Adaptations in the wall proteome of the clinical fungus Candida albicans in response to infection-related environmental conditions
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Chapter 1

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1. General introduction

1.1 General description of *Candida albicans*: taxonomy, genome, and biology

*Candida albicans* is an opportunistic pathogenic fungus which in immunocompromised individuals can cause life-threatening infections with a 40% mortality rate (Wey, Mori *et al.* 1988). Its infections are difficult to treat as *C. albicans* tends to acquire resistance to most antifungal drugs, including the commonly used azoles. *C. albicans* belongs to the genus *Candida* (order Saccharomycetales, class Saccharomycetes, kingdom Fungi, superkingdom Eukaryota). The binominal name *Candida albicans* was proposed by the Dutch mycologist Christine Marie Berkhout (1893–1932) who described the genus *Candida* in her doctoral thesis for the University of Utrecht in 1923. The name *Candida* has its origin in the Latin phrase “toga candida” which describes the white robe worn by candidates for the Roman Senate, whereas *albicans* comes from the Latin verb albicare, i.e., “to whiten”. Other researchers like Castellani, Bissereie, Malvoz, and Benham also significantly contributed to the characterization of *C. albicans* (reviewed by (Odds 1988)). The binominal name of the species was officially accepted at the 8th Botanical Congress in 1954. The genus *Candida* consists of a heterogeneous group of yeasts and contains approximately 150-200 species. Only a few species of this genus, including *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. krusei*, *C. lusitaniae*, *C. parapsilosis*, and *C. tropicalis*, are pathogenic. *C. albicans* is described as the most pathogenic species of the genus *Candida*.

The genome of *C. albicans* is highly variable due to the occurrence of chromosomal rearrangements like translocations, chromosome deletions and
trisomy, and chromosome length polymorphism (Chen, Magee et al. 2004; Chibana, Beckerman et al. 2000; Selmecki, Bergmann et al. 2005). In the absence of meiosis (see below) this creates genetic diversity and results in phenotypic changes, which play a role in the adaptation strategies of the fungus, including the acquisition of resistance to antifungal drugs (Selmecki, Forche et al. 2006). *C. albicans* is diploid. The genome size is ~14.3 Mbp divided over 8 chromosomes, which results in ~29 Mbp of nuclear DNA per diploid cell (*C. albicans* Genome Snapshot of CGD at http://www.candidagenome.org/cache/C_albicans_SC5314_genomeSnapshot.html).

The genome contains 8 chromosome pairs; chromosomes 1-7 have a constant size while chromosome R is polymorphic and varies in size. The genome of *C. albicans* has been sequenced by the Stanford Genome Technology Center and is available on the *Candida* Genome Database (http://www.candidagenome.org/). The current genome inventory of CGD (as of December 19, 2011) mentions 6202 ORFs, of which 1416 ORFs have been verified, 4634 ORFs are still uncharacterized, and 152 ORFs are considered to be dubious. An unusual feature of the *C. albicans* translation mechanism is that *C. albicans* translates codon CUG as serine rather than leucine (Santos, Keith et al. 1993; Santos and Tuite 1995). The genome of *C. albicans* is highly heterozygous, as many genes have two different alleles. Until recently it was believed that *C. albicans* existed as an obligate diploid, propagating only clonally and lacking a sexual cycle based on meiosis, which ensures recombination between homologous chromosomes and generates genetic diversity in eukaryotic organisms. Later research showed, however, that in *C. albicans* a special form of mating can take place, called a parasexual reproductive cycle because it is based on recombination between homologous chromosomes without meiosis (Forche, Alby et al. 2008). It is performed by opaque cells (Gow 2002; Gow,
Brown et al. 2000; Magee and Magee 2000) and requires switching of the morphological state of the cells from white cells to opaque cells (see below) in order to mate. This unique regulation of the mating program in C. albicans might play a role in limiting the mating process to restricted conditions or sites of the human body such as the skin. In the heterothallic parasexual cycle, diploid a/a and diploid α/α cells fuse to form tetraploid cells. Tetraploid cells undergo random chromosome loss leading to a gradual reduction of the ploidy of the cells, which might eventually produce diploid or close to diploid cells, a process which generates new phenotypes (Forche, Alby et al. 2008). A parasexual cycle has advantages compared to sexual reproduction: (1) it generates aneuploid strains with higher phenotypic diversity and showing chromosomal trisomy, which has been shown to be responsible for higher resistance of strains to antifungal azoles; (2) it does not require sporulation, a process that sexually reproductive fungi undergo. Spores are highly antigenic, thus bypassing spore formation increases the chances of survival in the host environment (Forche, Alby et al. 2008). Strain SC5314, which is the wild type strain that is commonly used in the laboratory and also in our studies, consists of diploid a/α cells which due to the presence of both mating alleles are unable to mate.

C. albicans can adapt to and grow in a wide range of environmental conditions. It is part of the natural human microflora, it exists on the skin, in the mucosal layers of the oral cavity and the gastro-intestinal track, and in the vagina. In the human body it is prevented from overgrowing the microflora by host immune factors such as pH, and limited nutrient and iron availability. C. albicans has the ability to grow over a pH range of 2-7.5. It grows well at temperatures of 20-38°C with a maximum temperature of 42-46°C and can survive a one-hour exposure to 70°C in rich
medium (reviewed by (Odds 1988)). *C. albicans* utilizes glucose as a carbon source but it also can assimilate n-alkanes and other non-fermentable carbon sources such as fatty acids and lactate. At physiological glucose concentrations (3-5 mM, equivalent to glucose concentrations of 0.06-0.1% in blood), *C. albicans* uses the glycolytic pathway and represses the glyoxylate pathway, which permits conversion of non-fermentable sources of carbon to glucose under glucose limitation. However, the glyoxylate pathway is activated upon phagocytosis by host cells, while in later stages of host colonization *C. albicans* mostly depends on glycolysis (Barelle, Priest *et al.* 2006). As a nitrogen source it utilizes ammonium, but it can also assimilate nitrogen from urea (Navarathna, Das *et al.* 2011), and from proteins such as BSA (Staib, Lermann *et al.* 2008) and mucin, due to the activity of secreted proteolytic enzymes such as Sap2 (described below), which digest proteins to easily assimilable peptides. *C. albicans* grows preferentially under aerobic conditions, but some reports mention growth under anaerobic conditions (Biswas and Chaffin 2005; Webster and Odds 1987). For example, it colonizes the intestines, an environment which is essentially anaerobic, and is also found in other niches deprived of oxygen like root canals (Baumgartner, Watts *et al.* 2000) and periodontal pockets (Urzua, Hermosilla *et al.* 2008). Collectively, these observations show that *C. albicans* can survive extreme conditions and explains why *C. albicans* is able to colonize different niches of the human body which widely vary in pH, and in oxygen and nutrient availability.

### 1.2 Morphological states of *C. albicans*

*C. albicans* is a polymorphic fungus. It can grow as yeast cells (also called blastospores or white cells to distinguish them from opaque cells), pseudohyphae,
or as hyphae, it can grow as opaque cells that, as mentioned in the previous section, are able to mate and it can form chlamydospores (Figure 1). This diversity of forms provides high flexibility to the fungus in adapting itself to external conditions and in overcoming host defense mechanisms against fungal infection during colonization of the human body.

**Figure 1** Morphological forms of *C. albicans* observed under different growth conditions. **A.** Yeast cells. New buds emerge from the mother cell; after formation of a septum in the bud neck region, the daughter cell can safely separate from the mother cell. **B.** Pseudohyphae. Similarly as in yeast cells, a septum is formed in the neck region, but after formation of a septum, newly formed cells stay attached to the mother cell and further elongate. **C.** Hyphae. Germ tubes emerge from yeast cells before the G1/S phase of the cell cycle; at this stage there is no septum formed, germ tubes elongate and only later a septum is formed in the hyphal tube. **D.** Opaque cells. They are able to mate in response to pheromones. They are called opaque cells, because they form opaque colonies, whereas yeast cells form white colonies. **E.** Chlamydospores. Thick-walled cells able to survive harsh environmental conditions.
Budding yeast cells have an oval shape; newly formed cells separate from the mother cell after a septum between the mother and daughter cell has been formed (Figure 1). It results in the formation of single cells. These cells form smooth white colonies on solid rich medium. Pseudohyphal cells have an elongated shape, always wider in the middle and more narrow at the ends. Newly formed cells stay attached to the mother cell after formation of a septum. These cells further elongate forming structures, which depending on the shape and length of the cells might very closely resemble true hyphae. Pseudohyphae are usually wider than hyphae, having a minimal diameter of 2.8 µm (Sudbery, Gow et al. 2004). Hyphal cells have a filamentous shape and are equally wide over the entire length of the hyphae. They can form branches or form very long unbranched hyphal tubes. In contrast to yeast cells, which form smooth colonies, hyphae, similarly as pseudohyphae, grow as wrinkled, stippled, ring-like or fuzzy colonies and can grow invasively into the substrate layer. In liquid culture they often form aggregates. Hyphae grown in serum-containing medium have a width of 2.5-3.5 µm, their extension rate very much depending on the degree of differentiation of the colony (~19 µm h\(^{-1}\) for undifferentiated colonies grown for 12 h and 22-46 µm h\(^{-1}\) for 3-5 day-old colonies) (Gow and Gooday 1982). Hyphae grown in buffered N-acetylglucosamine were shown to have a smaller width than hyphae grown in rich media, likely due to starvation (Sevilla and Odds 1986). Hyphae formation starts when new cells emerge from unbudded yeast cells (blastospores) after exposition to environmental conditions that induce hyphae formation. Unlike pseudohyphae, emerging hyphae (germ tubes) do not form a septum between the mother cell and the emerging tube, but further away in the germ tube, depending on the growth medium (10-15 µm in YPD-serum or 2.3 µm in defined medium) (Sudbery 2001; Sudbery, Gow et al.
The absence of a septum between the yeast cell and the germ tube is used in tests in order to distinguish pseudohyphae from hyphae.

