible $\leq$0.125 mg/L	% of Resistant $>$0.125 mg/L
C. albicans	804	99.4	0.6
C. glabrata	253	58.3	41.7
C. krusei	102	20.0	80.0
C. parapsilosis	660	99.3	0.7
C. tropicalis	253	93.4	6.6

Table 14.6 MIC_{50}, MIC_{90}, range and percentage of *Candida* spp. isolated from blood cultures with MICs $\geq$ 2.0 mg/L to caspofungin according EUCAST methodology (Unpublished results from Mycology Reference Laboratory of Spain)

Species	N	MIC_{50}	MIC_{90}	Range
C. albicans	410	0.06	0.25	0.015–16.0
C. glabrata	132	0.12	0.25	0.03–0.5
C. krusei	102	0.5	1.0	0.06–2.0
C. parapsilosis	262	1.0	1.0	0.06–8.0
C. tropicalis	104	0.12	1.0	0.03–32.0

isolates with MIC values above 4 mg/L are infrequent by both AST methods, CLSI and EUCAST. However, in this study the rate of resistance of *Candida glabrata* was 17.6% according CLSI breakpoints, somewhat lower than the 45.9% shown in Table 14.5 according to EUCAST breakpoints. It is well known that isolates of *C. glabrata* easily increase the expression of the genes encoding *CDR* efflux pumps, rendering them resistant to azole drugs. In addition, only 70.9% of *Candida krusei,* an intrinsically resistant species to fluconazole, are considered resistant following the breakpoints of CLSI. However, EUCAST breakpoints classified 100% of *C. krusei* isolates as resistant.

Regarding echinocandins, CLSI has produced a susceptibility breakpoint identical for all drugs in this class of antifungals (Table 14.1) whilst EUCAST has no recommendations so far, as several multicenter studies are now in progress. Table 14.6 shows the MIC_{50}, MIC_{90} and MIC range for blood cultures isolates of the main species of *Candida* from the Mycology Reference Laboratory of Spain.

Recently, several publications dealing with active population-based surveillance of candidemia have produced reliable data regarding species incidence rates and epidemiology of resistance (Almirante et al. 2005; Arendrup et al. 2008; Asmundsdottir et al. 2002; Bedini et al. 2006; Chen et al. 2006; Gonzalez et al. 2008; Hajjeh et al. 2004; Kao et al. 1999; Klingspor et al. 2004; Laupland et al. 2005; Odds et al. 2007; Poikonen et al. 2003; Rennert et al. 2000; Sandven et al. 2006; Takakura et al. 2004; Zepelin et al. 2007). However, data regarding resistance rates cannot be combined because the interpretation of the results has followed different criteria. In any case, species distribution is a valuable tool to choose the best treatment when an infection caused by *Candida* is suspected. Table 14.7 shows the species distribution in different countries where active population-based candidemia surveillance studies have been done. In all countries, *C. albicans* is the most prevalent species except in Mexico, where *C. parapsilosis* is more frequent. However, in many countries the percentage of species considered resistant or with potentially decreased susceptibility to fluconazole (i.e., *C. glabrata* and *C. krusei*) is higher than 15%, except in Spain (12%), Mexico (10.7%) and Israel (7.2%) (Table 14.7). Therefore, in many countries fluconazole cannot be used as empirical treatment anymore. This fact highlights the importance of a quick species identification of *Candida* isolates in order to provide the cheapest and most active antifungal treatment to the patient. In addition, AST of isolates has become an essential tool in those settings where invasive fungal infections are common.

Regarding other species of yeasts, the information is more limited because their incidence is much lower. In terms of clinical resistance, it is noteworthy that there is an emergence of clinical isolates of *C. neoformans* which are resistant to fluconazole as a consequence of antifungal therapy with this compound in different geographical regions (Hsueh et al. 2005; Perkins et al. 2005; Sar et al. 2004).

In the last few years, there has been a significant rise in the number of infections caused by other genera of yeasts, such as *Trichosporon* spp., *Rhodotorula* spp., *Geotrichum* spp., and *Saccharomyces cerevisiae*, so they are considered as emerging pathogens. Their susceptibility profiles are variable and often unpredictable. Some species of *Trichosporon* are resistant in vitro to amphotericin B, and *Rhodotorula* spp. seem to be insensitive in vitro to azole agents (Gomez-Lopez et al. 2005; Rodriguez-Tudela et al. 2005). In cases of deep infection due to those species, susceptibility testing seems to be essential in order to determine the best therapeutic alternative (see Chap 12).

14.3 Therapy of Yeast Infections

14.3.1 Therapy of Candida Infections

The drug of choice depends on the infecting species and the clinical setting (Gomez-Lopez et al. 2008b). *Candidemia and invasive candidiasis* have become increasingly important in the clinical setting in the last decade, as they are the most

Table 14.7 *Candida* species distribution from candidemia population-based surveillance studies

Reference	Country	Year	No isolates	*C. albicans*	*C. glabrata*	*C. krusei*	*C. parapsilosis*	*C. tropicalis*	Other	Polyfungal infections
10	Australia	2001–2004	1,095	47.3	15.4	4.3	19.9	5.1	5.9	2.2
36	Canada	1999–2004	209	51.1	21.5	4.7	6.2	5.7	10.5	NA
2	Denmark	2004–2008	1,089	59.8	20.5	4.1	4.0	4.6	7.1	4.0
51	Finland	1995–1999	479	70.0	9.0	8.0	5.0	3.0	5.0	NA
70	Germany	2004–2005	561	58.4	18.7	1.6	9.3	6.3	5.7	NA
4	Iceland	1980–1999	177	64.4	12.4	0.56	9.6	5.6	4.0	NA
53	Israel	1994	298	53.6	6.5	0.7	11.9	10.9	15.9	NA
7	Italy	2000–2003	94	40.0	12.8	3.2	22.3	16.0	5.3	9.3
68	Japan	2001–2002	535	40.7	17.9	2.4	23.0	11.6	4.3	NA
26	Mexico	2004–2007	398	31.9	8	2.7	37.9	14.8	4.6	NA
59	Norway	1991–2003	1,393	69.8	13.2	1.6	5.8	6.7	3.1	1.5
42	Scotland	2005–2006	300	52.0	22.7	1.0	11.7	6.0	8.0	NA
1	Spain	2002–2003	345	51.0	8.0	4.0	23.0	10.0	3.0	2.0
33	Sweden	1998–1999	191	67.0	15.7	1.0	7.3	2.1	4.1	2.7
32	USA	1992–1993	837	52.0	12.0	4.0	21.0	10.0	3.0	NA
27	USA	1998–2000	1,143	45.0	24.0	2.0	13.0	12.0	2.1	NA

common deep infection due to yeasts. *Candida* spp. are the fourth most common cause of bloodstream infections in the USA, but less so in Europe (Dimopoulos et al. 2008; Forrest et al. 2008; Hachem et al. 2008).

Initial therapy for candidemia and deep candidiasis remains controversial. It should involve an echinocandin compound, fluconazole, or lipid formulation of amphotericin B. Amphotericin B deoxycholate should be avoided due to its toxicity. The choice depends on the clinical status of the patient and other variables such as the knowledge of the antifungal susceptibility profile of the infecting isolate, the drug toxicity, and the patient's prior exposure to antifungal agents (Pappas et al. 2009).

Fluconazole is generally preferred for clinically stable patients. It remains the drug of choice for the majority of patients with uncomplicated *C. albicans*, *C. tropicalis*, and *C. parapsilosis* infections. It is also recommended for empirical treatment of suspected disseminated candidiasis in febrile non-neutropenic patients in many geographic areas where the rate of resistant species such as *C. krusei* and *C. glabrata* is below 10–15%.

The main alternatives to fluconazole are the echinocandins. Caspofungin and more recently micafungin and anidulafungin have become a first-line alternative to treat deep infections due to *Candida* including those by azole-resistant isolates (Kuse et al. 2007; Mora-Duarte et al. 2002; Reboli et al. 2007).

Liposomal amphotericin B and other lipid formulations of amphotericin B may be used in acutely ill patients or those with refractory disease. It is recommended for systemic candidiasis in neutropenic patients and for the treatment of infections due to *Candida* isolates either intrinsically resistant to azoles or after development of resistance under azole therapy (Pappas et al 2009).

Voriconazole has been shown as an effective alternative to fluconazole in the treatment of candidemia in non-neutropenic patients, particularly when the causative organism is intrinsically resistant to other azole antifungal agents (Kullberg et al. 2005).

Rates of mortality of invasive candidiasis are still very high and new alternative therapeutic approaches are being been developed. An approach to improve response rates is to combine more than one antifungal agent although clinical experience with combination therapy is limited (Cuenca-Estrella 2004). The most convincing clinical trial of combination therapy to treat invasive *Candida* infections showed that the combination of amphotericin B with fluconazole was not antagonistic, that its success rate was slightly better than that of fluconazole monotherapy, and that there was more rapid clearance from the bloodstream when the combination was used (Rex et al. 2003). Another alternative is Efungumab (Mycograb), a human recombinant monoclonal antibody fragment against heat-shock protein 90. It has been shown to act synergistically against a wide array of *Candida* spp. in combination with amphotericin B, although those findings need to be confirmed in further trials (Pachl et al. 2006).

The therapy of other *Candida* infections is based on the same criteria as for candidaemia and deep-seated infections (Gomez-Lopez et al 2008b). *Urinary candidiasis* must be treated with fluconazole (oral or intravenous) as it achieves

high urine concentrations. Rarely, amphotericin B or flucytosine are used, especially in patients with urologic infection due to non-*albicans Candida* species refractory to treatment. Long courses of therapy (7–14 days) are recommended. Usually, removal of urinary devices is helpful. Newer azole agents and echinocandins are not recommended for the treatment of urinary tract infections since they fail to achieve adequate urine concentrations (Malani and Kauffman 2007; Sobel et al. 2007).

Candida endocarditis is a rare entity which must be treated with combined medical and surgical therapy. Medical therapy includes amphotericin B with or without flucytosine at maximum tolerated doses for a minimum of 6 weeks. Recently, caspofungin has been used successfully to treat some cases of *Candida* endocarditis without valve replacement (Baddley et al. 2008; Pappas et al 2009).

Haematogenous Candida meningitis can be a manifestation of disseminated candidiasis in neonates and a complication of neurosurgical procedures in other groups of patients. It is associated with significant mortality and development of neurological abnormalities. All amphotericin B formulations, frequently used in combination with flucytosine, are useful for treating *Candida* meningitis. Recent clinical data suggest that echinocandins may have a role in the treatment of meningitis, but only at high doses (Hope et al. 2008).

Candida endophthalmitis usually develops as a result of haematogenous seeding. Intravenous heroin addicts, and patients who have either an intraocular lens implant or a corneal transplant can also develop this condition. Intravenous amphotericin B has long been considered the gold standard, but oral or intravenous fluconazole is frequently used for long treatments (Pappas et al 2009).

