Compartmentalization of metabolic pathways in Candida albicans: a matter of transport
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Introduction

“We propose that modern [Candida albicans] strains reflect early hominid evolution and have co-evolved with their host.”
Timothy J. Lott, Ruth E. Fundyga, Randall J. Kuykendall, Jonathan Arnold
Fungal Genetics and Biology, 2005.

The yeast Candida albicans is a human commensal that resides in the gastrointestinal tract and is part of the normal microflora of most humans (16). The interaction between C. albicans and its human host is thought to go back to the dawn of human existence, as nucleotide polymorphisms in modern C. albicans strains suggest a common ancestor 3-16 million years ago, which is around the same time as early hominid evolution (93). The authors therefore propose that the evolutionary relationship between modern C. albicans resembles early hominid evolution and thus suggest a long-lasting commensal relationship between fungus and host. It is thought that individuals are colonized with C. albicans at or near birth (128) and that from then on the fungus becomes part of the commensal microbial community in the oral cavity and gastrointestinal tract. However, environmental changes such as alterations in host immunity or physical perturbation of its niche, for example through surgery, can cause spreading through the body resulting in overgrowth. The transformation of the commensal C. albicans into the opportunistic pathogen has become more and more prevalent due to the rapid medical progression of the last century.

Biology and evolution of pathogenic fungi

“The potential benefits of sex are to purge the genome of deleterious mutations...”
Joseph Heitman
Current Biology, 2006.

The fungal kingdom

Fungi, or yeasts, are heterotrophic eukaryotic organisms and the fungal kingdom consists of the phyla of the Basidiomycetes, Zygomycetes, Chytrids and the Ascomycetes, the latter of which harbor 75% of all described fungi (Fig. 1). The different phyla of the fungal kingdom include mushrooms, black bread mold and bakers’ yeast, illustrating the morphological diversity amongst these organisms. Most fungi are living in soil or as symbionts of plants, animals or other fungi, some of them only becoming noticeable when fruiting, either as mushrooms or molds. Many fungal species are used as a direct source of food, such as mushrooms and truffles and in fermentation of various food products, such as wine, beer and soy sauce. One of their other virtues is that they can be used as sources for antibiotics and various enzymes, such as cellulases, pectinases, and proteases.

Several species of the fungi have adapted to a pathogenic lifestyle being able to infect animals or plants, the latter causing losses due to diseases of crops that have a large impact on human food supply and local economies (e.g., rice blast disease). However, of the total of 1.5 million fungal species a minority of only 150 species are pathogenic
to humans, animals or plants (82). Infection requires adaptation to enable growth in completely different conditions. The human body, for example, is a very hostile environment for the fungus and survival requires the ability to grow at 37°C, evade the immune system and to acquire nutrients in a competitive environment.

Three pathogenic lifestyles: Cryptococcus, Aspergillus and Candida

The three most common, successful systemic human fungal pathogenic species are Cryptococcus, *Aspergillus fumigatus* and *C. albicans* (Fig. 2). A unifying feature of these three major fungal pathogens is that they all mainly cause severe infections in immunocompromised hosts and only rarely in normal healthy hosts.

Cryptococcus is a ubiquitous basidiomycete that is associated with soil and pigeon guano. This pathogen comprises four defined serotypes (A-D) that have diverged over 10 to 40 million years into sibling species *C. neoformans* variety *grubii* (serotype A), *C. neoformans* variety *neoformans* (serotype D) and *C. gattii* (serotypes B and C), the latter of which has become endemic on Vancouver Island (72). Cryptococcus spores are readily aerosolized and deposited in alveoli of the lung. As these spores are very ubiquitous, most individuals have been exposed and the organism occurs in a dormant latent granulomatous form in the lymph nodes. Upon reactivated in response to immunosuppression, the organism disseminates to infect most prominently the central nervous system, including the
brain. The morphology of Cryptococcus during the infectious stage is yeast-like and characterized by a distinctive melanin capsule that surrounds the plasma membrane. Cryptococcus is a haploid organism with a bipolar mating-type system, $a$ and $\alpha$ (61). However, the vast majority of isolates are $\alpha$ and virulence is also linked to this mating type (81, 111).