The cell cycle progresses differently in hyphae compared to yeast and pseudohyphae (Sudbery, Gow et al. 2004). Formation and elongation of the germ tube begins before the G1/S phase of the cell cycle in contrast to the formation of a daughter cell in yeast and pseudohyphae. Germ tubes evaginate from yeast cells at random sites or show a bipolar pattern. In the G1 phase of the mother cell a large vacuole is formed while the newly formed germ tube elongates and contains most of the cytoplasm. Germ tubes have a basal band of septin bars located around the germ tube neck; further down the germ tube a true septin ring appears that marks the site of the first septum. Subsequently, the nucleus migrates along the germ tube to where the first nuclear division will take place across the septin ring. The first septum, which contains chitin, is thus formed in the germ tube and not at the neck between mother and daughter cell. Budding in yeast cells starts at the end of the G1 phase, new cells emerge in an axial or bipolar pattern, and a septin ring is formed in the neck region between mother and daughter cell, where the nuclear division takes place. After mitosis, the septin ring separates into two rings which marks the beginning of the formation of a primary septum located between the septin rings, similarly as in pseudohyphae. An important difference between the yeast and pseudohyphal cell cycle is that newly formed yeast cells have a shorter G2 phase because yeast cells switch earlier from polarized to isotropic growth (yeast buds grow in a polarized way only until they reach 2/3 of the mother size, then grow isotropically) which leads to the formation of a daughter cell that initially is smaller than the mother cell. Another difference is that the daughter cell becomes fully
separated from the mother cell. After the first cell cycle all pseudohyphal cells progress simultaneously through the next cell cycles in a unipolar pattern which leads to formation of branched structures. In hyphae the highly vacuolated mother cell remains in the G1 phase after completion of a cell cycle. After accumulating cytoplasm the mother cell can re-enter the cell cycle giving rise to a new germ tube or a branch. Hyphae grow in a polarized way which is a result of the organization of their actin cytoskeleton. Actin cortical patches are concentrated at the hyphal tip and actin cables are oriented towards the tip while in yeast their organization changes from polarized to isotropic.

Chlamydospores and opaque cells represent two more specialized and relatively rare morphological states. Chlamydospores have a spherical shape and thick walls (Akisada, Harada et al. 1983) and are formed in order to survive harsh conditions. It is possible that chlamydospores permit survival in niches where \textit{C. albicans} is exposed to farnesol, a quorum-sensing molecule that inhibits hyphae formation. Chlamydsospore formation can perhaps also play a role in survival upon exposure to reactive oxygen species secreted by host cells or by co-existing microorganisms (Martin, Douglas et al. 2005) as both farnesol and reactive oxygen species activate the same signalling pathway (Alonso-Monge, Navarro-Garcia et al. 2003). Opaque cells have received their name from the way they refract light (Soll 1992). They are more elongated compared to yeast cells, being three times larger than yeast cells, and they form flatter colonies than yeast cells (Slutsky, Staebell et al. 1987). Opaque cells are formed in vitro at temperatures below 37°C, suggesting that this form exists preferentially on the skin of the warm-blooded host. Further studies showed however that opaque forms also occur in vivo at 37°C and that their formation is induced by specific host factors like elevated CO$_2$ and lower oxygen concentrations compared
to atmospheric values and the presence of sugars released during proteolysis of host epithelium.

### 1.3 Transitions between morphological states.

*In vivo* and *in vitro* a morphological switch can occur between the different growth forms, in particular, between yeast, pseudohyphae and hyphae. Additionally, yeast and opaque forms can reversibly switch, and pseudohyphae can switch to chlamydospores (Ernst 2000). This morphological switch is under control of signalling pathways such as the Cek1 mitogen activated protein (MAP) kinase cascade and the cAMP-dependent protein kinase pathway which act via the transcriptional factors Cph1, Efg1, Tup1 and Rim101 (Brown and Gow 1999; Braun and Johnson 1997; Davis 2003; Liu, Kohler *et al.* 1994; Lo, Kohler *et al.* 1997). Double mutants deleted for *CPH1* and *EFG1* are unable to switch from yeast to hyphae while mutation of one of those genes does not fully block filamentation. For example, it was shown that the cph1/cph1 strain is unable to form hyphae on solid media but can form hyphae in liquid cultures. Transcriptional factors govern the morphological switch in response to different environmental factors (Biswas, Van Dijck *et al.* 2007).

Environmental conditions play an important role in determining the growth form of *C. albicans*. Often a combination of multiple factors is required to induce morphogenesis (Table 1). Up to now not all factors and their mode of action are well defined. Low glucose concentrations as present in the blood together with the presence of methionine induces hyphae formation while high glucose concentrations used in standard laboratory media (2%) promote yeast growth. This manner of
hyphae induction requires the presence of the receptor protein Gpr1 and activates the cAMP-dependent PKA signalling pathway (Maidan, De Rop et al. 2005). CO₂ dependent induction of hyphal growth is also governed by the cAMP signalling pathway (Klengel, Liang et al. 2005).

Table 1 Conditions inducing different morphological states of C. albicans. Often a combination of environmental factors is required to induce a full morphological switch. In general, acidic pH, 30°C, easily utilizable nitrogen sources and high glucose favor yeast formation and repress formation of hyphae, while 37°C, neutral pH, and starvation induce hyphae formation. Pseudohyphae are formed in intermediate conditions.

<table>
<thead>
<tr>
<th>Morphological forms</th>
<th>Inducing conditions</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast</td>
<td>30°C and rich nutrients</td>
<td>(Braun and Johnson 1997)</td>
</tr>
<tr>
<td></td>
<td>acidic pH</td>
<td>(Buffo, Herman et al. 1984; Herman and Soll 1984)</td>
</tr>
<tr>
<td></td>
<td>cell density&gt;10⁷ cells ml⁻¹</td>
<td>(Odds 1988)</td>
</tr>
<tr>
<td>Pseudohyphae</td>
<td>high phosphate</td>
<td>(Hornby, Dumitru et al. 2004)</td>
</tr>
<tr>
<td>Hyphae</td>
<td>35°C and rich medium</td>
<td>(Sudbery 2001)</td>
</tr>
<tr>
<td></td>
<td>temperature ≥37°C</td>
<td>(Lee, Shin et al. 1999)</td>
</tr>
<tr>
<td></td>
<td>37°C and serum</td>
<td>(Lee, Shin et al. 1999)</td>
</tr>
<tr>
<td></td>
<td>37°C and limiting amounts of nutrients</td>
<td>(Beheshti, Smith et al. 1975)</td>
</tr>
<tr>
<td></td>
<td>neutral pH</td>
<td>(Buffo, Herman et al. 1984; Herman and Soll 1984)</td>
</tr>
<tr>
<td></td>
<td>N-acetylglucosamine and 37°C</td>
<td>(Cassone, Sullivan et al. 1985; Naseem, Gunasekera et al. 2011)</td>
</tr>
<tr>
<td></td>
<td>other N-acetylhexosamine derivatives (N-acetylmannosamine, mucin, colloidal chitin, hyaluronic acid)</td>
<td>(Shepherd and Sullivan 1984; Sullivan and Shepherd 1982)</td>
</tr>
<tr>
<td></td>
<td>CO₂ in combination with 30°C and a source of nitrogen (serum, urea, asparagine, glutamine, acetamide)</td>
<td>(Sims 1986)</td>
</tr>
<tr>
<td></td>
<td>CO₂</td>
<td>(Klengel, Liang et al. 2005)</td>
</tr>
<tr>
<td></td>
<td>amino acids</td>
<td>(Lee, Buckley et al. 1975)</td>
</tr>
</tbody>
</table>
microaerobic (embedded) conditions (Brown, Giusani et al. 1999)
anaerobic conditions (Biswas and Chaffin 2005; Dumitru, Hornby et al. 2004)
nitrogen or glucose limitation (Csank, Schroppel et al. 1998; Hrmova and Drobnica 1981)
haemoglobin with low glucose or nitrogen, 37°C (Pendrak and Roberts 2007)
methionine and low glucose (Maidan, Thevelein et al. 2005)
proline and pH>5 (Holmes and Shepherd 1987)
tyrosol (Chen, Fujita et al. 2004)

**Chlamydospore**
- poor nutrients and low oxygen (Montazeri and Hedrick 1984)
- poor nutrients and detergent (Beheshti, Smith et al. 1975)
- farnesol (Martin, Douglas et al. 2005)

**Opaque form**
- 28-30°C in vitro (Rikkerink, Magee et al. 1988; Slutsky, Staebell et al. 1987)
- 37°C in vivo (host factors; high CO₂, N-acetylglucosamine from degradation of host mucin, anaerobic conditions) (Huang, Srikantha et al. 2009; Huang, Yi et al. 2010; Ramirez-Zavala, Reuss et al. 2008)
- low UV doses (Morrow, Anderson et al. 1989)