Mucosal candidiasis is a common condition in some groups of patients. Oropharyngeal candidiasis (OPC), esophageal candidiasis, and vulvovaginal candidiasis (VVC) are the most frequent yeasts infections in the Western world. OPC is common in infants, the elderly, and compromised patients, and occurs in association with serious underlying conditions including diabetes, leukemia, neoplasia, steroid use, antimicrobial therapy, radiation therapy, and HIV infection. *C. albicans* remains the most common species responsible for OPC (Vazquez et al. 2006).

OPC can be initially treated with topical azole compounds. Oral fluconazole (100 mg/day for 7–14 days) is effective as well. Itraconazole solution (200 mg/day for 7–14 days) is as efficacious as fluconazole. Amphotericin B, voriconazole, posaconazole, or echinocandins can be used to treat refractory infections.

Esophageal candidiasis occurs in patients with advanced HIV infections and those with chronic diseases previously treated with antibiotics or steroids. Recommended treatment is essentially the same as for oral candidiasis.

It is estimated that 75% of all women will experience at least one episode of VVC in their lifetime. Several factors are associated with increased rates of VVC including pregnancy, oral contraceptives with high estrogenic content, uncontrolled diabetes mellitus, corticosteroids use, antimicrobial therapy, and intrauterine devices, and other factors that alter the normal vaginal flora or change the avidity of epithelial cells for *Candida* spp.

Uncomplicated vaginal candidiasis may be treated with a short course of topical agents including azoles. Oral itraconazole and fluconazole formulations may be preferred since they can be given as a single dose. Complicated vaginitis requires therapy for more than 7 days, and for recurrent vaginitis, a 6-month treatment may be recommended. Azole treatment is not recommended for infections caused by fluconazole-resistant organisms such as *C. glabrata* or *C. krusei* (Sobel et al. 2004).

Superficial candidiasis can be observed on both the skin and the nails. *Candida* species have a predilection for warm and moist areas created by maceration and occlusion. Skin infections can cause diaper rash in infants, and intertrigo in the elderly and obese or diabetic adults. Topical azoles and polyenes are effective (Gomez-Lopez et al 2008b).

14.3.2 Therapy of Infections due to Cryptococcus

Cryptococcosis is mainly associated with HIV infection and decreased in CD4+ counts, although the disease could also occur after solid organ transplantation, solid tumor, hematological disease, or immunosuppressive treatments (Casadevall and Perfect 1998).

The clinical presentation could vary significantly. It is believed that the microorganism is acquired from the environment by inhalation, and produces a chronic or asymptomatic pneumonia. In case of immunosuppression, especially in HIV-positive patients, the fungus disseminates to different organs and produces a severe disease. In this last situation, the yeast preferentially resides in the central nervous system and can be found in the cerebrospinal fluid causing meningitis.

The most characteristic clinical outcome found during *C. neoformans* infection is meningitis, HIV infection being the main predisposing factor to suffer from this type of disease. Initial treatment based on amphotericin B and flucytosine combination decreased mortality of infected patients (Saag et al. 2000; Bicanic et al. 2008). Fluconazole orally administrated is used as a long-term suppressive therapy (Sugar and Saunders 1988). As a consequence, an initial treatment based on amphotericin B and flucytosine followed by a fluconazole maintenance treatment is the current standard for the management of cryptococcal meningitis in HIV-positive patients (Saag et al 2000).

With regard to other triazole compounds, itraconazole is recommended as an alternative maintenance treatment in those cases in which fluconazole cannot be administered (Saag et al. 1999). Posaconazole has shown a 50% efficacy in patients with cryptococcal meningitis (Pitisuttithum et al. 2005), and voriconazole had an efficacy rate of around 40% against cryptococcosis in patients who failed to respond to initial therapy (Perfect et al. 2003).

Cryptococcus gattii (formerly *C. neoformans* var. *gattii*) can cause infections as well but it is believed that it can affect immunocompetent individuals. Its importance significantly increased when it was found that *C. gattii* was the causative agent of the outbreak that occurred in the Vancouver Island, British Columbia, in

1999 (Bartlett et al. 2008). The clinical manifestations vary widely but lung and brain infections were the most common presentations. The management is similar to that of *C. neoformans* infection, although some differences might exist in the susceptibility profile in vitro, *C. gattii* being more resistant to azole agents (Gomez-Lopez et al. 2008a).

14.3.3 Therapy of Other Yeast Infections

In the last years, other genera of yeasts such as *Trichosporon* spp., *Rhodotorula* spp., *Geotrichum* spp., and *Saccharomyces* spp. have emerged as human pathogens. Many of these infections are associated with the presence of catheters and other intravenous devices. The optimal treatment for these infections has not been clinically determined, but according to the susceptibility profile in vitro, new azole compounds (in some cases in combination with amphotericin B) can be used to treat infections by *Trichosporon*, and combinations of amphotericin B and flucytosine are recommended for therapy of *Rhodotorula* (Gomez-Lopez et al. 2005; Paphitou et al. 2002). Both *Trichosporon* and *Rhodotorula* are basidiomycetous, so echinocandins are not recommended to treat these types of infections.

Geotrichum spp. are ascomycetous yeasts that can cause opportunistic mycoses. *Geotrichum capitatum* and *Geotrichum candidum* are the most common species. It seems that amphotericin B and voriconazole are the most active compounds in vitro and that echinocandins are inactive in vitro, but standard therapy for those infections has not yet been established (Etienne et al. 2008; Fianchi et al. 2008).

Finally, *S. cerevisiae* infection has been associated with catheter usage and other intravenous devices. Recently infections have occurred related to the administration of the probiotic Ultralevura, which contains freeze-dried yeast that is used to prevent *Clostridium difficile*-associated diarrhea. Few studies have addressed the susceptibility profile, but *S. cerevisiae* has high MIC values to fluconazole and itraconazole. Caspofungin is active in vitro against *S. cerevisiae* and could be a therapeutic alternative (Munoz et al. 2005).

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Chapter 15
Mechanisms of Multidrug Resistance in Fungal Pathogens

Sélène Ferrari and Dominique Sanglard

Abstract Fungal pathogens can develop resistance to antifungal agents as is the case with most microbes exposed to anti-infectives. Though the number of antifungal agents is limited, fungal pathogens have revealed a large pattern of resistance mechanisms. Among them, the phenomenon of multidrug resistance is of particular importance since interactions of the underlying molecular mechanisms can facilitate acquisition of resistance to multiple agents. This type of resistance is mostly mediated by efflux transporters of several classes. In this review, we have summarized the current understanding of this phenomenon at the clinical, cellular, and molecular levels and discussed its occurrence in several important fungal pathogens including *Candida*, *Aspergillus* and *Cryptococcus* spp.

15.1 Introduction

The fight against human fungal diseases necessitates the elaboration of therapeutic strategies. Besides a careful management of patients to minimize the risks of fungal infections, antifungal agents have a dominant role for either prophylaxis or therapeutic purposes.

Current available antifungal drugs belong to four major different classes: the polyenes, the azoles, the pyrimidine analogues and the echinocandins. Each of these classes contains different compounds with specific range of activities against the major fungal pathogens (polyenes: amphotericin B, nystatin; azoles: fluconazole, itraconazole. voriconazole; pyrimidine analogues: 5-flucytosine (5-FC); echinocandins: caspofungin, micafungin, anidulafungin), whose modes of action and activities against common fungal pathogens are described in several available reviews and are summarized here in Tables 15.1 and 15.2 (Akins 2005; Dodds Ashley et al. 2006;

S. Ferrari and D. Sanglard (✉)
Institute of Microbiology, University of Lausanne and University Hospital Center, Rue du Bugnon 48, 1011 Lausanne, Switzerland
e-mail: dominique.sanglard@chuv.ch

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Table 15.1 Mode of action of antifungal agents and their association to resistance mechanisms

Antifungal agent(s)	Mode of action and cellular target(s)	Occurrence of primary and acquired resistance mechanisms in clinical isolates				
		Impaired transport	Target alterations	Absence of target	Decreased toxic metabolite formation	Formation of multicellular complexes
Polyenes (Amphotericin B)	Binding to ergosterol			*ERG11* inactivation (Ca) *ERG6* inactivation (Cg)		Biofilms (Ca, Cd, Cg, Ck, Ct)
Azoles (Itraconazole, Fluconazole, Voriconazole, Posaconazole)	Inhibition of cytochrome P450 function:– 14α-lanosterol demethylase (*ERG11*)– sterol Δ^{22} desaturase (*ERG5*)	ABC-transporter upregulation (Ca, Cd, Ct, Crn, Cg) Major Facilitator upregulation (Ca, Ct, Cd)	*ERG11* mutations (Ca, Ct, Crn, Af) *ERG11* upregulation (Ca, Af)	*ERG11* inactivation (Ca)	*ERG3* non-sense mutation (Ca, Cd)	Biofilms (Ca, Cd, Cg, Ct)
Allylamines (Terbinafine)	Inhibition of squalene epoxidase (*ERG1*)					
Morpholines (Amorolfine)	Inhibition of sterol Δ^{14} reductase (*ERG24*) and of Δ^{7-8} isomerase (*ERG2*)					
5-Fluorocytosine (5-FC)	Inhibition of nucleic acids synthesis	Permease (*FCY2*) mutation (Ca)			*FUR1* mutation (Ca)	Biofilms (Ca, Cd, Cg, Ck, Ct)
Echinocandins (Caspofungin, Micafungin, Anidulafungin)	Inhibition of β -1,3 glucan synthase (*FKS* genes)		*FKS1* mutations (Ca, Ck, Cg, Cp, Af) *FKS2* mutations (Cg)			

Ca, *C. albicans*; Cd, *C. dubliniensis*; Ct, *C. tropicalis*; Cg, *C. glabrata*; Cp, *C. parapsilosis*; Ck, *C. krusei*; Af, *A. fumigatus*; Crn, *Cr. neoformans*

References for resistance mechanisms:

Biofilms (Ca, Cd, Cg, Ck, Ct) (Chandra et al. 2001; Ramage et al. 2002a, b; Seneviratne et al. 2008)

ABC-transporter upregulation (Ca, Cd, Ct, Crn, Cg) (Barchiesi et al. 2000; Moran et al. 1998; Posteraro et al. 2003; Sanglard et al. 1999; Sanglard et al. 1997; Sanglard et al. 1995; Torelli et al. 2008)

Major facilitator upregulation (Ca, Ct, Cd) (Barchiesi et al. 2000; Moran et al. 1998; Sanglard et al. 2001; Sanglard et al. 1999; Sanglard et al. 1995)