Figure 2. Reproduction in three pathogenic fungi

(A) *Cryptococcus neoformans* is a melanin-encapsulated basidiomycete. Yeasts of opposite mating type ($a$ and $\alpha$) can undergo diploidization and sporulation. A sexual cycle has recently been demonstrated in the laboratory. (B) The filamentous ascomycete *Aspergillus fumigatus* continuously sporulates. A sexual cycle has recently been demonstrated in the laboratory. (C) The diploid ascomycete *Candida albicans* has a parasexual cycle and is normally found as an $a/\alpha$ heterozygote. Mating-type locus (MTL) homozygosis leads to white $a/a$ and $\alpha/\alpha$ cells that can switch to the mating-competent opaque phenotype. Cell fusion produces an $a/a/\alpha/\alpha$ tetraploid that under certain conditions undergoes chromosomal loss to return to the diploid state.

*A. fumigatus* is a filamentous ascomycete that shares the inhalation route of infection with Cryptococcus. People are daily exposed to *A. fumigatus* spores, but only severely immunocompromised are at high risk for disease. Alveolar macrophages play an important role in preventing infections initiated from spores that can lead to fungal balls in the lungs. A high incidence of Aspergillosis is observed among organ transplant recipients, especially lung transplants. The increased prevalence of infections could be caused by increased exposure of the organ to the environment, impaired defenses or reduced cough and mucociliary clearance (19, 142). *A. fumigatus* is thought to have a heterothallic sexual cycle and population genetic studies reveal a 1:1 distribution of the two mating types in nature providing evidence of recombination (117). Very recently such a sexual cycle for *A. fumigatus* was demonstrated in the laboratory (112). However, isolates worldwide share considerable genetic similarity, which could be indicative of a principally clonal mode of reproduction or inbreeding (134).
C. albicans is a polymorphic diploid ascomycete that is a commensal in healthy human hosts, but an opportunistic pathogen when the immune system is weakened. Several clades exist worldwide: North American clades I-III and European and African-specific clades, but no correlation has been found between host factors such as sex and age and strain type (143). C. albicans multiplies primarily in clonal mode of reproduction (44, 92) and relative high levels of non-homologous mitotic recombination, translocation and aneuploidy are observed (21). However, lately more and more evidence has been put forward for a cryptic sexual or parasexual cycle that entails mating and genome reduction without meiosis (63). Although C. albicans harbors both alleles of the mating-type locus and is an a/α diploid that would not be expected to mate (62), mating competent a/a and α/α homozygous strains exist in clinical isolates as 5-7% of the population (85, 88). A switch from the regular “white” morphology to the “opaque” cell type was shown to govern mating efficiency (103). C. albicans mating appears to occur robustly on skin, where the temperature is 31-32ºC, a prerequisite for stable opaque cell formation (83). Mating on skin may facilitate genetic exchange between isolates from different hosts, promote transfer between hosts or avoid immune surveillance during mating.

Interestingly, all three major systemic human pathogens have retained the machinery needed for a sexual cycle, yet all three limit their access to sexual reproduction. This suggests that it might confer an advantage to be able to create clonal populations in response to constant environment and recombining populations in response to changing environments. Another advantage of reduction of sexual reproduction is limitation of immunogenic spore formation. Sporulation might be reduced if continuous spreading from a nutrient-rich environment like the body is not necessary (48).

Yeasts as model organisms
Since ancient times the yeast Saccharomyces cerevisiae has been used for baking and brewing, but in the last century it was recognized as a suitable unicellular model organism to study eukaryotic cells, partially because of its compact genome of about 6000 genes (compared to the estimated 20,000-25,000 protein-coding human genes) and relatively easy genetic manipulation. Many cellular processes are conserved between yeast and human and even an estimated 20% of human gene defects leading to disease have yeast counterparts. Because of the diversity among fungi, different species were found most suitable to study different aspects of eukaryotic cell behavior. For example Neurospora crassa, the orange bread mold, is mainly used for genetic studies on meiosis, metabolic regulation, and circadian rhythm and Schizosaccharomyces pombe or fission yeast for studies on cell cycle, cell polarity, RNAi and centromere structure. It is tempting to extrapolate findings from one organism to the other, but the observation that for example S. cerevisiae and C. albicans are evolutionary as distant as human and fish, is important to keep in mind during inter-species comparison.
Epidemiology of *C. albicans*