Conditions driving a morphological switch that cells encounter during colonization of the human body are various pHs (Table 2), 37°C and 30°C (skin), haemoglobin, serum, low glucose concentration (5 mM), low iron concentration, low oxygen/higher CO₂ levels compared to atmospheric values, amino acids resulting from tissue degradation, and the mucosal surface (Table 1). That is why the ability to switch between morphological forms which are adjusted to encounter specific environmental conditions is a crucial adaptation which allows *C. albicans* colonizing...
the human body. The ability to respond to different pHs is crucial for colonization of the host as different niches in the host environment vary in pH (Table 2). These adaptations include morphological switches, adjusting of intracellular processes like transcription and signal transduction pathways (Xu, Smith et al. 2004), incorporation of cell wall proteins (CWPs) which mediate cell integrity and virulence, secreted proteolytic enzymes which degrade host cells in order to penetrate tissues and to access nutrients (described below). It was shown that cells unable to adapt to changes in external pH are avirulent in a mouse model of systemic infection and cannot grow in vitro at different pHs (De Bernardis, Muhlschlegel et al. 1998). The pH has a strong influence on the yeast-to-hyphae transition. As hyphae are considered to be the more invasive form, acidic pH plays a protective role against Candida infections. For example, an acidic pH forms a natural chemical barrier in part of the gastro-intestinal track, in the vagina, and on the skin. Additionally, the solubility of the trace element iron, which is required for cell growth, is strongly pH-dependent, making it less accessible for C. albicans cells at neutral pH in extra- and intracellular fluids. The response to pH is governed by the Rim101 zinc finger transcriptional factor which is a homolog of Rim101/PacC of S. cerevisiae and Aspergillus nidulans, which are involved in the alkaline response pathway (Espeso and Penalva 1996; Li and Mitchell 1997; Tilburn, Sarkar et al. 1995). Rim101 and its putative membrane sensor Dfg16 are required for growth at neutral pH and under iron-limited conditions and Rim101 regulates genes responsible for high affinity iron uptake under alkaline conditions and growth in an iron-limited environment (Bensen, Martin et al. 2004; Thewes, Kretschmar et al. 2007). In A. nidulans activation of Rim101/PacC requires removal of the C-domain (Diez, Alvaro et al. 2002; Orejas, Espeso et al. 1995), which is obtained at ambient pH via signal transduction proteins
such as the plasma membrane proteins: PalH/Rim21, Pall/Rim9, PalF/Rim8, and the endosomal membrane complex proteins PalB/Rim13, PalA/Rim20, and PacC/Rim101 (Calcagno-Pizarelli, Negrete-Urtasun et al. 2007). *C. albicans* Rim101 is higher expressed in response to alkaline pH and activated through proteolytic removal of its C-terminal domain by Rim13 (Li, Martin et al. 2004) together with Rim8 and Rim20. It was however shown that also the full length of Rim101 can bind promoters of regulated genes (Baek, Martin et al. 2006). Rim101 positively regulates expression of alkali-induced CWPs (Pra1, Phr1), represses acidic CWPs (Phr2) and induces hyphae formation. Rim101 directly binds to the *PHR1* (Ramon and Fonzi 2003) and *PHR2* promoters (Baek, Martin et al. 2006). Additionally, a Rim101-independent pathway regulates the cellular response to alkaline pH (Davis, Wilson et al. 2000). In *S. cerevisiae*, Rim101 is involved in maintaining ion homeostasis by positive regulation of ion pump activity. *RIM101* mutants show a growth defect in alkaline pH which might be caused by insufficient transport of protons from the cells and incompletely maintaining the proton gradient in order to import molecules like iron, copper, phosphate, nucleotides, and glucose inside the cell which leads to starvation (Lamb, Xu et al. 2001).

**Table 2** pHs of different niches in the human body

<table>
<thead>
<tr>
<th>Niche of the human body</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duodenum</td>
<td>7.0 - 8.5</td>
</tr>
<tr>
<td>Blood</td>
<td>7.4</td>
</tr>
<tr>
<td>Oral cavity</td>
<td>6.5 - 7.5</td>
</tr>
<tr>
<td>Small intestine</td>
<td>4.0 - 7.0</td>
</tr>
<tr>
<td>Large intestine</td>
<td>4.0 - 7.0</td>
</tr>
<tr>
<td>Sweat and sebum (acid mantle)</td>
<td>4.0 - 6.0</td>
</tr>
<tr>
<td>Vaginal cavity</td>
<td>4.2</td>
</tr>
<tr>
<td>Stomach</td>
<td>1.5 - 4.0</td>
</tr>
</tbody>
</table>
The ability to switch between different morphological forms is essential for colonization of the host. Yeast cells or pseudohyphae unable to switch to the hyphal form are less virulent in the mouse model of systemic infection (Braun and Johnson 1997; Lo, Kohler et al. 1997; Rocha, Schroppel et al. 2001). Much research emphasizes the role of the hyphal form in colonization of the host environment as hyphae are able to grow invasively and penetrate tissues, leading to systemic infection. Yeast cells mainly mediate colonization by attachment to the substrate while hyphae progress by invading the epithelium. Both forms have cell wall-bound adhesins from the Agglutinin Like Sequence (Als) gene family, and Eap1 (Enhanced Adherence to Polystyrene), which mediate attachment to host epithelium and abiotic surfaces and mediate cell aggregation, facilitating formation of aggregates which allow attachment of larger amounts of fungal cells to the host surface. The differences in function of yeast and hyphae are also due to differences in binding specificity of their cell surfaces. Yeast cells have CWPs such as the Als1 adhesin which mediates attachment to host epithelium. Hyphae mediate attachment and/or invasion of host epithelium which is obtained by induced endocytosis by epithelial cells (invasion into epithelial cells) or by growth between cells by proteolysis of host proteins (Zhu and Filler 2010). Invasion into epithelial cells is mediated by Als3 which is mainly present in the cell wall of hyphae. Other hyphae- associated proteins like Hwp1 (Hyphal Wall Protein) mediate firm attachment to the host surface by becoming covalently linked to host epithelial cells, a process that is mediated by a host transglutaminase.

One of the mechanisms allowing attachment to host tissues and abiotic surfaces is hydrophobicity of the cell surface. This cell surface property depends on phosphodiester linked, acid-labile β-1,3-linked oligomannoside chains in cell wall
mannoproteins (Masuoka and Hazen 1997). Studies on hydrophobic properties of cells indicate that exponentially growing cells at 23°C (yeast cells) are more hydrophobic than cells grown at 37°C and that after a shift to 37°C the cells become temporarily also more hydrophobic before germ tube formation (Hazen and Hazen 1988). Hydrophobic cells are more resistant to phagocytosis by host cells and more competent to switch their morphology to hyphae (Hazen, Wu et al. 2001). On the other hand, another work shows that hyphal cells show better attachment to host tissues than yeast cells (Rotrosen, Calderone et al. 1986). Yeast cells are more easily recognized by macrophages because they expose beta-glucan on the surface of the bud scars which is recognized by the macrophage receptor Dectin 1 (Gantner, Simmons et al. 2005). This glucan moiety is normally hidden under a layer of cell surface proteins and is hidden in hyphae as they do not have bud scars.

*C. albicans* forms biofilms, which are communities of cells encased within a self-produced extracellular matrix. Biofilms are formed on biotic surfaces such as mucus and abiotic surfaces such as medical devices, joints, catheters, and dental implants. Biofilms have received close interest because biofilm cells are generally more resistant to external conditions including antifungal treatment. As a result, formation of biofilms can lead to systemic infection and create life-threatening disease. *In vitro*, a biofilm is formed in three steps (Chandra, Kuhn et al. 2001): (1) attachment of yeast cells to a surface, followed by (2) development of different morphological states, mainly filamentous forms, and formation of an aggregated structure, (3) formation of an extracellular matrix surrounding the cells such that in a mature biofilm the cells are encased in matrix material. The production of matrix material is increased by flow of the medium (Al-Fattani and Douglas 2006). *In vivo, C. albicans*
forms mixed biofilms together with other members of the microflora and contains host components (Dongari-Bagtzoglou, Kashleva et al. 2009). Multilayer biofilm formation is under control of cell-cell signalling involving the quorum-sensing molecule farnesol. Farnesol is secreted by planktonic cells and its concentration depends on cells density. Low cell density and low farnesol concentration allow hyphae formation and biofilm development, but high cell densities block development of biofilms by inhibition of hyphae formation (Ramage, Saville et al. 2002). Extracellular matrix material is composed of 40% of carbohydrates (mainly glucose and small amounts of galactose and mannose), 5% of proteins, 3% of hexosamine, 0.5% of phosphorus and 0.1% uronic acid (Al-Fattani and Douglas 2006). Water channels in the matrix allow circulation of nutrients between cells. Amazingly, C. albicans biofilms can be up to 1,000-fold more resistant to antifungal drugs than planktonic cells (Lamfon, Porter et al. 2004). C. albicans growing as biofilms is resistant to almost all antifungals like azoles and amphotericin B, which target the membrane, but stays sensitive to echinocandins that target cell wall glucan formation and also to lipid formulations of amphotericin B (Kuhn, George et al. 2002). The physical properties of the extracellular matrix contribute to the high resistance of biofilms to antifungal drugs due to decreased diffusion of drugs through biofilm and binding of drugs to matrix material (Al-Fattani and Douglas 2006; Vediyappan, Rossignol et al. 2010). Higher resistance of biofilm cells to azoles like fluconazole is also a result of increased expression of genes encoding the plasma membrane efflux pumps Cdr1, Cdr2, and Mdr1, changes in sterol composition, and expression of cell surface proteins (Basso, Gast et al. 2010; d'Enfert 2006; Li, Svarovsky et al. 2007; Mukherjee and Chandra 2004; Nobile, Andes et al. 2006; Tsao, Rahkhoodaee et al. 2009).
1.4 Utilization of host iron by *C. albicans*

Iron is an essential component for almost all living organisms. It is a cofactor of a variety of metalloproteins, oxygen-transporting proteins and redox proteins required for cellular respiration, oxidation and reduction. Iron occurs in a wide range of oxidation states from -2 to +6, the most common ones being the +2 (ferrous iron) and the +3 (ferric iron) oxidation state. Free iron is toxic due to the Fenton reaction in which ferrous iron reacts with $\text{H}_2\text{O}_2$ and produces toxic hydroxyl radicals. Iron occurs in the form of iron salts, and is associated with organic acids and bound to proteins. Under atmospheric oxygen conditions soluble bioavailable ferrous iron undergoes spontaneous oxidation to ferric iron which precipitates. In order to utilize iron, living organisms must therefore possess mechanisms which allow them to reduce the ferric form to the ferrous form.