ERG11 mutations (Ca, Ct, Crn, Af) (Diaz-Guerra et al. 2003; Mann et al. 2003; Marichal et al. 1999; Martinez et al. 2002; Perea et al. 2002; Sanglard et al. 1998; Vandeputte et al. 2005)

ERG11 upregulation (Ca, Af) (Dunkel et al. 2008a, b; Frade et al. 2004; Perea et al. 2002)

ERG11 inactivation (Ca) (Sanglard et al. 2003)

ERG6 inactivation (Cg) (Vandeputte et al. 2007; Vandeputte et al. 2008)

ERG3 non-sense mutation (Ca, Cd) (Miyazaki et al. 1999; Pinjon et al. 2003; Sanglard et al. 2003)

Permease (FCY2) mutation (Ca) (Whelan 1987)

FUR1 mutation (Ca) (Dodgson et al. 2004; Hope et al. 2004)

FKS1 mutations (Ca, Ck, Cg, Cp, Af) (Cleary et al. 2008; Douglas et al. 1997; Garcia-Effron et al. 2008; Kahn et al. 2007; Park et al. 2005; Rocha et al. 2007)

FKS2 mutations (Cg) (Katiyar et al. 2006; Thompson et al. 2008)

Table 15.2 Activities of antifungal agents against fungal pathogens

Organism	Antifungal agent								
	AmB	FLC	ITZ	VOR	POS	ANF	CSF	MCF	5-FC
Aspergillus species									
A. flavus	±	−	+	+	+	+	+	+	−
A. fumigatus	+	−	+	+	+	+	+	+	−
A. niger	+	−	±	+	+	+	+	+	−
A. terreus	−	−	+	+	+	+	+	+	−
Candida species									
C. albicans	+	+	+	+	+	+	+	+	+
C. glabrata	+	±	±	+	+	+	+	+	+
C. krusei	+	−	±	+	+	+	+	+	±
C. lusitaniae	−	+	+	+	+	+	+	+	+
C.parapsilosis	+	+	+	+	+	±	±	±	+
C. tropicalis	+	+	+	+	+	+	+	+	+
Other species									
Cryptococcus neoformans	+	+	+	+	+	−	−	−	+
Coccidioides species	+	+	+	+	+	±	±	±	−
Blastomyces	+	+	+	+	+	±	±	±	−
Histoplasma species	+	+	+	+	+	±	±	±	−
Fusarium species	±	−	−	+	+	−	−	−	−
Scedosporium apiospermum	±	−	±	+	+	−	−	−	−
Scedosporium prolificans	−	−	−	±	±	−	−	−	−
Zygomycetes	±	−	−	−	+	−	−	−	−

AmB amphotericin B; *FLC* fluconazole; *ITZ* itraconazole; *VOR* voriconazole; *POS* posaconazole; *ANF* Anidulafungin; *CSF* Caspofungin; *MCF* Micafungin, *5-FC* 5-Flucytosine; + activity; − no activity; ± intermediate activity

Ghannoum and Rice 1999; Sanglard and Odds 2002; White et al. 1998). As summarized in Table 15.1, antifungal agents target different cellular components important for the growth and survival of fungal pathogens: while azoles and amphotericin B interfere with fungal sterols, the other classes target nucleic acid metabolism (5-FC) and cell wall integrity (echinocandins). The activity of antifungal agents may largely differ between fungal species. For example, fluconazole, an agent widely used to treat candidiasis, is active against several *Candida* species, while inactive against *C. krusei*. In addition, the same agent is not active against most filamentous fungi, while voriconazole, which shares basic structural features of fluconazole, is more active against these species (Table 15.2).

When exposed to antifungal agents, fungal pathogens will respond by using different cellular processes. Short (a few minutes) or long (several hours) drug exposure can result in the reversible activation of signaling pathways and in reversible changes of the transcriptional activity of several genes. These cellular responses help fungal pathogen to adapt to drug stress. The use of microarrays enabling the transcriptional analysis of entire genomes as well as proteome analysis has provided several clues on how fungal pathogens react to the presence of a given drug (Barker et al. 2004; Hooshdaran et al. 2004; Liu et al. 2005; Rogers and Barker 2002; Rogers and Barker 2003).

Fungal pathogens can also undergo genome modifications in the form of mutations arising from positive genetic selection due to the presence of antifungals. This

selection results in acquisition of drug resistance allowing growth at drug concentrations that are otherwise detrimental to the pathogen. However, because some agents exhibit no activity against some pathogens (see Table 15.2), resistance can be intrinsic due to the presence of specific traits in their genomes.

Antifungal resistance is reflected *in vitro* by an increase in minimal inhibition drug concentrations (the MIC, for minimal inhibitory concentration), as compared to MICs measured in control cells that are susceptible to drugs. Because it is measured out of a clinical context, it is referred to as microbiological resistance (Rex et al. 1997). *In vivo* resistance refers to development of antifungal resistance in a clinical environment. *In vitro* measures of antifungal resistance can predict, more or less with confidence, the outcome of a treatment with a given agent. Clinical breakpoints (MIC values above which clinical resistance can be predicted) have been proposed for several agents (Arikan 2007; Rodriguez-Tudela et al. 2007). Clinical resistance, which correlates to *in vitro* measured resistance has been documented for several antifungal agents in most major pathogens (Kontoyiannis and Lewis 2002). With the increasing number of immuno-compromised patients worldwide, antifungal treatments have increased and this has been paralleled by an increase in the incidence of antifungal resistance. A well known example was the occurrence of azole resistance in HIV-positive patients with oropharyngeal candidiasis treated with fluconazole: the incidence of azole resistance in this population was reaching 20% until the introduction of highly active antiretroviral therapy (HAART) that has reduced their immunodeficiency and susceptibility to fungal infections (Martins et al. 1998). Echinocandins have been used in hospitals for several years and cases of resistance are still poorly reported.

Resistance to antifungal drugs in fungal pathogens occurs by specific resistance mechanisms according to different molecular principles including transport alterations, target alterations, utilization of compensatory and catabolic pathways and lastly presence of complex multicellular structures (biofilms). The occurrence of these mechanisms along with their known basis, are summarized in Table 15.2. One can observe from Table 15.2 that resistance mechanisms based on transport alterations have a dominant position. Transport alterations in several fungal pathogens originate from changes in the activity of two major drug transporter families, the ATP Binding Cassette (ABC) transporters and the Major Facilitator transporters. Due to the importance of this resistance mechanism and their association with multidrug resistance, this review will emphasize on this mechanism and on the regulatory circuits important for its control.

15.2 Resistance Mechanisms Mediated by Antifungal Efflux: Azoles as an Exemplary Case

In order to inhibit cell growth, azole antifungal agents have to reach intracellular concentration levels capable of blocking the activity of every target molecule. The mechanism of azole entry in the fungal cell is still not totally understood, but current models support the idea of passive diffusion through the cell wall and cell

membrane (Sanglard 2002). However, it is now well established that fungal cells possess in their plasma membrane active efflux mechanisms that extrude azoles out of the cells. In yeasts, azole-resistance often results from an increased azole efflux, impairing accumulation of azole within the cells, as compared to susceptible strains (Sanglard et al. 1995).

15.2.1 Upregulation of Membrane Efflux Transporters

The interplay between azole resistance and efflux has been extensively investigated for fluconazole and *C. albicans* (Lupetti et al. 2002; Sanglard and Odds 2002; White et al. 1998).

Azole resistance in *C. albicans* clinical isolates involves efflux of fluconazole out of the cell through the constitutive high expression of two types of multidrug efflux transporters: the ATP-binding cassette (ABC) transporters encoded by *CDR* (*Candida* drug response) genes, which use adenosine triphosphate (ATP) as energy source and the Major Facilitators (MFS) to which *MDR1* (multidrug resistance 1) is belonging. This transporter class uses a proton gradient across the membrane as the driving force for transport (Panwar et al. 2008). Overexpression of efflux pumps encoded by *CDR1, CDR2,* and *MDR1* genes has been shown to be one of the most frequent mechanisms of fluconazole resistance. For example, *CDR* genes have been shown to be upregulated in 64% of the strains among a collection of azole-resistant *C. albicans* clinical isolates, *MDR1* overexpressed in 21% and both types of pumps in 3% (Calabrese et al. 2000). Upregulation of *MDR1* leads to resistance to both fluconazole and voriconazole, while upregulation of the highly homologous *CDR1* and *CDR2* genes leads to resistance to multiple azoles including fluconazole, voriconazole, posaconazole and ravuconazole (Li et al. 2004; Marr et al. 1998; Sanglard et al. 1995, 1996, 1997). Azole resistance driven by enhanced efflux has also been characterized in other fungal pathogens including *C. glabrata, C. tropicalis, C. dubliniensis, C. neoformans* and *A. fumigatus,* in which homologues of multidrug transporter genes have been identified and linked to resistance (see below).

Azole resistance of *Aspergillus* species appears to be strongly associated with modifications of lanosterol 14-α sterol demethylase, the target enzyme of azoles (*CYP51*) (Mellado et al. 2007). However, decreased intracellular accumulation of azoles has been linked to decreased susceptibility to itraconazole in *A. fumigatus* laboratory-derived itraconazole resistant strains (Manavathu et al. 1999). Moreover, Nascimento et al. (2003) found that, in addition to a mutation in *CYP51*, itraconazole-resistant isolates of *A. fumigatus* also exhibited high expression levels of two efflux pump genes, *Afu-MDR3* and *Afu-MDR4*, belonging to the MFS and ABC-transporter family, respectively. Finally, itraconazole is able to induce the *A. fumigatus* ABC-transporter gene *atrF* (Slaven et al. 2002). However, the role of these transporters in the resistance of clinical isolates to itraconazole is still not known in detail.

Besides the constitutive upregulation observed in azole-resistant clinical isolates, transient upregulation of multidrug transporter genes can also occur when fungal pathogens are exposed to different drugs. In *C. albicans*, the ABC-transporter genes *CDR1* and *CDR2* can be upregulated by short-term exposure to steroids, antifungal agents (azoles, terbinafine and amorolfine) or other metabolic inhibitors (De Micheli et al. 2002). Expression of the major facilitator gene *MDR1* can be stimulated specifically by the addition of agents such as benomyl or H_2O_2, which have no effect on *CDR* genes expression (Gupta et al. 1998; Rognon et al. 2006).