“We have administered approximately $10^{12}$ cells of Candida albicans orally to a healthy volunteer. (…). The volunteer (W.K., one of the authors) is a 36-year-old surgeon …”

W. Krause, H. Matheis and K. Wulf


One of the main differences between *C. albicans* and the other described pathogenic fungi is that, in contrast with an environmental habitat and an inhalation route of infection of the latter, the normal habitat of *C. albicans* is the human body. *C. albicans* resides in the gastrointestinal tract and oral cavity as part of the normal microflora in an estimated 70% of the human population (16, 133). The commensal lifestyle of the fungus is associated with normal immune response of the host and characterized by nutrient acquisition, adherence and maintenance. While *in vitro* growth at 37°C is associated with hyphal formation, this seems not always to be the case within the body, as *C. albicans* is mostly found as yeast cells in the gut (22). In the 1960s, a bold experiment of oral self administration of 80 grams of *C. albicans* proved that the fungus can transmigrate from gut to blood (77). Candida infections can be divided into two groups: superficial mucosal Candida infections and systemic infections. The mucosal infections include thrush of the mouth and throat, vaginal infections, diaper rash and skin infections. The systemic infections occur almost exclusively in immunocompromised hosts and can result in deep mycosis in which the fungus has invaded the organs. Besides immune-incompetence, other factors can also contribute to candidiasis: natural factors comprise microbial infections other than fungi, diabetes, pregnancy, infancy or old age. Dietary factors include nutrient excess (carbohydrate-rich diets) or vitamin deficiencies. Trauma, burns, wounds, occlusions or prosthetics like dentures also lead to increased *C. albicans* infections. Certain medical treatments can contribute to candidiasis such as antibiotics that result in reduction of bacterial competitors, immunosuppressive drugs that alters host defenses or medical devises such as catheters (125). The latter is probably associated with the formation of microbial biofilms (see below). Lately a rise in hospital infections by non-albicans Candida species like *C. tropicalis* and *C. lusitaniae* was described by several studies in the United States (1, 55) and Europe (123, 157, 158), showing that also previously considered non-pathogenic Candida species are able to cause infections.

Virulence factors

“Specific inactivation of the gene(s) associated with the suspected virulence trait should lead to a measurable loss in pathogenicity or virulence (…). Specific inactivation or deletion of the gene(s) should lead to loss of function in the clone.”

Koch’s second postulate adapted for molecular biology

Stanley Falkow

Reviews of Infectious Diseases, 1988

Although *C. albicans* is a member of the normal commensal flora of the gastrointestinal tract of most humans, it has the additional ability to invade other parts of the body
and cause superficial mucosal infections or severe systemic infections. The traits that contribute to these infectious processes are called virulence factors and are defined by the postulates of Koch adapted for molecular biology (31, 32). The molecular postulates state, among other things, that disruption of a virulence gene should lead to measurable loss of pathogenicity and that re-introducing the virulence gene should restore pathogenicity. The interpretation of the postulates is however open to discussion, as some genes that are essential for growth under normal physiological conditions would also classify as virulence factors using this definition.

**Models to assay C. albicans virulence**

In the search for *C. albicans* virulence factors, several different virulence models have been used of which the mouse survival test is the most widely accepted. In this acute lethality test, a *C. albicans* yeast cell suspension is injected into the tail veins of several mice and survival/lethality of the animals is assayed over a period of three to four weeks (108). It is thought that this test most closely resembles hematogenously disseminated candidiasis in patients, however it is blind to virulence genes associated with the actual process of dissemination from gut to bloodstream. Therefore during the last years more specific models have been developed in which the role of virulence factors in certain aspects of *C. albicans* infection can be monitored more closely. Some of the *in vivo* models include the murine/rat vaginitis model (18, 122) and the mouse model of gastrointestinal (GI) colonization and fungemia (76). Examples of *in vitro* models are invasion of reconstituted human oral or vaginal epithelial cells (138).

**Figure 3. Candida albicans, a dimorphic fungus**

Morphology of the polymorphic fungus *C. albicans* during growth in rich glucose medium at 28°C (yeasts) and 37°C (hyphae).