As both host and pathogen need iron to maintain vital processes, iron accessibility is strictly controlled by the host as a defense mechanism against microbial infections, and microbial pathogens need effective iron acquisition mechanisms for survival in most host environments. Human iron is present in the body in amounts of 3-5 g in adults and is bound to iron-binding proteins in order to store and release iron in specific niches of the body. *C. albicans* as an opportunistic pathogen encounters both iron deprivation conditions, for instance, in the bloodstream ($\sim 10^{-24} \text{ M for Fe}^{3+}$) where iron is bound to human proteins, and an iron-rich environment during colonization of the gastrointestinal track where iron is accessible ($\sim 15 \text{ mg/day equivalent to 0.27 mmol/day in humans}$) due to incomplete absorption of dietary iron (Miret, Simpson *et al*. 2003). In humans there are iron transport proteins (siderophilins), iron storage proteins, heme-proteins, iron-sulfur
proteins and others that might serve as sources of iron for pathogenic microorganisms (Almeida, Wilson et al. 2009; Mietzner and Morse 1994).

Siderophilins have high affinity for iron \( (K_d < 1 \times 10^{-20} \text{ for ferric iron}) \), and are present in the human body as lactoferrin and transferrin. Transferrin binds iron reversibly depending on pH; this feature is important for the function of transferrin as it is responsible for transport of iron between cells. Transferrin is present mostly in serum, plasma, lymphatic and cerebrospinal fluids, and in saliva. Lactoferrin is present in secreted fluids like milk, saliva, and tears, and is present on mucosal surfaces in order to inhibit microbial growth. The iron storage protein ferritin is responsible for sequestering, storage, and detoxification of iron and is responsible for maintaining intracellular iron homeostasis. It binds around 30% of body iron. Ferritin is found mostly in the spleen, liver, and in bone marrow. Haemoglobin and myoglobin contain ferrous iron in their prosthetic sites. Haemoglobin is associated with erythrocytes and contains 60% of the iron pool in humans, whereas myoglobin is found in muscle tissue. Iron-sulfur proteins contain iron bound through cysteiny1 residues or incorporate inorganic sulfur in coordination sites. Iron-sulfur proteins have various functions: ferredoxin-like proteins take part in mitochondrial respiration, aconitase participates in the Krebs cycle and converts citrate into isocitrate, and xanthine oxidase can oxidize xanthine into uric acid (Almeida, Wilson et al. 2009). Other non-heme and non-sulfur proteins are represented by for example oxygenases and reductases.

Pathogenic microorganisms have developed different mechanisms of iron acquisition depending on which form of iron is more accessible in certain niches of the host. In *C. albicans* there are three systems for high affinity iron acquisition: 1)
siderophore uptake, 2) the reductive system, and 3) iron acquisition from haemoglobin. Siderophores are low molecular weight non-protein, non-porphyrin ferric iron-chelating compounds (Mietzner and Morse 1994) produced by microorganisms in order to acquire iron from the environment and capture iron from host proteins. In *C. albicans* there is no evidence for the existence of genes that might encode siderophores (reviewed by (Almeida, Wilson et al. 2009)) but *C. albicans* has the ability to utilize siderophores of other microorganisms (Lee and Han 2006). *C. albicans* possesses a single siderophore transporter (Sit1/Arn1) localized in the plasma membrane (Ardon, Bussey et al. 2001; Hu, Bai et al. 2002). It has been shown that *C. albicans* can also utilize iron from haemoglobin or heme (Moors, Stull et al. 1992; Pendrak, Chao et al. 2004). Iron utilization from haemoglobin requires binding of *C. albicans* to erythrocytes through a complement receptor-like molecule (Moors, Stull et al. 1992), proteolysis of erythrocytes, and binding of haemoglobin by cell surface receptors from the haemoglobin-binding family Rbt5 and Rbt51/Pga10 (Weissman and Kornitzer 2004). Rbt5-bound haemoglobin enters the cells by endocytosis. Rbt5 is higher expressed in hyphae than in yeast cells, suggesting that haemoglobin is more efficiently utilized by hyphae, a morphological form induced by the neutral pH of blood. On the other hand, *RBT5* was shown to be up-regulated in yeast cells that undergo iron starvation, both on the transcriptional and translational level (Lan, Rodarte et al. 2004; Sosinska, de Groot et al. 2008, see chapter 2). In the vacuoles haemoglobin is degraded and iron is released from heme via heme oxygenase Hmx1 resulting in the formation of α-biliverdin (Pendrak, Chao et al. 2004). Reductive high affinity iron uptake requires reduction of ferric iron by externally localized ferric reductase; ferrous iron is then transported into the cell via an oxidation/internalization complex.
consisting of the multicopper oxidase Fet3 and the high affinity ferric iron permease Ftr1 (Knight, Lesuisse et al. 2002).

Reductive iron uptake in C. albicans shows considerable complexity as there are several genes encoding its components (Almeida, Wilson et al. 2009). For the reductase there are 17 homologous proteins, for the oxidase there are 5 predicted ORFs and for the permease there are 4 proteins that are differently regulated. Ftr1 is upregulated upon iron starvation and is responsible for high affinity iron uptake while Ftr2 is active when the iron concentration in the medium is high. The reductive iron uptake pathway allows iron acquisition from the environment and from host proteins like transferrin (Knight, Vilaire et al. 2005) and ferritin, as C. albicans was shown to bind ferritin via the hyphal surface adhesin Als3, followed by local acidification of the environment which allows release of iron from ferritin (Almeida, Brunke et al. 2008). Importantly, a C. albicans strain deleted for FTR1 was not able to develop systemic infection in mice (Ramanan and Wang 2000), showing that iron acquisition in the host environment determines survival and pathogenicity of C. albicans.

1.5 Effect of oxygen deprivation on C. albicans

Oxygen is an essential molecule for cellular respiration in aerobic organisms. In addition to cellular respiration, oxygen is required for important cellular processes such as the synthesis of sterols, unsaturated fatty acids, and high-affinity iron utilization (described above) (Berg, Tymoczko et al. 2007; Kosman 2003; Schweizer 2004) . Respiration is an essential process, which generates energy in the form of adenosine triphosphate (ATP) from nutrients. During glycolysis glucose is converted to pyruvate. Under aerobic conditions the end-product of glycolysis – pyruvate is oxidized to acetyl-CoA. Acetyl-CoA enters the Krebs cycle (tricarboxylic acid cycle)
inside the mitochondrial matrix and is oxidized to CO$_2$ with reduction of nicotinamide adenine dinucleotide (NAD$^+$) to NADH. NADH is further used in the electron transport chain which generates a proton gradient used for phosphorylation of adenosine diphosphate (ADP) to ATP. Oxygen is used as a final acceptor of electrons in the electron transport chain where it combines with two protons to form H$_2$O. Under limited oxygen conditions pyruvate enters a different metabolic pathway-fermentation, which also provides energy but less efficiently than respiration. In *C. albicans*, pyruvate is converted to ethanol (Synnott, Guida *et al.* 2010). During this process NADH is oxidized to NAD$^+$ and can be re-used in glycolysis to obtain energy from glucose. In sterol synthesis oxygen is needed as a substrate for squalene monooxygenase (Erg1) (Favre and Ryder 1997; Ryder and Dupont 1984) and lanosterol 14-α demethylase (Erg11) (Kirsch, Lai *et al.* 1988). Oxygen regulates the biosynthesis of unsaturated fatty acids by regulating the activity of stearoyl desaturase Ole1, whose end product is oleic acid (C$_{18:1}$) (Krishnamurthy, Plaine *et al.* 2004). The plasma membrane of *C. albicans* is bilayered and contains in addition to saturated fatty acids also unsaturated fatty acids and sterols which are asymmetrically distributed between the two leaflets of the membrane, and furthermore lipid rafts (detergent-insoluble membrane structures), consisting of lipids and proteins (Ghannoum, Swairjo *et al.* 1990; Marriott 1975; Murayama, Negishi *et al.* 2006). Changes in the lipid composition leads to alterations in fluidity of the plasma membrane and thus affects membrane proteins involved in cell wall synthesis and trafficking of CWPs (Mishra, Bolard *et al.* 1992). Impaired activity of Erg1 leads to increased sensitivity of cells to drugs (Pasrija, Krishnamurthy *et al.* 2005). Erg11 is a direct target ofazole antifungals (Favre, Didmon *et al.* 1999; Van den Bossche, Marichal *et al.* 1990). Azoles affect membrane stability and lead to
increased expression of proteins from the **Secreted Asparagine Protease (SAP)** family and alterations in the wall proteome (Copping, Barelle *et al.* 2005). As low oxygen levels affect sterol synthesis in yeast (Bien and Espenshade 2010; Blatzer, Barker *et al.* 2011; Hughes, Todd *et al.* 2005; Setiadi, Doedt *et al.* 2006), this might lead to changes in the cell wall proteome as well (Sorgo, Heilmann *et al.* 2011). **Sterol regulatory element binding proteins (SREBP)** are conserved fungal transcriptional factors which regulate sterol synthesis in response to hypoxia (Bien and Espenshade 2010). In *C. albicans* ergosterol synthesis and the response to azoles are governed by the same transcriptional factor Upc2 (Silver, Oliver *et al.* 2004). Mimicking hypoxic conditions by lowering sterol levels induces genes that encode CWPs with a CFEM domain and are under control of the Upc2 and Bcr1 transcription factors (Synnott, Guida *et al.* 2010). *C. albicans* encounters limited oxygen conditions during colonization of the intestinal track, when invading host tissues and during biofilm formation. Thus, adaptation to low oxygen is important for successful colonization of host niches. Adaptation to hypoxic conditions requires up-regulation of genes involved in fermentation including glycolytic genes (Bonhomme, Chauvel *et al.* 2011; Setiadi, Doedt *et al.* 2006), genes involved in sulfur metabolism, iron uptake (Stichternoth and Ernst 2009), ergosterol biosynthesis, hypha-specific genes, and down-regulation of genes involved in oxidative phosphorylation (Setiadi, Doedt *et al.* 2006). Oxygen limitation also results in changed expression and incorporation of CWPs ((Setiadi, Doedt *et al.* 2006), see chapter 2).
1.6 The cell wall of *C. albicans*