15.2.2 Efflux Mediated by ABC Transporters

ABC proteins are generally made up of two trans-membrane domains (TMDs) and two cytoplasmic nucleotide binding domains (NBDs). Typically, the TMDs are composed of 12 trans-membrane α-helical segments (TMS) while the NBDs contain both α-helices and β-sheets (Fig. 15.1). The conserved NBDs located at the cytoplasmic periphery posses ATPase activity. The NBDs of all ABC transporters, irrespective of their origin and nature of transport substrate, share extensive amino

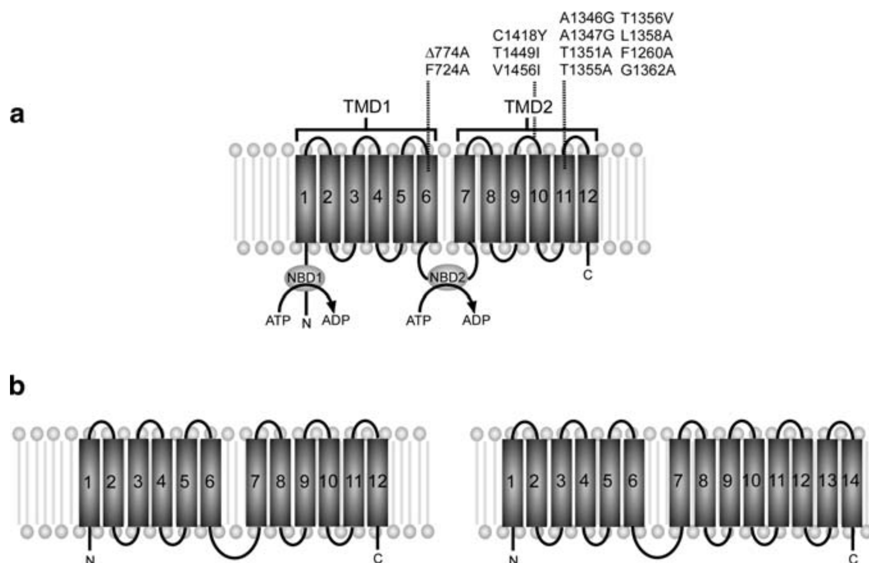

Fig. 15.1 Schematic organization of multidrug transporters. Panel A: Cdr1p as an exemplary ABC-transporter**.** Cdr1p possesses two trans-membrane domains (TMD1 and TMD2) and two nucleotide binding domains (NBD1 and NBD2). The TMDs are composed of 12 trans-membrane α-helical segments numbered from 1 to 12 (TMS). Mutational analysis of Cdr1p has revealed several residues critical for efflux function. The point mutations introduced in Cdr1p are indicated (adapted from (Prasad et al. 2006)). Panel B: Organization of Major Facilitors with 12- and 14-TMS models. Mdr1p from *C. albicans* possesses 12 TMS

acid sequence identity and typical motifs. NBDs of ABC transporters have a β-sheet sub-domain containing the typical Walker A and Walker B motifs, a feature of all ATP requiring enzymes (see below), along with an α-helical sub-domain that possesses the conserved ABC signature sequence. NBD protein sequences possess conserved amino acid stretches critical for functionality including the Walker A motif, with a consensus sequence GxxGxGKS/T (where 'x' represents any amino acid), the Walker B motif (hhhhD, where 'h' represents any aliphatic residue) and an ABC signature LSGGQQ/R/KQR. It is believed that the formation of substrate binding site(s) results from the association of several TMSs, but this feature is probably not sufficient for substrate transport across the membrane phospholipid bilayer (Gaur et al. 2005; Prasad et al. 2006). Transport of ABC transporter substrates across the membrane requires energy from the hydrolysis of ATP carried out at the NBDs with a highly mechanistic conservation. This is reflected by the conservation of the domain architecture exiting among all ABC transporters. The substrate specificity of ABC transporters is highly variable due to the high divergence of primary sequences.

C. albicans possesses two highly homologous ABC-transporters, Cdr1p and Cdr2p, which are composed of two homologous halves, each made up of a hydrophilic, cytoplasmic NBD and TMD composed by six TMS, a so-called (NBD-$TMD_6)_2$ topology (Fig. 15.1). Cdr1p and Cdr2p overexpression is responsible for azole resistance in many clinical isolates recovered from patients receiving long-term antifungal therapy (Sanglard et al. 1995, 1996, 1997). The *CDR1* gene is a homolog of the *S. cerevisiae* pleiotropic drug resistance gene *PDR5* and was originally cloned for its ability to complement the *S. cerevisiae* cycloheximide hypersusceptible *pdr5* mutant (Prasad et al. 1995). Expression of *CDR1* in this *pdr5* mutant also increased resistance to many other drugs, suggesting that *CDR1* was also a multidrug resistance gene. The nature of Cdr1p substrates varies enormously as it includes structurally unrelated compounds such as azoles, lipids, and steroids (Shukla et al. 2003, 2006). Among ABC transporter genes, *CDR1* has been shown to play a key role in azole resistance in *C. albicans* as deduced from its high level of expression found in several azole-resistant clinical isolates. For instance, deletion of both alleles of *CDR1* in *C. albicans* results in high intracellular fluconazole levels. The *cdr1*Δ/Δ mutant strain is hypersensitive to azoles, terbinafine, amorolfine and several other metabolic inhibitors (cycloheximide, brefeldin A and fluphenazine). *CDR1* disruption does not affect susceptibility to amphotericin B or 5-FC (Sanglard et al. 1996).

How substrate specificity and substrate efflux is controlled in the *C. albicans* Cdr1p is a major topic of investigation. Mutational analysis of Cdr1p and evaluation of efflux activity using model compounds (for example, rhodamine 6G) has been performed and revealed several critical residues for efflux (see Fig. 15.1). These residues were located in TMS6 and TMS11 as well as in external cytoplasmic loops (Saini et al. 2005; Shukla et al. 2003).

Intriguingly, *CDR1* and *CDR2* exhibit allelic polymorphisms in *C. albicans* clinical isolates. In one study, six non-synonymous single nucleotide polymorphisms (NS-SNPs) were detected. Cdr1p function was not, however, affected by these

substitutions (Haque et al. 2007). Another study revealed extensive allelic heterozygosity for *CDR2* (Holmes et al. 2006). *CDR2* alleles showed different abilities to transport azoles when individually expressed in *S. cerevisiae*. Two NS-SNPs contributed to functional differences between the proteins encoded by the two *CDR2* alleles. Phylogenetic analysis identified 33 codons in *CDR2* in which amino acid allelic changes could be selectively advantageous. In contrast, all codons in *CDR1*, a gene located approximately 100 kb upstream of *CDR2*, were under purifying selection. The results of this study suggested that *CDR2,* a gene probably duplicated from its ancestor (*CDR1*), was still undergoing evolutionary pressure and function optimization, a feature that is known from other duplicated genes of eukaryotic genomes (Holmes et al. 2006). Recent work also demonstrated differences between Cdr1p and Cdr2p in terms of their relative quantity and of their azole efflux capacities (Holmes et al. 2008). Consistent with this observation, *CDR1* was shown by genetic demonstration to mostly contribute to azole resistance of clinical isolates as compared to *CDR2* (Holmes et al. 2008; Tsao et al. 2009). Whether other ABC transporters (apart from *CDR1* and *CDR2*) among the remaining 26 *C. albicans* putative members (Gaur et al. 2005) contribute to azole resistance has been addressed by several studies but none have yet been identified (Balan et al. 1997; Franz et al. 1998a, b).

In other *Candida* species, functional homologues of *CDR1* and *CDR2* have been described and associated with drug resistance. In *C. glabrata*, the constitutive high expression of ABC-transporter genes *CgCDR1*, *CgCDR2* (also known as *PDH1*), and *CgSNQ2* plays a dominant role in azole resistance (Bennett et al. 2004; Miyazaki et al. 1998; Sanglard et al. 1999, 2001; Torelli et al. 2008; Vermitsky and Edlind 2004). Each of these genes can be upregulated in azole-resistant clinical isolates. The upregulation of *CgCDR1*, *CgCDR2,* and *CgSNQ2* is associated with mutations in a transcriptional regulator (see below), however, it can also be due to mitochondrial deficiencies. *C. glabrata* is prone to spontaneous or induced mitochondrial modifications which themselves result in strong transcriptional changes that include multidrug resistance genes and therefore antifungal resistance (Brun et al. 2003, 2004; Sanglard et al. 2001).

In contrast to *C. albicans CDR* genes, ABC transporter genes of *C. glabrata* are not coordinately expressed in azole-resistant clinical isolates indicating that different combinations of ABC transporters genes can lead to azole resistance (Ferrari et al. 2009). Although *CgCDR1/2* and *CgSNQ2* genes have all been reported to be involved in multidrug resistance, resistance spectra associated with these genes are different. Disruption of *CgCDR1* and *CgCDR2* leads to increased intracellular accumulation of fluconazole and to hypersusceptibility to cycloheximide, chloromphenicol, and several azoles (Izumikawa et al. 2003; Sanglard et al. 1999, 2001; Sanguinetti et al. 2005) while *CgSNQ2* mediates resistance to azoles and specifically to 4-nitroquinoline-*N*-oxide (4-NQO) (Torelli et al. 2008).

Other ABC-transporters from *C. dubliniensis* (*CdCDR1* and *CdCDR2*), *C. krusei* (*ABC1* and 2), *C. tropicalis* (*CDR1*-homolog) and from *C. neoformans* (*CnAFR1*, antifungal resistance 1) were reported as upregulated in azole-resistant isolates (Barchiesi et al. 2000; Katiyar and Edlind 2001; Moran et al. 1998; Pinjon et al.

2005; Posteraro et al. 2003). *ABC1* from *C. krusei* was expressed in *S. cerevisiae* and shown to confer resistance to azoles. However, since Erg11p from *C. krusei* is less sensitive to azoles than other fungal orthologues, *ABC1* is not the only mediator of azole resistance in this yeast species (Lamping et al. 2009). In *A. fumigatus*, *atrF,* and *AfuMDR4* are upregulated in itraconazole-resistant strains (Nascimento et al. 2003). To date, only *CdCDR1* and *CnAFR1*, from among the non-*C. albicans* species, have been experimentally associated with azole resistance (Moran et al. 1998; Posteraro et al. 2003).

15.2.3 Efflux Mediated by MFS Transporters

The MFS superfamily is ubiquitously present in all kingdoms of life. They are involved in the symport, antiport, or uniport of various substrates. Most MFS proteins vary between 400 and 600 amino acid residues in length and possess either 12 or 14 putative TMS with an intercalating cytoplasmic loop (Fig. 15.1). The MFS superfamily consists of 61 families. MFS transporters are energized by the electrochemical proton-motive force, composed of an electrical potential ($\Delta\Psi$) and a chemical proton gradient ($\Delta pH = pH_{external} - pH_{internal}$). In yeast, the MFS-MDR transporters function by proton antiport and are classified into two groups: the drug: H+ antiporter-1 (12 TMS) DHA1 family, and the drug:H+ antiporter-2 (14 TMS) DHA2 family (Gaur et al. 2008; Sa-Correia et al. 2009).