**Morphology**

*C. albicans* is a polymorphic fungus that has at least three distinct morphological growth forms: yeast, pseudohyphae and hyphae. Additionally it has the ability to form conidia. Yeast and pseudohyphae of *C. albicans* are similar to those of *S. cerevisiae* in shape and size. Yeast cells grow by asymmetric budding, forming smooth, round colonies, while pseudohyphal cells grow in a unipolar pattern and their colonies are fibrous and rough. Pseudohyphal cells remain attached after cytokinesis, forming branched chains of
elongated buds. Hyphae are narrower than pseudohyphal cells (about 2 μm) and have parallel walls with no obvious constriction at the site of septation (Fig. 3) (150).

Growth in the yeast form is associated with low temperatures (28ºC) and low pH. Hyphal growth is stimulated by higher temperatures (37ºC), high pH and is associated with stress conditions like nutrient starvation and reactive oxygen species. Phagocytosis of yeast cells by macrophages also results in induction of hyphal formation. Switching between yeast and hyphal growth is essential for virulence when tested in the mouse model of infection. A cph1/efg1 null mutant that cannot form hyphae and is therefore locked in the yeast phase is avirulent in this model (87), however, transcription of hyphal-associated genes might be affected in this strain, as Cph1 and Efg1 are both transcription factors involved in the initiation of the hyphal program. Therefore avirulence of another null mutant, hgc1, which is thought to be solely affected in hyphal development, poses a better argument that hyphae formation specifically is essential for virulence (175). Switching between yeast and hyphal forms seems essential for virulence, as also a strain with an increased tendency to form hyphae showed attenuated virulence (11, 12). The fact that, in general, the hyphal form of *C. albicans* is associated with pathology, is an exception among pathogenic fungi. Dimorphic pathogenic ascomycetes like *H. capsulatum*, *P. brasiliensis*, *C. immitis*, *S. schenkii*, *P. marneffei*, *B. dermatitides* grow as filaments at 25ºC in their natural habitat soil, but start growing as yeasts after the temperature shift to 37ºC that is associated with infection of the body (8, 100). Apparently the commensal yeast *C. albicans* has reversed the yeast-hyphal program to enable permanent association with its human host.

**Invasion**

Ultrastructural studies of specimen from humans and experimental animals have suggested that *C. albicans* hyphae are indeed the invasive form of the organism, because hyphae are found within epithelial cells, whereas yeasts are generally found either on the epithelial cell surface or between these cells (127, 139). Hyphae are hypothesized to be essential for crossing epithelial layers and for invasion of tissue. One of the read-out parameters of the mouse model of infection is the organ fungal burden, as measured by colony forming units retrieved from shredded organ material. Several models for hyphal invasion are in use, for example systems that either monitor the penetration (crossing) of a layer of epithelial cells (138) or of the chick chorioallantoic membrane in (41). The importance of the hyphae for invasive growth might partially lie in the ability to build up sufficient physical pressure to disrupt cell-cell adhesion in epithelial layers of tissues. For the plant pathogen *Magnaporthe grisea* it was suggested that turgor build-up in hyphae sprouting from the infectious appressoria is essential to invade plant tissue (171). An interesting mechanism of invasion of epithelial cells is the induction of epithelial cell endocytosis by *C. albicans*. Both yeasts and hyphae can stimulate epithelial cells to produce pseudopods that surround the organism and lead to endocytosis, thereby displaying a more sophisticated way of breaching the epithelial layer (4, 118).

**Adhesins**

In addition to the merely physical and morphological differences between *C. albicans* yeast and hyphae, a wide variety of different genes are specifically expressed after initiation of the hyphal program. The *C. albicans* genome encodes multiple cell wall adhesins, some specifically expressed by yeasts and others by hyphae. The best-known hyphae-associated
Adhesins include the single hyphal wall protein (Hwp1) and the Als and Ewa families of adhesion proteins (106). The different adhesins determine the stickiness of hyphae and they are thought to play a role in vivo in the attachment of hyphae to epithelial cells. The adhesins have been widely studied and show a wide variety in length and binding capacities. Because many of the adhesins have overlapping functions, their individual contribution to virulence is difficult to address. Transformation and characterization of S. cerevisiae with a C. albicans adhesin of choice has proven a powerful tool to understand the capacities of these virulence-associated proteins (56).