1.6.1 Introduction

The cell wall of *C. albicans* consists of an internal skeletal layer of polysaccharides and an external layer of proteins. It is responsible for counteracting the internal turgor pressure, maintaining cell shape and for contact with the environment. The ability of the cell wall to adapt to changes in the environment is an important feature which guarantees correct formation of the wall and survival of the cells in a variety of external and internal conditions including different host environments, progression through the cell cycle, and mode of growth (Figure 2). Host-associated stress factors include low oxygen conditions, acidic pH, iron depletion, presence of ROS (discussed above), carbon deprivation which cells encounter after phagocytosis by host macrophages or neutrophils (Lorenz, Bender *et al.* 2004; Piekarska, Mol *et al.* 2006), presence of host antimicrobial peptides secreted in saliva (Kavanagh and Dowd 2004) and defensins secreted by epithelial cells, and antifungal agents like azoles affecting sterol synthesis and echinocandins, which inhibit the synthesis of β-1,3-glucan, and thus weaken the cell wall (Liu, Lee *et al.* 2005). The temperature of the human body is rather not considered as a stress condition because *C. albicans* can survive at higher temperatures up to 45°C. The response by *C. albicans* to stress conditions often includes dramatic changes in the transcript levels of cell wall protein-encoding genes and the corresponding protein levels (Bensen, Martin *et al.* 2004; Copping, Barelle *et al.* 2005; Lan, Rodarte *et al.* 2004; Setiadi, Doedt *et al.* 2006).
Figure 2 Factors influencing the cell wall composition of *C. albicans*. AMPs, anti-microbial peptides.

1.6.2 Molecular organization of the cell wall

The cell wall of different growth forms of *C. albicans* is similar although the best characterized walls are from yeast and hyphal cells, and less research has been done in order to analyse the cell walls of chlamydospores and opaque cells. Figure 3 shows a molecular model of the *C. albicans* cell wall. The internal part of the cell wall consists of polysaccharides to which externally CWP s are attached. The inner polysaccharide layer consists of β-1,6-glucan, β-1,3-glucan, and small amounts of chitin (Chauhan, Li *et al.* 2002; Elorza *et al.* 2006; Kapteyn, Hoyer *et al.* 2000; Nather and Munro 2008; Ruiz-Herrera) as described in Table 3.

Table 3 Composition of the cell wall of *C. albicans*

<table>
<thead>
<tr>
<th>Component</th>
<th>% of cell wall dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>mannoproteins</td>
<td>35-40</td>
</tr>
<tr>
<td>β-1,3-glucan</td>
<td>40</td>
</tr>
<tr>
<td>β-1,6-glucan</td>
<td>20</td>
</tr>
<tr>
<td>chitin</td>
<td>1-2</td>
</tr>
</tbody>
</table>

The external protein coat is around 100 nm thick (Tokunaga, Kusamichi *et al.* 1986). The protein coat consists of glycosylphosphatidylinositol-anchored proteins (GPI-CWPs) and non-GPI proteins such as Pir (proteins with internal repeats).
proteins. GPI-CWPs vary in size and represent the majority of CWPs. The protein coat of the cell wall is strengthened by disulfide bonds formed between cysteine residues of GPI-CWPs (Figure 3).

Figure 3 Molecular model of the *C. albicans* cell wall. In addition to the components shown here the cell wall also contains dityrosine and under some growth conditions the cell walls possess fimbrial structures which can be removed from the cell walls by reducing agents (Chauhan, Li *et al.* 2002; Hazen and Hazen 1992; Tronchin, Bouchara *et al.* 1988).

It was shown in *S. cerevisiae* that disulfide bonds restrict cell wall permeability (de Nobel, Klis *et al.* 1990). GPI-CWPs are covalently linked through their GPI glycan to β-1,6-glucan molecules which are in turn covalently linked to nonreducing ends of β-1,3-glucan. β-1,6-glucan forms a highly branched, flexible nonregular structure which offers multiple nonreducing ends as acceptor sites for attachment of GPI-CWPs. β-1,3-glucan forms a more rigid structure, which through its nonreducing ends is linked to both β-1,6-glucan and chitin. Chains of β-1,3-glucan are less branched because of regions of nonsubstituted glucose residues which allow aligment of β-1,3-glucan chains and their interconnection through hydrogen bonds between glucose molecules. The β-1,3-glucan network is additionally strengthened through cross-links formed by glutamine-dependent transesterification involving the
internal repeats in Pir-CWPs (Ecker, Deutzmann et al. 2006; Kapteyn, Hoyer et al. 2000) and possibly also by the presence of a dityrosine-containing polymer. The helical structure of β-1,3-glucan allows the cell wall to extend its size due to turgor pressure of living cells. The thickness of the internal polysaccharide layer of cell is about 60–70 nm, but probably depends on growth conditions (Tokunaga, Kusamichi et al. 1986). All possible protein-polysaccharide connections found in *C. albicans* and *S. cerevisiae* are shown in Figure 4.

**Figure 4** Connections between proteins and polysaccharides of the cell wall. Components of the cell wall are connected through glycosidic linkages marked by arrows; the direction of the arrows points to a nonreducing end of the polysaccharides. Except for Pir-CWPs the alkali sensitive linkage (ASL) connecting some GPI-CWPs with β-1,3-glucan is not well understood. Under stress conditions additional connections are formed between chitin and β-1,6-glucan.

Similar components as present in the cell wall also appear in the extracellular matrix of biofilms although their exact composition, origin, and relationship with the cell wall is not well defined (Al-Fattani and Douglas 2006). Interestingly, the polysaccharide network of the cell wall is flexible and can be rearranged in response
to environmental conditions; for example, in *S. cerevisiae* at acidic pH more chitin becomes directly connected to β-1,6-glucan.

**1.6.3 GPI-modified proteins**

The main class of CWPs are GPI proteins. Those proteins are usually mannosylated and phosphorylated and as a consequence positively charged ions and proteins get easily bound to the cell walls (Cutler 2001; Horisberger and Clerc 1988). There are 115 predicted GPI proteins (Plaine, Walker *et al.* 2008). Their amino acid sequence contains an N-terminal signal peptide which is followed by the functional domain, a spacer domain, and terminates in a GPI anchor addition signal. The spacer domain often contains repeats (for example, in case of Als proteins) and serine- and threonine-rich sequences for the attachment of O-mannosyl side-chains, which result in the formation of rod-like structures. This defined molecular organization of GPI-CWPs facilitates the development of algorithms that predict if a protein can be classified as a GPI-CWP (De Groot, Hellingwerf *et al.* 2003; Eisenhaber, Schneider *et al.* 2004). GPI proteins follow the secretory pathway during which they become modified. The N-terminal signal peptide of GPI proteins directs proteins to the endoplasmic reticulum (ER) where the signal peptide is removed and the C-terminal sequence for attachment of a GPI anchor is substituted by a GPI anchor. In the ER GPI proteins are glycosylated on specific asparagine residues (*N*-linked) or on serine or threonine residues (*O*-linked). These carbohydrate chains are extended and modified in the Golgi (reviewed by (Cutler 2001; Ernst and Prill 2001)). In the plasma membrane some GPI proteins are retained in the outer leaflet of the membrane, while others are incorporated in the cell wall (GPI-CWPs) (Mao, Zhang *et al.* 2008). GPI proteins localized to the cell wall have a truncated GPI anchor.
(deprived of lipid) and are via their GPI remnant linked to β-1,6-glucan. GPI proteins that are localized in the plasma membrane possess a dibasic motif close to and in front of the omega amino acid (Frieman and Cormack 2003). Other amino acid sequences also can influence the localization of GPI proteins (Hamada, Terashima et al. 1998; Hamada, Terashima et al. 1999).

Release of GPI-CWPs from the cell wall can be obtained by chemical or enzymatic methods (Figure 5). These methods allow isolation of CWPs (after HF-pyridine or phosphodiesterase treatment (Kapteyn, Montijn et al. 1996) or of CWPs complexed with (partially degraded) cell wall polysaccharides (after β-1,3- or β-1,6-glucanase or chitinase treatment (Kapteyn, Hoyer et al. 2000; Kapteyn, Ram et al. 1997). Peptide fragments of CWPs for mass spectrometric analysis (MS) can also be obtained by direct treatment of isolated cell walls with trypsin. Table 4 shows the GPI-CWPs which have been experimentally proved to be localized in the cell wall. Proteins were identified by MS analysis or immunological analysis (Eap1, Hyr1, Hwp1, Rbt5, Pga10 or in combination with tagging with green florescent protein: Pga59 and 62), by immunogold labeling (Sap9 and 10), expression in S. cerevisiae and probing of cells surface with antibody- or ligand-coated magnetic beads (Als family of CWPs and Csa1) (Lamarre, Deslauriers et al. 2000; Sheppard, Yeaman et al. 2004). Detection of some CWPs is difficult due to low abundance of some CWPs in cell walls, high glycosylation of CWPs, which blocks the accessibility of proteins for trypsin digestion and hampers the detection of tryptic peptides because they have a m/z out of the range of detection of the mass spectrometer (Hwp1, Pga62). As a result, the tryptic peptides of the majority of CWPs are obtained from the functional domain of protein which generally is less glycosylated.
**Figure 5** Structural organization of GPI-CWPs and their linkage to the cell wall. Chemical and enzymatic methods are available to remove cell wall fractions.