The MFS transporter gene *MDR1* (formerly *BEN*^{r} for benomyl resistance) of *C. albicans* is a member of the DHA1 family and was originally cloned for its ability to confer resistance to several compounds including benomyl, methotrexate, cycloheximide, benztriazole and 4-NQO when expressed in *S. cerevisiae* (Ben-Yaacov et al. 1994; Fling et al. 1991). Whereas *C. albicans* is naturally resistant to these drugs, deletion of both *MDR1* alleles renders the cell susceptible to all these drugs except benomyl. Clinical *C. albicans* isolates overexpressing *MDR1* are more resistant to drugs such as fluconazole, 4-NQO, cerulenin and brefeldin A, as compared to matched isolates with no *MDR1* detectable expression (Wirsching et al. 2001). In contrast to the overexpression of *CDR* genes from which resistance to many different azoles can result, overexpression of *MDR1* appears to be specific to fluconazole transport and is not associated with cross-resistance to other azoles (Sanglard et al. 1995, 1996, 1997). The functional domains required for substrate recognition and drug efflux of Mdr1p are still poorly characterized. One study has addressed the functional relevance of a specific domain in Mdr1p referred to as the antiporter motif (G(X6)G(X3)GP(X2)GP(X2)G), which is located in TMS5 (Pasrija et al. 2007). Alanine scanning mutagenesis on the 21 residues of TMS5 had different impact on the function of Mdr1p. The TMS5 variants were assigned to four categories related to the level of drug resistance and efflux activity. All but 11 residues modified Mdr1p activity. Interestingly, all the amino acid residues modifying Mdr1p activity were clustered in a helical wheel projection of TMS 5. The clustering of mutation-sensitive residues on the same face of the helix further

confirmed that these residues are important for the structural and functional role of the transporter (Pasrija et al. 2007).

Homologues of *MDR1* in *C. dubliniensis* and *C. tropicalis*, named *CdMDR1* and *CtMDR1*, respectively, are upregulated in azole-resistant strains obtained from HIV-positive patients treated with azoles or by *in vitro* exposure in laboratory conditions, respectively. (Barchiesi et al. 2000; Wirsching et al. 2001). Moreover, since the *CdCDR1* gene is inactivated by a point mutation in many *C. dubliniensis* strains and that *CdCDR2* is poorly expressed, *CdMDR1* overexpression is the major remaining mechanism of fluconazole resistance in this species (Moran et al. 1998, 2002; Wirsching et al. 2001). In contrast, *CdMDR1* inactivation does not cause increased susceptibility to amorolfine, terbinafine, fluphenazine, and benomyl (Wirsching et al. 2001). Overexpression of *CtMDR1* confers resistance to the structurally unrelated drugs 4-NQO, cerulenin, and brefeldin A in clinical isolates. In *C. glabrata*, the *MDR1* homologue is *CgFLR1*, which itself is closely related to *FLR1* in *S. cerevisiae* (Chen et al. 2007). *FLR1* is under the control of the transcription factor *YAP1* and is able to confer fluconazole resistance when *YAP1* is activated (Alarco et al. 1997). Although *CgFLR1* can mediate fluconazole resistance when expressed in *S. cerevisiae*, the significance of *CgFLR1* in azole resistance has not been yet demonstrated as it is probably masked by the vast majority of azole-resistant isolates upregulating ABC-transporters (Sanglard et al. 1999). In *A. fumigatus*, *in vitro* generated itraconazole-resistant isolates show constitutive high expression level of the MFS transporter *AfuMDR3* (Nascimento et al. 2003).

Another *C. albicans* gene encoding MFS transporter, *FLU1* (fluconazole resistance), has been isolated by complementation of an azole hypersusceptible *S. cerevisiae pdr5Δ* mutant (Calabrese et al. 2000). The expression of *FLU1* in this strain mediated not only resistance to fluconazole but also to cycloheximide among the different drugs tested. The disruption of *FLU1* in *C. albicans* had only a slight effect on fluconazole susceptibility, but disruption of *FLU1* in a mutant with deletions in several multidrug efflux transporter genes, including *CDR1*, *CDR2,* and *MDR1*, resulted in enhanced susceptibility to several azole derivatives, demonstrating that *FLU1* can mediate azole resistance in *C. albicans*. *FLU1* overexpression has not yet been identified as a cause of azole resistance in clinical isolates (Calabrese et al. 2000).

The availability of fungal genomes enables one to address the involvement of other potential MFS transporters in antifungal resistance. In a recent study, 95 MFS ORFs were identified in the *C. albicans* genome, which were grouped in 17 subfamilies. The drug: H^+ Antiporter-1 (DHA1) family, to which the fluconazole efflux transporters *MDR1* and *FLU1* belong, is the second largest gene family after the sugar transporter family (Gaur et al. 2008). This family contains transporters such as *TMP1* and *TMP2*. Interestingly, the inactivation of these transporters in *C. albicans* results in hypersusceptibility to a number of unrelated compounds such as cycloheximide, 4-nitroquinoline-*N*-oxide and 1,10-phenanthroline. Both genes are also upregulated in response to these drugs, suggesting that they may function as multiple drug efflux pumps (Sengupta and Datta 2003). Genomes of other fungal

species such as *A. fumigatus* contain as many as 295 MFS transporters (data available at http://www.membranetransport.org/), among which 60 have been grouped in the category of multidrug efflux transporters. This large numbers of ORFs and their role in the development of antifungal resistance will represent a challenge for future research in this area.

15.3 Factors Modifying the Activity of Multidrug Transporters

15.3.1 Plasma Membrane Composition and Impact on Transporter Functions

Due to their cellular localization, multidrug transporters establish a close interaction with membrane lipids. It is also known that yeast transporters such as Pdr5p or Cdr1p and Cdr2p can translocate phospholipids between the two monolayers of the plasma membrane (Panwar et al. 2008). The function of multidrug transporters is dependent on the nature and the physical state of the surrounding lipids. Functionality of these transporters can be thus modulated by the lipid environment when it is altered by specific mutations (Mukhopadhyay et al. 2004). Moreover, specific transporters can be recruited to the so-called detergent-resistant membrane (DRM) microdomains or "lipid rafts". Lipid rafts are enriched with sterols and allow interactions between different partners of metabolic cascades, ensuring their efficiency (Mukhopadhyay et al. 2004). *C. albicans* Mdr1p and Cdr1p are preferentially localized within rafts when expressed in *S. cerevisiae* (Pasrija et al. 2008). Using this yeast as a model for *C. albicans* and exploiting the availability of the *S. cerevisiae* mutant collection, it has been possible to address the importance of membrane composition by expressing the *C. albicans* membrane proteins in known *S. cerevisiae* mutants. For example, Cdr1p is inappropriately targeted when sphingolipid biosynthesis is compromised in *sur4*Δ, *fen1*Δ, and *ipt1*Δ mutants, or ergosterol biosynthesis in *erg24*Δ, *erg6*Δ, and *erg4*Δ mutants. In these mutants, Cdr1p accumulates in intracellular cell compartments. In contrast, Mdr1p displayed no sorting defects in the same mutant backgrounds and showed normal activity (Pasrija et al. 2008). Therefore, although both multidrug transporter types are directed to the same subcellular compartment, the functional lipid requirements are very different and suggest that Cdr1p function will be more sensitive to imbalance in the plasma membrane composition. No data addressing the role of lipid environment on transporter function are yet available for Cdr2p.

15.3.2 Posttranslational Modifications

Like other proteins produced in living cells, multidrug transporters can undergo posttranslational modifications. It has been shown that CgCdr1p and CgCdr2p, when expressed in *S. cerevisiae,* can be phosphorylated and thus decrease the

ATPase activity of CgCdr1p and the drug efflux activity of CgCdr2p (Wada et al. 2005). CgCdr2p phosphorylation was PKA-dependent, while that of CgCdr1p was PKA-independent. Multiple phosphorylation sites exist in these proteins, but a few have been identified experimentally. Using specific p-Ser or p-Thr antibodies, some of these sites and among them CgCdr1p Ser^{307} (located near NBD1) and Ser^{484} (located downstream of NBD1 but near to TMS1) were dephosphorylated after glucose depletion and rephosphorylated during glucose exposure or under stress (Wada et al. 2002). The phosphorylation sites near NBD1 of CgCdr1p suggest that this domain is an important sensor of physiological stimuli.

Ubiquination of membrane proteins has been long recognized as a prerequisite for their targeting vacuoles or proteasomes and represent another possible mode of selective modification or inactivation of multidrug transporters (Dupre et al. 2004). Until now, only Pdr5p from *S. cerevisiae* has been demonstrated to undergo ubiquination before internalization within subcellular compartments for degradation (Egner and Kuchler 1996). This process presents the means by which multidrug transporter activity can be rapidly modified when required. Although it is likely to be a conserved mechanism, transporter ubiquination has not yet been demonstrated in pathogenic yeast.

15.4 Molecular Mechanisms of Multidrug Transporter Regulation

15.4.1 Experimental Approaches for the Identification of cis- and trans-Elements Regulating Multidrug Transporters Expression

15.4.1.1 Model Systems and Their Application to Fungal Pathogen Research

The identification of *trans*-acting factors regulating ABC-transporters in pathogenic fungi relied first on the well described PDR network involved in multidrug resistance in *S. cerevisiae*. The two Zn_2-Cys_6 transcription factors *PDR1* and *PDR3* are master regulators of this network and control multidrug resistance by modifying the expression of several ABC- (*PDR5*, *SNQ2* and *YOR1*) and MFS- (*FLR1*, *TPO1*) transporters as well as other genes determining cell membrane composition (*PDR16*, *RSB1*, *LPT1*). *In silico* searches for *PDR1*/*PDR3* homologues in the genomes of pathogenic fungi has been performed, and so far only one functional homolog, *CgPDR1* from *C. glabrata* has been described (Vermitsky and Edlind 2004). CgPdr1p has 40% and 35% identity with Pdr1p and Pdr3p, respectively, and complements a *pdr1*Δ S. cerevisiae mutant strain (Tsai et al. 2006). Similar to *S. cerevisiae*, the expression of *C. glabrata CgCDR1/2* and *CgSNQ2* genes is regulated by CgPdr1p (Vermitsky and Edlind 2004). Deletion of *CgPDR1* in *C. glabrata* azole-resistant clinical isolates leads to a loss of *CgCDR1*, *CgCDR2*,

and *CgSNQ2* regulation and to a sharp increase in azole susceptibility, indicating that *CgPDR1* is the main regulator of efflux-mediated azole-resistance in *C. glabrata* (Torelli et al. 2008; Vermitsky et al. 2006). Deletion of *CgPDR1* also abolishes *CgCDR1* and *CgCDR2* upregulation in the presence of fluconazole (Vermitsky et al. 2006). It is now well established that *S. cerevisiae* Pdr1p and Pdr3p act through *cis*-acting sites present in the promoters of target genes. The consensus motif is named PDRE (for pleiotropic drug resistance element) and is present in several ABC-transporter gene promoters such as *PDR5*, *SNQ2,* and *YOR1* (MacPherson et al. 2006). In *C. glabrata*, a genome-wide study identified genes regulated by *CgPDR1* and, by analysis of the promoters, the sequence 5′-TCC(GA)(CT)GAA-3′ was identified as a strong candidate for *C. glabrata* PDRE. This sequence is found in the promoters of *CgCDR1, CgCDR2* and *CgSNQ2* genes, suggesting that CgPdr1p binds directly to PDRE sequences to regulate transcription of target genes (Torelli et al. 2008; Tsai et al. 2006; Vermitsky et al. 2006). *CgPDR1* contains a PDRE in its promoter suggesting an auto-regulation of its transcription. Consistent with this observation, upregulation of *CgCDR1* and *CgCDR2* is correlated in some azole resistant strains with an increase of *CgPDR1* expression (Tsai et al. 2006; Vermitsky et al. 2006). However, although promoters regions of *CgCDR1*, *CgCDR2*, *CgSNQ2* and *CgPDR1* genes all contain PDRE, they are not simultaneously expressed in clinical azole-resistant isolates (Ferrari et al. 2009). CgPdr1p acts as nuclear receptor by directly binding to diverse drugs and xenobiotics, such as azoles, to activate expression of efflux pump genes resulting in multidrug resistance (Thakur et al. 2008). A small portion of the activation domain of CgPdr1p binds directly to the KIX domain of the Mediator co-activator subunit CgGal11p in a xenobiotic-dependent manner in order to activate transcription of target genes (Thakur et al. 2008).

There are no close homologues of *PDR1* or *PDR13* in the *C. albicans* genome. To aid their identification, two distinct genetic screens were carried out in order to complement a *S. cerevisiae pdr1/pdr3* mutant strain for drug resistance. These screens led to the identification of five genes *FCR1* and *FCR3* (fluconazole resistance) and *CTA4*, *ASG1* and *CTF1* (Coste et al. 2008; Talibi and Raymond 1999). Although expression of these genes in a *S. cerevisiae* strain lacking *PDR1* or *PDR3* could confer resistance to azoles, deletion of *CTA4*, *ASG1* and *CTF1* in *C. albicans* had no effect on fluconazole susceptibility. In contrast, deletion of *FCR1* resulted in a mutant hyperresistant to fluconazole, and thus *FCR1* may act as a negative regulator of fluconazole susceptibility (Coste et al. 2008; Talibi and Raymond 1999). In another attempt, a *C. albicans* genomic library was screened for high *CDR1* reporter activity using a *LacZ* reporter system under the control of the *CDR1* promoter prompting the isolation of the *CaNTD80* gene with similarity to *NTD80* of *S. cerevisiae*. This gene encodes a meiosis- specific transcription factor. Disruption of *CaNDT80* was shown to affect basal expression levels of *CDR1* in *C. albicans* and to reduce the ability of this gene to be transiently upregulated by miconazole (Chen et al. 2004). However, there is presently no evidence that this transcriptional regulator is responsible for the constitutive overexpression of efflux pumps in clinical isolates.

15.4.1.2 Systematic Analysis of *cis*-Acting elements in the Promoters of Multidrug Transporters

The successful isolation of regulators of multidrug resistance in *C. albicans* was based on different strategies. One emerged through the analysis of *cis*-acting elements in *CDR1*/*CDR2* and *MDR1* (see below), while the other was deduced from genome-wide transcriptional analysis of *MDR1*-upregulating strains (see Sect. 15.4.1.3).

The systematic molecular dissection of the *CDR1* and *CDR2* promoters identified five distinct regulatory elements: the BBE (basal expression element) responsible for basal expression, the DRE (drug-responsive element) required for the response to drugs such as fluphenazine and oestradiol, two SREs (steroid responsive element) involved in the response to steroid hormones and the NRE (negative regulatory element) (De Micheli et al. 2002; Gaur et al. 2004; Karnani et al. 2004). Internal deletions of the BEE and DRE in the *CDR1* promoter affect basal *CDR1* expression and drug-induced expression, respectively. Conversely, the deletion of the NRE leads to an increase in the basal expression of *CDR1*. In contrast to *CDR1*, the *CDR2* promoter only contains the DRE element (De Micheli et al. 2002). Among these different *cis*-acting elements, only the DRE was shown to be involved not only in the transient upregulation of both *CDR1* and *CDR2* in response to inducers but also in their constitutive high expression in azole-resistant clinical isolates (De Micheli et al. 2002). The DREs present in the promoter of *CDR* genes contain two CGG triplets, which are potentially recognized by Zn_2-Cys_6 transcription factors (Hikkel et al. 2003; Kren et al. 2003; Mendizabal et al. 1998; Schjerling and Holmberg 1996). In order to isolate regulators of *CDR1* and *CDR2*, the *C. albicans* genome was searched for genes encoding proteins with Zn_2-Cys_6 fingers. Interestingly, three of these genes were arranged in tandem near the mating locus (*MTL*) (see also Fig. 15.2), the homozygosity of which is linked to the development of azole resistance in *C. albicans* (Rustad et al. 2002). Deletion of one of these genes, *TAC1* (transcriptional activator of *CDR* genes), in an azole-susceptible strain led to increased drug susceptibility and to loss of transient *CDR1* and *CDR2* upregulation in the presence of inducers. In azole-resistant *C. albicans,* clinical isolates deletion of *TAC1* abolishes *CDR1* and *CDR2* expression and therefore drug resistance demonstrating that *TAC1* is the main mediator of ABC transporter-mediated azole resistance due to the upregulation of ABC transporter in *C. albicans* (Coste et al. 2004). Tac1p acts by direct binding to the DRE present in the promoter region of both efflux pump genes and induces their expression in response to steroid and several toxic chemicals (Coste et al. 2004; De Micheli et al. 2002). Tac1p is not involved in the basal expression of *CDR1* and the transcription factor regulating *CDR1* expression through the BEE element remains to be identified.

Functional dissection studies of the *MDR1* promoter have identified two distinct regulatory regions. The BRE (benomyl response element) also called MDRE (*MDR1* drug resistance element) is responsible for the constitutive high expression of *MDR1* in fluconazole-resistant isolates (Harry et al. 2005; Riggle and Kumamoto 2006;

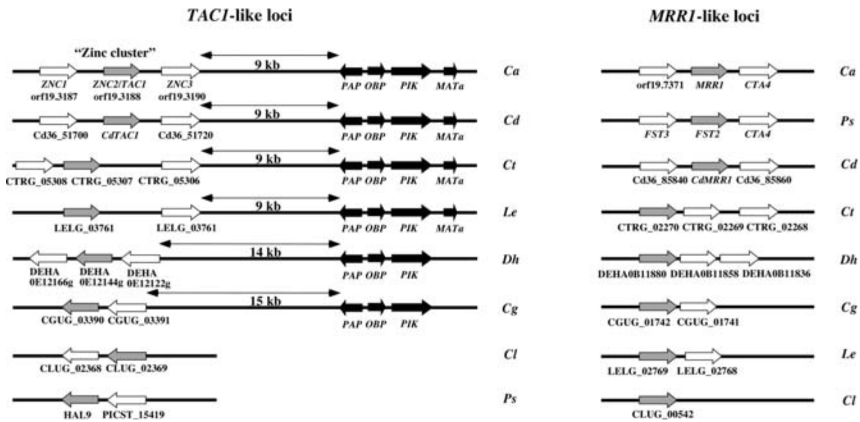

Fig. 15.2 Genomic loci containing *TAC1*- and *MRR1*-like genes in several fungal species. Grey arrows indicate the most similar ORFs to *TAC1* (left panel) and *MRR1* (right panel) in corresponding loci. Genome data were obtained from the CandidaDB database (http://genodb.pasteur.fr/) or from *Candida dubliniensis* GeneDB (http://www.genedb.org/genedb/cdubliniensis/index.jsp). The distance between the zinc finger ORF and the first ORF of the *MTL* locus is indicated by arrows and given in kb. Abbreviations: *Ca: C. albicans; Cd: C. dubliniensis; Ct: C. tropicalis; Cg: C. guilliermondii; Cl: C. lusitaniae; Le: L. elongisporus; Dh: D. hansenii; Ps: P. stipitis*

Rognon et al. 2006). This region is also necessary for the inducible expression of *MDR1* in response to benomyl (Harry et al. 2005; Rognon et al. 2006). The second element, the HRE (H_2O_2 response element) is involved in the response to oxidative stress agents. In contrast to the BRE, the HRE is not required for constitutive upregulation of *MDR1* in azole-resistant isolates. The HRE region contains two YRE (*YAP1* response element) motifs and the BRE/MDRE contains a perfect match for the Mads-box transcription factor Mcm1p (Harry et al. 2005; Nguyen et al. 2001; Riggle and Kumamoto 2006). However, there is no direct evidence either for interactions between Yap1p and the HRE and between Mcm1p and the BRE or for the involvement of Yap1p and Mcm1p in the inducible or constitutive expression of *MDR1*.

15.4.1.3 Transcriptional Profiling for Regulator Identification

The molecular basis for the constitutive upregulation of the Major facilitator gene *MDR1* has been recently elucidated. A genome-wide study was undertaken to compare the transcriptional profiles of three different *C. albicans* clinical isolates overexpressing *MDR1* in order to identify genes commonly upregulated with *MDR1*. One of the genes of interest was orf19.7372 since it contained a Zn_2-Cys_6 zinc finger motif of the same type as *TAC1*. Because inactivation of orf19.7372 caused loss of *MDR1* upregulation, the transcription factor was called Mrr1p (multidrug resistance regulator) (Morschhauser et al. 2007). *MRR1* inactivation in

azole-resistant isolates resulted in the loss of *MDR1* expression and increased susceptibility to fluconazole, cerulenin and brefeldin A (Morschhauser et al. 2007). Deletion of *MRR1* in a drug-susceptible strain abolished *MDR1* upregulation in the presence of inducing chemicals like benomyl and H_2O_2, thus demonstrating that Mrr1p mediates both inducible *MDR1* expression and constitutive *MDR1* overexpression in drug-resistant strains (Morschhauser et al. 2007). A *MRR1* homolog has been identified in *C. dubliniensis* and was shown responsible for *CdMDR1* expression (Schubert et al. 2008). Although Mrr1p has not been yet shown to act directly at the *MDR1* promoter the protein is able to heterologously activate the *MDR1* promoter in *C. albicans*, suggesting that Mrr1p and CdMrr1p recognize the same binding site(s), which should be present in the *MDR1* promoters of both species (Schubert et al. 2008).