**Table 4** CWPs experimentally proven to be linked to the cell wall, their function and regulation. The CWPs presented here were identified by tryptic digestion and MS either directly or after extraction from isolated cell walls, by immunoanalysis after their release from the cell wall using cell wall polysaccharide-degrading enzymes or HF-pyridine or by conjunction with green fluorescent protein tagging (Pga59 and Pga62), by immunogold-labeling (Sap 9 and Sap10), or by cloning in *S. cerevisiae* and binding of the transformed cells to antibody- or ligand- coated magnetic beads.

<table>
<thead>
<tr>
<th>Protein</th>
<th>No. of aa</th>
<th>Region of detectable tryptic peptides*</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GPI-CWPs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Als1¹</td>
<td>1260</td>
<td>79-311</td>
<td>Promotes adhesion to epithelial and endothelial cells when expressed in <em>S. cerevisiae</em> (Fu, Rieg <em>et al</em>. 1998), adherence in <em>in vivo</em> catheter model (Nobile, Schneider <em>et al</em>. 2008) and human umbilical vein endothelial cells (Zhao, Oh <em>et al</em>. 2004), presence of amyloid forming sequence (Otoo, Lee <em>et al</em>. 2008)</td>
</tr>
<tr>
<td>Protein</td>
<td>Accession</td>
<td>Coverage</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
<td>----------</td>
<td>-------------</td>
</tr>
</tbody>
</table>
| Als3$^1$ | 1155 | 77-311 | Adhesion to human umbilical vein endothelial cells, buccal epithelial cells, buccal reconstituted human epithelium (Zhao, Oh et al. 2004)  
- Promotes endocytosis by binding cadherins (Phan, Myers et al. 2007)  
- Required for binding of ferritin (Almeida, Brunke et al. 2008)  
- Presence of amyloid forming sequence (Otoo, Lee et al. 2008)  
- Induced in germ tubes and in hyphal cultures (Bahn, Molenda et al. 2007; Sorgo, Heilmann et al. 2010)  
- Promotes biofilm formation (Nobile, Schneider et al. 2008)  
- Higher incorporation levels in cell walls of hyphae (Heilmann, Sorgo et al. 2011)  
- N-terminal sequence used as vaccine antigen (Spellberg, Ibrahim et al. 2008)  
- Down regulated by fluconazole (Sorgo, Heilmann et al. 2011)  
- High level of expression (Hoyer, Green et al. 2008) |
| Als4$^1$ | 2100 | 77-91 | Upregulated in surface grown cells and in the presence of fluconazole (Sorgo, Heilmann et al. 2011)  
- Intermediate level of expression (Hoyer, Green et al. 2008) |
| Als5$^1$ | 1347 | No data | Adhesion to host epithelium, endothelium, mediates cell adherence, role in invasion due to promoting of endocytosis (Gaur and Klotz 1997; Gaur, Klotz et al. 1999)  
- Contains amyloid forming sequence (Garcia, Lee et al. 2011; Otoo, Lee et al. 2008)  
- Cell wall location based on expression in *S. cerevisiae* (Sheppard, Yeaman et al. 2004)  
- Low level of expression (Hoyer, Green et al. 2008) |
### General introduction

<table>
<thead>
<tr>
<th>Protein</th>
<th>Accession</th>
<th>Expression</th>
<th>Observations</th>
</tr>
</thead>
</table>
| Als6$^1$ | 1366      | No data    | Lower level of adhesion, compared to Als1, 3, 5 (Sheppard, Yeaman et al. 2004)  
|         |           |            | Cell wall location based on expression in *S. cerevisiae* (Sheppard, Yeaman et al. 2004)  
|         |           |            | Low level of expression (Hoyer, Green et al. 2008)  
| Als7$^1$ | 1568      | No data    | Lower level of adhesion, compared to Als1, 3, 5 (Sheppard, Yeaman et al. 2004)  
|         |           |            | Highly variable repeat domain leads to existence of different protein properties (Zhang, Harrex et al. 2003)  
|         |           |            | Down regulated in biofilms (Garcia-Sanchez, Aubert et al. 2004)  
|         |           |            | Cell wall location based on expression in *S. cerevisiae* (Sheppard, Yeaman et al. 2004)  
|         |           |            | Low level of expression (Hoyer, Green et al. 2008)  
| Als9$^1$ | 1889      | No data    | Lower level of expression, compared to Als1, 3, 5 (Sheppard, Yeaman et al. 2004)  
|         |           |            | Cell wall location based on expression in *S. cerevisiae* (Sheppard, Yeaman et al. 2004)  
|         |           |            | Intermediate level of expression (Hoyer, Green et al. 2008)  
| Cht2$^2$ | 583       | 22-299     | Chitinase  
|         |           |            | N-terminal GH18 (glycoside hydrolase) domain (Cantarel, Coutinho et al. 2009)  
|         |           |            | Down-regulated by fluconazole (Sorgo, Heilmann et al. 2011)  
|         |           |            | Up-regulated by fluconazole (Sorgo, Heilmann et al. 2011)  
| Csa1/ Wap1$^4$ | 1018 | No data    | Identified by screening a genomic library in *S. cerevisiae* (Lamarre, Deslauriers et al. 2000)  
|         |           |            | Up-regulated at alkaline pH, Rim101 dependent (Bensen, Martin et al. 2004; Ramon and Fonzi 2003)  
|         |           |            | Deletion negatively affects biofilm formation and results in increased cell surface hydrophobicity (Perez, Pedros et al. 2006; Perez, Ramage et al. 2011)  
| Eap1    | 653       | No data    | Isolated as cell wall adhesin by screening a genomic library in *S. cerevisiae* (Li and Palecek 2003)  
|         |           |            | N terminal domain mediates cell adherence,
<table>
<thead>
<tr>
<th>Protein</th>
<th>Start</th>
<th>End</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ecm33[^b]</td>
<td>423</td>
<td>139-341</td>
<td>Cell wall integrity (Martinez-Lopez, Park et al. 2006)</td>
</tr>
<tr>
<td>Hwp1</td>
<td>634</td>
<td>No data</td>
<td>N-terminal domain serves as a substrate for epithelial transglutaminases, which results in covalent attachment of hyphae to host epithelial cells (Staab, Bradway et al. 1999)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Hyphal induced (Staab, Ferrer et al. 1996), regulated by Efg1, Tup1 and Rbf1 transcriptional factors (Sharkey, McNemar et al. 1999)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>N-terminal 14-mer peptide used as vaccine antigen (Xin, Dziadek et al. 2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Required for biofilm formation, Bcr1-regulated (Nobile, Nett et al. 2006)</td>
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<td></td>
<td></td>
<td></td>
<td>Involved in mating (Ene and Bennett 2009)</td>
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<td></td>
<td></td>
<td></td>
<td>Down-regulated by fluconazole (Sorgo, Heilmann et al. 2011)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Induced by low oxygen (Sosinska, de Groot et al. 2008)</td>
</tr>
<tr>
<td>Hyr1[^b]</td>
<td>919</td>
<td>143-286</td>
<td>Induced in hypha (Bailey, Feldmann et al. 1996)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mediates resistance to phagocytosis (Luo, Ibrahim et al. 2010)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Member of the Hyr-like family which also occurs in other pathogenic Candida spp. (Butler, Rasmussen et al. 2009)</td>
</tr>
<tr>
<td>Ihd1[^c]</td>
<td>392</td>
<td>+[^#]</td>
<td>Induced during hyphal development, morphologically regulated (Nantel, Dignard et al. 2002)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Unknown function</td>
</tr>
<tr>
<td>Pga4[^b]</td>
<td>451</td>
<td>42-385</td>
<td>Transglucosidase with an N-terminal GH7 (glycoside hydrolase) domain (Cantarel, Coutinho et al. 2009)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Belongs to the Gas1 family, which is widespread in fungi (Popolo and Vai 1999)</td>
</tr>
<tr>
<td>Pga10[^d]</td>
<td>250</td>
<td>47-60</td>
<td>N-terminal CFEM domain</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Role in virulence: heme-bound iron utilization (Weissman and Kornitzer 2004)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Loss of function results in fragile biofilms (Perez, Pedros et al. 2006)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Induced by ketoconazole (Liu, Lee et al. 2005), and low oxygen (Sosinska, de Groot et al. 2008)</td>
</tr>
<tr>
<td>Pga30[^g]</td>
<td>277</td>
<td>123-149</td>
<td>Unknown function</td>
</tr>
</tbody>
</table>
### General introduction