15.4.2 Coactivators in the Regulation of Multidrug Resistance Genes

In eukaryotes, transcription factors are part of larger complexes, which are necessary for gene transcription and this is also the case for regulators of multidrug resistance. In this perspective, a recent study has demonstrated the role of the Mediator complex in the transcriptional response of multidrug transporter genes in *S. cerevisiae* and *C. glabrata*. One of the subunit of this complex, Gal11p, was shown to bind Ppdr1p from *S. cerevisiae*. Pdr1p itself acts as a nuclear receptor and, in the drug-bound stage, stimulates its association with Gal11p as a step for Mediator recruitment (Thakur et al. 2008). This binding is crucial for the upregulation of *PDR1* target genes. This process was conserved in *C. glabrata*, but it is still hypothetical in the other relevant fungal pathogens.

The mediator complex can associate with other transcriptional activators; one of them is called the SAGA complex. Recently, the transcriptional adapter Ada2p, which is part of the SAGA co-activator complex, has been shown to bind directly to both *CDR1* and *MDR1* promoters in *C. albicans* (Sellam et al. 2009). Ada2p is recruited to the *MDR1* promoter in an Mrr1p-dependent manner indicating that Ada2p functions as a co-activator for Mrr1p. In contrast, the transcription factor recruiting Ada2p to the *CDR1* promoter is still unclear. Nevertheless, deletion of *ADA2* reduces the transient upregulation of *MDR1* and abolishes *CDR1* expression in response to fluconazole (Sellam et al. 2009). Given these novel observations, it is possible that an association between the Mediator- and SAGA complexes might occur at promoters of *TAC1*- and *MRR1*-regulated genes. Together with transcription factors, these complexes help the recruitment of the RNA polymerase, which itself results in the transcription of target genes (Daniel and Grant 2007).

Despite all these recent discoveries, the molecular details that dictate the function of the transcriptional machinery regulating multidrug transporters are still at a preliminary stage but constitute a strong basis for future studies.

15.4.3 Conservation of Multidrug Resistance Regulators in Pathogenic Fungi

Following *TAC1* and *MRR1* identification in *C. albicans*, the search for functional homologues in other fungal species became possible due to availability of genome data. A striking feature of *TAC1* is that it is situated near the mating type locus on Chr. 5 within a group of two other genes (the so-called "zinc cluster") with the same zinc finger signature (Fig. 15.2). Curiously, *MRR1* is also located within a similar cluster of zinc finger encoding genes but on another chromosome (Chr. 3). It is likely that both clusters emerged from independent gene duplication events. Gene duplications found in genomes are indicative of acquisition of novel functions originating from an ancestor gene. Under selective pressure, acquisition of novel functions can arise from gene duplication as an evolutionary process. This process has been illustrated by *CDR1* and *CDR2* (see above) (Holmes et al. 2006). It is intriguing that two major regulators of drug resistance are found within such clusters. The genes of these clusters may actually still undergo selective evolution. This property may be needed for genes participating in multidrug resistance, which eventually leads to protection of fungi against a large variety of toxic compounds. The zinc finger proteins flanking *TAC1* and *MRR1* have not yet been systematically investigated, with the exception of *CTA4,* which is located near *MRR1* in *C. albicans* (Fig. 15.2). This gene was isolated in a genetic screen for complementation of *PDR1*/*PDR3* function in *S. cerevisiae*, but was later found to be involved in nitrosative stress response but not in antifungal resistance in *C. albicans* (Chiranand et al. 2008; Coste et al. 2008). Until now, inactivation of genes of these two clusters did not reveal a role in multidrug resistance (Coste et al. 2004, 2008). Blast searches performed with *TAC1* and *MRR1* identified putative homologues in *C. dubliniensis*, *C. tropicalis*, *C. parapsilosis*, *C. lusitaniae*, *C. guilliermondii*, *Debaryomyces hansenii*, *Lodderomyces elongisporus* and *Pichia stipitis*. It was difficult to identify clear homologues of *TAC1*/*MRR1* in other clinically relevant species such as *A. fumigatus* or *Cr. neoformans* due to low similarity scores of blast searches. Mapping of the *TAC1*/*MRR1* homologues in the contig assemblies revealed various degrees of synteny in the loci containing these genes (Fig. 15.2). The physical linkage between the mating locus of *TAC1*-like genes is conserved for most investigated species with the exception of *C. lusitaniae* and *P. stipitis*. *D. hansenii* and *C. guilliermondii* have the particularity that they lack, as far as genome data can predict, a *MATa* locus next to the group of genes normally present in *MTLa* (*PAPa*, *OBPa*, *PIKa* and *MATa*). As shown in Fig. 15.2 (right panel), the *MRR1*-like loci are also conserved among the most investigated species, however, the cluster has been reduced for *C. guilliermondii* and *L. elongisporus* and is lost in *C. lusitaniae*. *C. lusitaniae* is therefore the most divergent species in the conservation of the tandemly arranged clusters of both transcription factors. Future studies are still needed to verify if all above-mentioned *TAC1*- and *MRR1*-like genes actively participate in multidrug resistance of respective species.

15.4.4 Gain-of-Function Mutations in Transcription Factors Regulating Efflux Genes

TAC1, *MRR1,* and *CgPDR1* gene encode transcriptional regulators regulating efflux pump expression in both azole-susceptible and azole-resistant clinical strains of *C. albicans* and *C. glabrata* respectively. However, alleles isolated from azole-resistant isolates can confer constitutive high expression of efflux pumps and thus azole resistance when expressed in an azole-susceptible backgrounds, indicating that *TAC1*, *MRR1,* and *CgPDR1* alleles isolated from azole-resistant strains differ from those found in azole-susceptible strains (Coste et al. 2004, 2006a, b; Dunkel et al. 2008a, b; Ferrari et al. 2009; Morschhauser et al. 2007; Torelli et al. 2008; Tsai et al. 2006; Vermitsky et al. 2006). This was first demonstrated in *C. albicans* in which two types of *TAC1* alleles can be isolated from clinical isolates: wild-type alleles, which confer transient *CDR1* and *CDR2* upregulation in response to drugs, and hyperactive alleles, which can be isolated from azole-resistant strains and can confer constitutive high *CDR1* and *CDR2* expression and therefore drug resistance to a mutant strain lacking *TAC1* (Coste et al. 2004, 2006a, b). Sequencing of these alleles revealed that wild-type and hyperactive alleles differ by single point mutations leading to single amino acid substitutions defined as gain-of-function (GOF) mutations. The presence of a GOF mutation alone can account for the constitutive high expression of *CDR* genes, as introduction of a GOF mutation in a wild type allele confers hyperactivity to *TAC1* (Coste et al. 2004, 2006a, b). In addition, wild type and hyperactive alleles are co-dominant. It is only when hyperactive alleles are in the homozygous state that their phenotypes are fully expressed (Coste et al. 2006a, b). Large-scale sequencing of *TAC1* alleles from *C. albicans* clinical isolates allowed, up to now, the identification of 39 hyperactive alleles harboring 16 different GOF mutations at 12 distinct positions along *TAC1*. Three other GOF mutations introduced by random mutagenesis were also able to confer hyperactivity to a *TAC1* wild type allele (Coste et al. 2004, 2006a, b, 2009; Znaidi et al. 2007). The majority of these GOF mutations (15) are located in the C-terminal portion of *TAC1* corresponding to a putative transcriptional activation domain, while the remaining mutations are situated in the MHR and the N-terminal part of the protein, a region with no defined function (Fig. 15.3). Interestingly, five mutations introduced by random mutagenesis abolished completely the transcriptional activity of *TAC1* even in the presence of inducers. These mutations were termed LOF (loss-of-function) mutations and were located both at the N- and C- terminal parts of the protein (LOF mutations are indicated by grey bars in Fig. 15.3). How these mutations affect the transcriptional activity of *TAC1* remains unknown. Although other transcription factors have been shown to regulate *CDR1* expression (Chen et al. 2004; Gaur et al. 2004), only mutations in Tac1p have been found to mediate efflux pump overexpression in clinical *C. albicans* isolates.

Similar to *CDR1/2* upregulation by Tac1p, *MDR1* overexpression is also caused by GOF mutations in its regulator, Mrr1p (Morschhauser et al. 2007). So far, 14 distinct GOF have been identified in *MRR1* on 13 distinct positions spanning

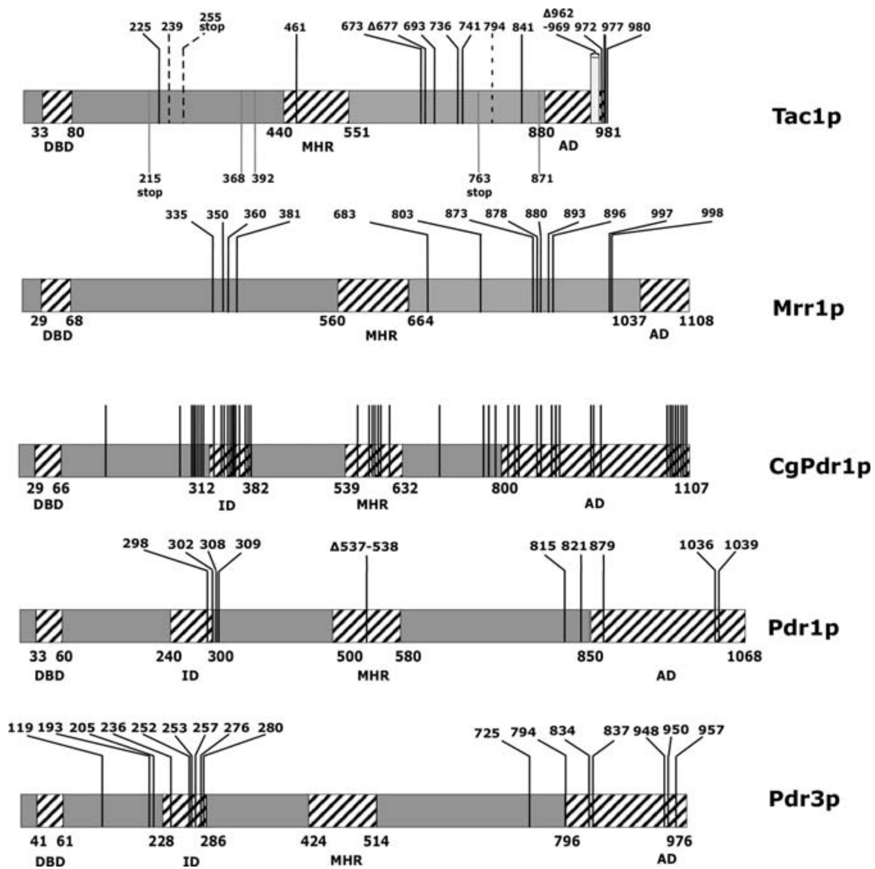

Fig. 15.3 Gain-of-function mutations in transcription factors regulating efflux genes. GOF mutations (black bars) identified in the transcription factors Tac1p, Mrr1p, CgPdr1p, Pdr1p and Pdr3p. Hatched bars in Tac1p signify that GOF mutations were obtained by random mutagenesis. The GOF mutation deleting amino acid position 962 to 969 is indicated by a rectangle. *TAC1* random mutagenesis allowed the identification of 5 LOF mutations (indicated by grey bars) (Coste et al. 2009). Data relevant for *MRR1*, *CgPDR1*, *PDR1* and *PDR3* were obtained from published reports (Dunkel et al. 2008a, b; Ferrari et al. 2009; Kolaczkowski and Goffeau 1997; Mizoguchi et al. 2002; Morschhauser et al. 2007; Nourani et al. 1997; Vermitsky and Edlind 2004)

throughout the open reading frame (Fig. 15.3) (Dunkel et al. 2008a, b). The hyperactive phenotype appears to be dose-dependent as the presence of two instead of one *MRR1* mutated allele results in increased drug resistance (Morschhauser et al. 2007). As for Tac1p, the functional domains of Mrr1p are still unknown. It is therefore difficult to speculate about the molecular mechanism underlying Mrr1p hyperactivity. Nevertheless, GOF mutations were also identified in *CdMRR1*, the *MRR1* homolog of *C. dubliniensis*, and expression of mutated *CdMRR1* in a *C. albicans mrr1*Δ/Δ deletion strain results in *MDR1* overexpression, indicating

that GOF mutations affect similarly the activity of Mrr1p in both *C. albicans* and *C. dubliniensis* (Schubert et al. 2008).

C. glabrata differs from *C. albicans* with respect to the diversity of GOF mutations in *CgPDR1*. The number of GOF mutations in *CgPDR1* is much higher than in Tac1p and Mrr1p from *C. albicans* (Ferrari et al. 2009). On the other hand, because CgPdr1p is closely related to the well-known *S. cerevisiae* Pdr1p and Pdr3p transcription factors, functional domains can be deduced using sequence comparisons. Three studies have identified four separate amino acid substitutions in CgPdr1p of azole-resistant strains, which are responsible for constitutive high expression of ABC-transporter genes and of *CgPDR1* itself (Schubert et al. 2008; Torelli et al. 2008; Tsai et al. 2006; Vermitsky et al. 2006). Large-scale analysis of *CgPDR1* alleles from *C. glabrata* clinical isolates allowed the identification of 70 alleles, among which only 12 were wild type and 58 were hyperactive alleles. These 58 hyperactive alleles contain 58 distinct GOF mutations yielding 57 single amino acid substitutions located at 51 different positions localized along the protein with some "hot spots" near the N-terminal inhibitory domain, the central MHR (Middle Homology Region) and the C-terminal activation domain (Ferrari et al. 2009). The localization of CgPdr1p mutations is similar to gain-of-function mutations described in *S. cerevisiae* homologues Pdr1p/Pdr3p (Fig. 15.3).

Most GOF mutations in *PDR3* map to the inhibitory activation domains of the protein (Nourani et al. 1997). In contrast, *PDR1* mutations are scattered throughout the entire protein with some hot spots at the C-terminus (Carvajal et al. 1997). *PDR1/PDR3* GOF mutations are distributed in specific protein domains as reported from several studies (Carvajal et al. 1997; Mizoguchi et al. 2002). Figure 15.3 summarizes the distribution of GOF mutations among several Zn_2-Cys_6 regulators of drug resistance isolated in several yeast species. At least two "hot spots" for GOF mutations can be clearly identified. One region covers the domain referred to as the transcriptional inhibitory domain and the other region is closer to the C-terminal region of the transcription factor corresponding to the activation domain. *CgPDR1* GOF mutations cover, in addition, a region corresponding to the MHR. GOF mutations of yeast Zn_2-Cys_6 transcription factors involved in other types of stresses target the above-mentioned domains also. For example, GOF mutations of the *S. cerevisiae* War1p, which is involved in weak acid stress response, were reported in the MHR and activation domains (Gregori et al. 2007). The molecular mechanisms behind the significance of these "hot spots" remain to be investigated in the future.

Given the high diversity of GOF mutations found in *CgPDR1,* it is unlikely that all mutations similarly affect the transcriptional activity of CgPdr1p in order to mediate ABC-transporter gene overexpression. Hence, GOF mutations in the inhibitory domain might impair transcriptional inhibition and those in the activation domain might induce hyperactivation as proposed for *PDR3* in *S. cerevisiae*. The effect of the GOF mutations present in the MHR might be enlightened by the recent finding that CgPdr1p acts as a nuclear receptor by direct binding to azoles and xenobiotics to activate expression of efflux pump genes (Thakur et al. 2008). For instance, the xenobiotic binding domain of *S. cerevisiae* Pdr1p has been mapped between amino acids 352 and 543, which corresponds by sequence alignment to the

MHR region of CgPdr1p. GOF mutations in the MHR might bypass the requirement of azole binding in order to activate efflux pump gene transcription. Moreover, once bound to azole, CgPdr1p binds directly to the KIX motif of the Mediator subunit Gal11p in order to activate transcription of target genes. The CgPdr1p portion interacting with the KIX domain has been mapped in the activation domain between amino acids 1074–1104 (Thakur et al. 2008). Interestingly, nine different GOF mutations were identified within this short motif. It has thus been proposed that GOF mutations within this motif might modify the interaction with the KIX domain leading to CgGal11p recruitment in the absence of drug binding.

Another feature of *CgPDR1* GOF mutations is that they induce distinct patterns of ABC-transporter constitutive overexpression. For example, the P822L substitution is responsible for the constitutive overexpression of *CgSNQ2*, but has no effect on the expression of *CgCDR1* and *CgCDR2* (Torelli et al. 2008). In addition, two distinct mutations in the CgPdr1p KIX-interacting motif, E1083Q and D1082G, drive selective upregulation of *CgCDR1* alone or of both *CgCDR1* and *CgCDR2,* respectively, indicating that mutations within a domain do not yield similar ABC-transporter expression patterns (Ferrari et al. 2009). Nevertheless, *CgPDR1* GOF mutations have differentiated effects on target genes including the major ABC-transporters involved in azole resistance. The precise molecular mechanism of this selective upregulation remains to be determined.

15.4.5 Chromosomal Rearrangements Affecting Multidrug Transporter Expression

In *C. albicans* high levels of azole resistance usually develop gradually as a result of sequential alterations because of the continuous pressure exerted by the drug. The acquisition of GOF mutations in *TAC1* represents only a first step in the development of azole resistance. As *TAC1* wild type and hyperactive alleles are co-dominant, two *TAC1* hyperactive alleles are required in order to develop high levels of azole resistance (Coste et al. 2004, 2006a, b). The acquisition of a second *TAC1* hyperactive allele results either from the acquisition of a GOF mutation in the second allele or from loss-of-heterozygosity (LOH) via mechanisms including, chromosome loss and reduplication or mitotic recombination. These chromosome alterations can be accompanied by acquisition of additional chromosomal elements by isochromosome formation, resulting in an increased *TAC1* copy number. Moreover, isochromosome formation from Chromosome 5, increases the copy number not only of *TAC1*, but also of the resistance gene, *ERG11*, which is also present on this chromosome (Coste et al. 2007; Selmecki et al. 2006). All these mechanisms contribute to elevated drug resistance in *C. albicans* (Coste et al. 2006a, b, 2007).

Consistent with the observations made with *TAC1*, the presence of one mutated *MRR1* allele leads to intermediate resistance levels, while the presence of two mutated *MRR1* alleles results in higher *MDR1* transcript and to further increased fluconazole resistance (Dunkel et al. 2008a, b; Franz et al. 1998a, b). Similar to

TAC1, most clinical isolates overexpressing *MDR1* contain two *MRR1* alleles (Dunkel et al. 2008a, b). The acquisition of the second allele occurs by two types of LOH events: loss and reduplication of Chr. 3, on which *MRR1* is located, and mitotic recombinations. In azole-resistant clinical isolates, *MRR1* overexpression seems not be associated with an increase in the copy number of *MRR1,* suggesting that the development of high *MDR1* expression levels do not involve the acquisition of extra chromosomal elements (Dunkel et al. 2008a, b).

15.5 Strategies to Combat Multidrug Resistance in Yeast

The identification of important mediators of multidrug resistance clearly opens up the possibility of designing inhibitors to disable their function. The inhibition of multidrug transporters and of their regulators could in principle disarm fungal pathogen not only for their capacity to extrude antifungal drugs but also for their capacity to respond transiently to drug stress. Combining antifungal agents and inhibitors of multidrug resistance is one means of limiting the impact of antifungal drug resistance. Several drug efflux transporters have been investigated by heterologous expression in *S. cerevisiae,* where they confer increased resistance to azoles (Lamping et al. 2007). This provides a system to screen for pump inhibitors that chemosensitize to azoles strains overexpressing pumps (Niimi et al. 2004). In the past years, several authors have reported the identification of peptide-based transporter inhibitors, phenothiazine- or quinazolinone-derivatives using chemosensitisation assays that were designed with *S. cerevisiae* and also validated using clinical fungal isolates (Kolaczkowski et al. 2009; Lemoine et al. 2004; Niimi et al. 2004; Schuetzer-Muehlbauer et al. 2003; Tanabe et al. 2007; Watkins et al. 2004). On the other hand, regulators of multidrug resistance have also been used for designing cell-based assays enabling discovery of inhibitors (Kozovska and Subik 2003). The recent finding that PDR regulators (Pdr1p, Pdr3p) are functioning like nuclear receptors for ligands opens novel opportunities to block their activity (Monk and Goffeau 2008; Thakur et al. 2008). It is conceivable that ligands can be designed to inhibit their coupling to basal elements of the transcriptional machinery, including the Mediator complex. Future studies will be necessary to evaluate this possibility and to implement this concept in other fungal pathogens.

15.6 Conclusion

This review has highlighted the current status in the understanding of multidrug resistance in fungal pathogens. Within the last years, our understanding of this important process has progressed considerably. What originated as clinical observations made on specific fungal pathogens have now been dissected at the molecular level. Multidrug transporters from two families participate in the development

of multidrug resistance. With increasing numbers of fungal genomes becoming available, other transporters of these families have been revealed and further investigations will be needed to help characterize these proteins and their potential role in multidrug resistance. We are facing several other challenges in this research area, one of which is to improve structure–function relationships of known transcriptional activators. It is still not clear how transcriptional regulators of multidrug resistance can be activated by external stimuli and how this activation can recruit the transcriptional machinery that ultimately results in enhanced gene expression of target genes. Likewise, the effect of GOF mutations on the transcriptional activators, the resulting increase of gene expression and the other partners involved in this process still remain incompletely characterized, even though breakthroughs have recently been published. Moreover, the list of transcriptional activators involved in mediation of multidrug resistance in fungal pathogens is still not exhausted. With more sophisticated analysis tools and powerful genetics implemented in fungal pathogens, our field of investigation is still largely open to discoveries.

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