- The Pga30-like gene family is enriched in pathogenic *Candida* spp. (Butler, Rasmussen *et al.* 2009)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Start</th>
<th>End</th>
<th>Description</th>
</tr>
</thead>
</table>
| Pga31*| 352   | 102-162 | - Unknown function  
- Up regulated by the cell wall perturbing agent Congo red (Sorgo, Heilmann *et al.* 2011) |
| Pga45 | 462   | 351-364 | - Unknown function  
- Conserved in *Candida* spp. |
| Pga59*| 113   | No data | - Role in cell wall integrity: gene inactivation causes increased sensitivity to cell wall perturbing drugs and alterations in cell wall structure (Moreno-Ruiz, Ortu *et al.* 2009)  
- Cell wall localization determined by GFP fusion (Moreno-Ruiz, Ortu *et al.* 2009)  
- Mature protein predicted to consist of 74 residues with three cysteine residues; has potential sites for *N*- and *O*- glycosylation (Moreno-Ruiz, Ortu *et al.* 2009) |
| Pga62*| 213   | No data | - Role in cell wall integrity: gene inactivation causes increased sensitivity to cell wall perturbing drugs and alterations in cell wall structure (Moreno-Ruiz, Ortu *et al.* 2009)  
- Cell wall localization determined by GFP fusion (Moreno-Ruiz, Ortu *et al.* 2009)  
- Contains two tandem repeats similar to the 3-cysteine domain of Pga59 (Moreno-Ruiz, Ortu *et al.* 2009) |
| Phr1* | 548   | 71-452 | - Transglycosylase involved in cross-linking of β-1,3- and β-1,6-glucans (Fonzi 1999), N-terminal GH72 domain followed by an 8-cysteine domain (pfam07983) in the C-terminal half, belongs to the Gas1 family (Popolo and Vai 1999)  
- Required for maintenance of the hyphal form of growth, systemic infection, adhesion to abiotic surfaces and invasion of epithelial cells (Calderon, Zavrel *et al.* 2010)  
- Up-regulated by fluconazole (Sorgo, Heilmann *et al.* 2011) |
<p>| Phr2* | 544   | 23-269 | - Transglycosylase involved in cross-linking of β-1,3- and β-1,6-glucans (Fonzi 1999), N-terminal GH72 domain followed by an 8-cysteine domain (pfam07983) in the C-terminal half, belongs to the Gas1 family (Popolo and Vai 1999) |</p>
<table>
<thead>
<tr>
<th>Gene</th>
<th>Start</th>
<th>End</th>
<th>Summary</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rbt1</td>
<td>721</td>
<td>262-271</td>
<td>Repressed by Tup1 (Tup1 is a transcriptional factor- a negative regulator of hyphal growth (Braun, Head et al. 2000)), regulated by the Cph1, Cph2, Efg1 transcriptional factors regulating hyphal growth (Lane, Birse et al. 2001). Required for systemic infection (Braun, Head et al. 2000). N-terminal Flo11 domain (pfam10182).</td>
</tr>
<tr>
<td>Rbt5</td>
<td>241</td>
<td>37-92</td>
<td>Repressed by Tup1 (Tup1 is a transcriptional factor- a negative regulator of hyphal growth (Braun, Head et al. 2000)). Involved in haemoglobin utilization (Weissman and Kornitzer 2004). N-terminal CFEM domain. Regulated by Rim101 (Bensen, Martin et al. 2004) and transcriptional regulator of biofilm formation Bcr1 (Nobile and Mitchell 2005). Deletion negatively affects biofilm formation and results in increased cell surface hydrophobicity (Perez, Pedros et al. 2006; Perez, Ramage et al. 2011). Protein levels increase upon oxygen and iron deprivation (Sosinska, de Groot et al. 2008) and by fluconazole (Sorgo, Heilmann et al. 2011).</td>
</tr>
<tr>
<td>Rhd3 /Pga29</td>
<td>204</td>
<td>16-150</td>
<td>Repressed during hyphal development (Nantel, Dignard et al. 2002). One of the most abundant CWPs in the yeast form (De Boer et al. 2010). Probably structural protein, highly O-mannosylated, deletion does not change cell wall architecture but decreases mannan content of the cell walls and causes alteration of the cell surface leading to a reduced proinflammatory immune response likely due to...</td>
</tr>
</tbody>
</table>
**General introduction**

<table>
<thead>
<tr>
<th>Protein</th>
<th>MW (Da)</th>
<th>ORF</th>
<th>Description</th>
</tr>
</thead>
</table>
| Sap9\textsuperscript{12} | 544 | +\# | Secreted aspartyl protease  
Required for cell wall integrity, separation of cells during budding, role in adhesion to epithelial cells and epithelial cell damage (Albrecht, Felk \textit{et al.} 2006)  
Yapsin-like protein (Gagnon-Arsenault, Tremblay \textit{et al.} 2006; Krysan, Ting \textit{et al.} 2005)  
Mainly found in the plasma membrane  
Up-regulated by fluconazole (Sorgo, Heilmann \textit{et al.} 2011) |
| Sap10\textsuperscript{12} | 453 | No data | Secreted aspartyl protease  
Required for cell wall integrity, separation of cells during budding, role in adhesion to epithelial cells and epithelial cell damage (Albrecht, Felk \textit{et al.} 2006)  
Yapsin-like protein (Gagnon-Arsenault, Tremblay \textit{et al.} 2006; Krysan, Ting \textit{et al.} 2005)  
Found in the plasma membrane and in the cell wall (Albrecht, Felk \textit{et al.} 2006) |
| Sod4\textsuperscript{13} | 232 | 16-117 | Copper-zinc-dependent superoxide dismutase; contributes to clearance of ROS (Frohner, Bourgeois \textit{et al.} 2009)  
Down-regulated in hyphae (Heilmann, Sorgo \textit{et al.} 2011) |
| Sod5\textsuperscript{13} | 228 | 26-140 | Copper-zinc-dependent superoxide dismutase; contributes to clearance of ROS (Frohner, Bourgeois \textit{et al.} 2009)  
Up-regulated by fluconazole (Sorgo, Heilmann \textit{et al.} 2011) and in hyphae (Heilmann, Sorgo \textit{et al.} 2011; Martchenko, Alarco \textit{et al.} 2004) |
| Ssr1 | 234 | 23-79 | N-terminal CFEM domain  
Localized in the cell wall (Garcera, Martinez \textit{et al.} 2003) |
| Utr2\textsuperscript{3} | 470 | 75-310 | Putative transglycosylase (Cabib, Farkas \textit{et al.} 2008; Pardini, De Groot \textit{et al.} 2006), GH16 domain (Cantarel, Coutinho \textit{et al.} 2009)  
Conserved in ascomycetes and basidiomycetes |
| Ywp1 | 533 | 93-349 | Yeast wall protein 1  
Up-regulated in exponentially growing yeast cells, but down regulated in stationary phase and in hyphae  
Inactivation increases adherence and facilitates biofilm formation (Granger, Flenniken \textit{et al.} 2008) |
### Chapter 1

<table>
<thead>
<tr>
<th>Protein</th>
<th>Accession</th>
<th>Start-End</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mp65\textsuperscript{14}</td>
<td>378</td>
<td>128-371</td>
<td>Putative transglycosylase with a C-terminal GH17 domain (Cantarel, Coutinho \textit{et al.} 2009), vaccine candidate (Pietrella, Lupo \textit{et al.} 2008), deletion causes a defect in hyphal formation, diminished adherence to abiotic surfaces, and reduced virulence (Sandini, La Valle \textit{et al.} 2007)</td>
</tr>
<tr>
<td>Pir1\textsuperscript{15}</td>
<td>346</td>
<td>19-346</td>
<td>Potential (\beta)-1,3-glucan crosslinking protein, essential for cell-wall integrity (Ecker, Deutzmann \textit{et al.} 2006; Klis, Ram \textit{et al.} 2007; Martinez, Castillo \textit{et al.} 2004), S. cerevisiae homolog induced by low pH (Kapteyn, ter Riet \textit{et al.} 2001), induced by hypoxia (Sosinska, de Groot \textit{et al.} 2008 and low pH (Sosinska, de Koning \textit{et al.} 2011)</td>
</tr>
<tr>
<td>Sim1\textsuperscript{16}</td>
<td>372</td>
<td>32-265</td>
<td>C-terminal SUN domain; required for septation; synthetic lethality with SUN41 results in osmoremediable wall rupturing close to the septum (Firon, Aubert \textit{et al.} 2007), induced by hypoxia (Setiadi, Doedt \textit{et al.} 2006; Sosinska, de Groot \textit{et al.} 2008)</td>
</tr>
<tr>
<td>Tos1</td>
<td>468</td>
<td>288-370</td>
<td>N-terminal DUF2403 domain (glycine rich domain) and C-terminal DUF2401 domain (putative TOS1-like glycosyl hydrolase domain)</td>
</tr>
</tbody>
</table>


\# A. Sorgo, C. Heilmann, & G. Sosinska, personal observations

Protein families (based on (Klis, de Koster \textit{et al.} 2011)):

\textbf{1 Als (Agglutinin like sequence) family}, 8 members; all possess an adhesion domain (Pfam PF11766) at the N-terminus, followed by a short high-amyloid-potential sequence (Otoo, Lee \textit{et al.} 2008); role in host cell adherence (Grubb, Murdoch \textit{et al.} 2008; Hoyer 2001); each family member has a four-domain structure: 1) the Ig domain of 300 amino acids, a region with three tandem Ig-like sequences
followed by a short high-amyloid-potential sequence; 2) a 127-residue threonine-rich conserved domain, 3) a region consisting of a variable number of 36-residue, threonine rich containing tandem repeats, 4) the stem domain, a highly glycosylated serine/threonine-rich domain of low structural complexity and variable length (Otoo, Lee et al. 2008; Rauceo, De Armond et al. 2006).

2 **Cht family**, 4 members (Cht1, Cht2, Cht3, Cht4) (Dunkler, Walther et al. 2005; McCreath, Specht et al. 1995; McCreath, Specht et al. 1996), chitinase

3 **Crh family**, 3 members (Crh11, Crh12, Utr2), transglycosylases involved in linking β-glucan and chitin

4 **Rbt5 family**, 4 members (Rbt5, Csa1, Pga7, Pga10), CFEM domain with conserved 8 cysteine motif, iron utilization

5 **Ecm33 family**, 3 members (Ecm33, Ecm331, 19.4955), role in cell wall integrity

6 **Hyr/Iff family**, 8[11] members (Hyr1, Hyr3, Hyr4, Iff3, Iff4, Iff5, Iff6, Iff9) [Iff8, Iff11, Flo9], three members do not possess a GPI anchor attachment site, predicted exo-α sialidase/sialic acid binding domain (Pfam PF11765)

7 **Ihd1 family**, 6 members (Ihd1, Pga15, Pga41, Pga42, Pga50, Pga61), unknown function, possess a conserved seven-cysteine pattern at their N-terminus

8 **Phr family**, 3 [4] members (Phr1, Phr2, Pga4) [Pga5], transglycosylases involved in elongation of glucan, Pga5 is a predicted member

9 **Pga30 family**, 3 members (Pga30, Pga31, Rhd3/Pga29), Pga30 and Pga31 have an unknown function
10. **Pga59 family**, 2 members (Pga59, Pga62), abundant CWPs masking the glucan layer


12. **Yapsin family**, 2 members (Sap9, Sap10), involved in cell wall integrity (Gagnon-Arsenault, Tremblay *et al.* 2006; Krysan, Ting *et al.* 2005)

13. **Sod4 family**, 2 members (Sod4, Sod5), superoxide dismutases, role in protection against oxidative stress

14. **Mp65 family**, 3 members (Mp65, Scw4, Scw11), transglycosylases

15. **Pir family**, one member in *C. albicans*, protein with internal repeats, similar structure in *S. cerevisiae* Pir CWPs (5 members) (Ecker, Deutzmann *et al.* 2006)

16. **Sun family**, 2 members (Sim1, Sun41), cell separation

1.6.4 Pir CWP

Pir1 is a putative cross-linking CWP that contains a C-terminal conserved 4-Cys pattern. Whereas most CWPs are coat proteins, Pir1 is an exception. It is mainly localized in the internal skeletal layer, where it is believed to act as a cross linking protein, and it is essential for cell wall integrity (Ecker, Deutzmann *et al.* 2006; Kapteyn, Hoyer *et al.* 2000; Klis, Ram *et al.* 2007; Martinez, Castillo *et al.* 2004). It can be released from β-1,3-glucan by mild alkali treatment as it is linked via alkali-labile ester linkages between the gamma-carboxyl group of glutamic acids originating from specific glutamines, and hydroxyl groups of glucose residues of β-
1,3-glucan chains (Ecker, Deutzmann et al. 2006; Mrsa, Seidl et al. 1997). In *S. cerevisiae* there are 5 Pir proteins (Pir1-5). In *C. albicans* there is one Pir-CWP, Pir1, and its function is essential (Martinez, Castillo et al. 2004). In addition, *C. albicans* has a predicted Pir-like protein, called Pir32, that has not been studied in detail. Pir proteins share a common structure; they contain a four-cysteine domain of unknown function, and a repeat domain (PFam00399), in which specific glutamine residues are involved in cross linking β-1,3 glucan (Figure 6). The wall levels of Pir proteins are increased upon stress conditions in *S. cerevisiae* like low pH (Kapteyn, ter Riet et al. 2001); in *C. albicans* the levels of Pir1 are increased in cell walls of yeast cells, grown at low pH and under hypoxic conditions (Sosinska, de Groot et al. 2008), and after treatment of cells with fluconazole (Sorgo, Heilmann et al. 2011).

**Figure 6** Model of the mature form of Pir CWPs and its possible role in cross linking of β-1,3 glucan. The central glutamine residue in the core sequence of the repeat domain is probably involved in making cross-links. X- any hydrophobic amino acid.

1.6.5 General and specific properties of cell wall proteins

The cell wall surface of *C. albicans* is occupied by 1.8–2.5x10⁶ covalently bound CWP molecules or 27–37x10³ CWPs µm⁻² (Klis, Sosinska et al. 2009), similar as in *S. cerevisiae* (Dranginis, Rauceo et al. 2007; Yin, de Groot et al. 2008). Most CWPs stay permanently in the cell wall once they are incorporated (E. Mol personal observations, (Ruiz-Herrera, Martinez et al. 2002)). Only a slow and limited release of wall-bound CWPs into the medium takes place over time, which accounts for
about 20% of the initial number of CWPs, probably as a result of (i) local severage of the cell wall between mother and daughter cell during cell separation, and (ii) some extension and remodeling of the mother cell wall during each subsequent cell cycle (E. Mol personal observations, (Hiller, Heine et al. 2007; Sorgo, Heilmann et al. 2010). Cell wall linked proteins in the external layer of the cell wall determine several general properties of the cell surface: a) cell wall permeability, b) cell surface charge, c) cell surface hydrophobicity, d) adhesion, e) interaction with the host, f) biofilm formation. Cell wall permeability is restricted due to the dense packing of CWPs, the presence of bulky N-linked protein side-chains, and the formation of intermolecular disulfide bridges (de Nobel, Klis et al. 1990; Zlotnik, Fernandez et al. 1984). Especially, some very abundant, highly glycosylated CWPs function as masking coat proteins. This protects the cell wall polysaccharides against degradation by foreign glycanases and shields β-glucan from detection by the mammalian β-glucan receptor dectin-1 present in leucocytes (Esteban, Popp et al. 2011; Gantner, Simmons et al. 2005; Gow, Netea et al. 2007; Wheeler and Fink 2006; Zlotnik, Fernandez et al. 1984). Dectin-1 recognizes and binds to β-glucan exposed in birth and bud scars of yeast cells (Gantner, Simmons et al. 2005). Negative charges on the cell surface are due to the presence of phosphodiester bridges in N-linked carbohydrate side-chains of CWPs (Cutler 2001; Fradin, Slomianny et al. 2008; Horisberger and Clerc 1988). Cell surface hydrophobicity depends on the properties of some CWPs and is important for the attachment of C. albicans cells to hydrophobic surfaces and also to host cells. For example, the CWP Eap1 mediates adherence to styrene, a hydrophobic polymer derived from ethenylbenzene (Li and Palecek 2003), and to mammalian cells due to the presence of a serine/threonine-rich domain containing tandem repeats (Li and Palecek 2008). Adhesion is mediated
by adhesins from the Als family of CWPs and by Hwp1. Als proteins possess a four-domain structure in which threonine-rich tandem repeat domains play a role in cell aggregation. Als proteins can bind to various mammalian cells (Klotz, Gaur et al. 2004) and have the ability to form cells aggregates which are important for the formation of a cohesive biofilm and the formation of aggregates consisting of bacterial and fungal cells, which is mediated by an amyloid-like sequence (Fowler, Koulov et al. 2007; Garcia, Lee et al. 2011; Otoo, Lee et al. 2008). The CWP Hwp1 also plays a role in adhesion to host cells as its N-terminal domain is recognized as a substrate by host transglutaminases at the epithelial surface (Staab, Bradway et al. 1999), and also in biofilm formation (Nobile, Schneider et al. 2008). Other functions of CWPs are remodeling and maintaining the structure of the cell wall (Pir1, and several carbohydrate-active enzymes, which posses a known or predicted glycosylase/transglycosylase domain). It is not known if the location of the carbohydrate-active enzymes is in an external layer of the cell wall as part of the protein coat and thus away from their potential substrates. Localized externally they could also be involved in formation of biofilm matrix material, which contains similar components as the cell wall. For example, loss of the putative cell wall (trans)glycosidase Sun41, a non-GPI CWP, results in strongly decreased biofilm formation on an abiotic surface (Hiller, Heine et al. 2007; Norice, Smith et al. 2007). The exact role of proteins from the yapsin family (Sap9 and 10), which posses proteolytic activity, in cell wall maintenance is not known although their loss affects cell wall structure (Gagnon-Arsenault, Tremblay et al. 2006).

An important role of CWPs in *C. albicans* is adaptation of the fungus to the host environment. This can be achieved by general cell surface properties of CWPs as mentioned above but also more specifically by invasion-related CWPs like Als3,
which facilitates endocytosis (Phan, Myers et al. 2007), by CWPs that cope with oxidative stress originating from innate immune cells (Sod4 and 5 (de Groot, de Boer et al. 2004; Fradin, De Groot et al. 2005; Martchenko, Alarco et al. 2004)), by CWPs involved in iron acquisition (Als3, Rbt5) (Sosinska, de Groot et al. 2008; Weissman and Kornitzer 2004) and by CWPs that play a role in biofilm formation.

Outline of the thesis

The main aim of this work was to elucidate the adaptation of the pathogenic fungus *C. albicans* to factors encountered in the host environment in terms of changes in its cell wall proteome and cell wall properties. An additional aim was to describe the cell wall proteome of the non-pathogenic and only distantly related fungus *Schizosaccharomyces pombe*.

**Chapter 1** contains the general introduction and consists of three parts: 1) general information about *C. albicans* including its taxonomy, its genome, and its biology, 2) morphological states of *C. albicans* and its regulation, 3) iron utilization by *C. albicans*, 4) effect of oxygen deprivation on *C. albicans*, 5) the cell wall of *C. albicans*.

In **chapter 2** I describe the adaptation of *C. albicans* to low oxygen and elevated CO₂ levels, low pH and 37°C, conditions which the cells encounter *in vivo* during vaginitis. Changes in cell wall composition were investigated using qualitative mass spectrometry, immunological analysis and by measuring the resistance of intact cells to cell wall-degrading enzymes.
Chapter 3 investigates the effects of ambient pH on *C. albicans* morphology and composition of the cell wall proteome. I developed an *in vitro* model of mucosal infections at pH 4 and pH 7 resembling the environmental conditions of different niches in the human body where *C. albicans* causes infection (vaginitis and oral infection). The changes in composition of the cell wall proteome were measured using qualitative MS (ESI-QTOF), quantitative FT-MS in combination with metabolic labelling of the cells, and immunological analysis.

Chapter 4 describes the effect of thermal stress on *C. albicans*. As high temperatures tend to cause cell wall stress and 37°C is a natural temperature for *C. albicans* living in the human host, we investigated the effect of 42°C on changes on growth and composition of the cell wall. The changes in cell wall composition were determined by measuring the sensitivity of intact cells to cell wall-perturbing drugs, by measuring the chitin content in cell walls, by qualitative MS analysis of the cell wall proteome, and by immunological analysis.

Chapter 5 reveals the cell wall proteome of the fission yeast *S. pombe* by using MS and an immunological approach. It shows that *S. pombe* similarly as *C. albicans* and model yeast *S. cerevisiae* contains GPI- and non GPI-proteins which are covalently linked to the cell wall.

Chapter 6 contains a discussion of the results presented in this thesis.

References


General introduction


Chapter 1
General introduction