**Secreted enzymes**

In 1965 it was shown that C. albicans has the ability to utilize protein as the sole nitrogen source and that the fungus achieves this by expressing proteolytic activity (146). This activity was demonstrated to be encoded by a family of at least 10 members of secreted aspartic proteinases (SAPs) (57, 136). Sap1 to Sap8 are excreted in the extracellular space while Sap9 and Sap10 are membrane-anchored glycoprophosphatidylinositol (GPI) proteins and most of these lytic enzymes display an optimal activity in the acidic pH range. Other pathogenic Candida species including C. dubliniensis, C. tropicalis and C. parapsilosis also express proteolytic activity in vitro (reviewed by 137). Sap activity was shown to be higher in fungi isolated from oral diseased states (80, 113) while Sap5 was shown to be specifically involved in systemic C. albicans infections. The fact that C. dubliniensis lacks SAP5 may partially explain why this species is rarely involved in systemic infections (105).

Another class of C. albicans lytic enzymes of which some members are excreted are the phospholipases (PLs) that can hydrolyze one or more ester linkages in glycerophospholipids, a major component of biological membranes. The PLs can be divided in different subclasses based on the mode of action and the target within the phospholipid molecule: PLA, PLB, PLC and PLD (reviewed by 137). The primary activity detected in the supernatants of C. albicans cultures is due to PLB activity. The PLs are not only considered virulence factors in C. albicans, but also in other pathogenic fungi, e.g. A. fumigatus and C. neoformans. Blood isolates from systemic C. albicans infections produce significantly more extracellular phospholipases than commensal strains (67). Disruption of PLB1, one of the two PLB-class enzymes, leads to diminished virulence (67). The exact contribution of the PLs to virulence of C. albicans is not precisely known, but they have been implicated in invasion of reconstituted human oral epithelium (69). The contribution of the PLs to digest the phospholipids of the epithelial cell surface would enable C. albicans invasion (33).

C. albicans is able to grow in media with triolein (a triglyceride with three oleic acid chains) as sole carbon source, suggesting that the organism can hydrolyze the ester bonds between the glycerol and fatty acid side chains (141). The C. albicans genome encodes several excreted lipases and esterases that have the ability to catalyze the hydrolysis of ester bonds of mono-, di- and triacylglycerol or even phospholipids. One of the differences between these two classes of enzymes is that lipases can act on insoluble triacylglycerol, while esterases can only act on soluble substrates. The first identified C. albicans lipase was named Lip1 and nine new family members were additionally characterized (36, 58). Not much is known about the function of the lipases in vivo, but
transcription data indicate that the expression of this gene family is highly flexible and can be lipid-independent (147). Whether these lipases contribute to \( C. \textit{albicans} \) nutrition or invasion \textit{in vivo} remains to be established.

**Growth as a virulence factor**

According to the strict definition of a virulence factor as proposed in the molecular postulates, the ability of a pathogenic organism to grow can also be considered a virulence factor. \( C. \textit{albicans} \) is a strict commensal and has rarely been isolated from the environment. In the body it encounters many different niches during different stages of the commensal-pathogenic life cycle. As a commensal, \( C. \textit{albicans} \) grows in the oral cavity and in the gastrointestinal tract, while as a pathogen the fungus invades oral and vaginal epithelial surfaces and in severely immunocompromised individuals it spreads through the bloodstream to the organs. All these niches vary with respect to physiology, \( \text{pH} \), availability of nutrients and type of immunological defense (15). After colonization of a niche, \( C. \textit{albicans} \) probably alters it by metabolizing available nutrients, thereby altering the ambient \( \text{pH} \) and damaging host tissues. Nutrient acquisition during these different states of maintenance, invasion and colonization requires a custom-made metabolic adaptation.

**Biofilm formation**

Biofilms are a protected niche created by interaction between microorganisms forming a community that can consist of one or more species. \( C. \textit{albicans} \) is able to form biofilms on indwelling medical devices like dental implants, catheters, heart valves and ocular lenses. Catheter-associated candidemia has a high mortality rate (40%) (110) and biofilms are particularly harmful because they convey resistance to antifungals.

![Figure 4. Biofilm formation by \( C. \textit{albicans} \).](image)

Figure adapted from (10). Biofilm formation is depicted in four stages: (1) substrate adherence in which \( C. \textit{albicans} \) yeast cells attaches to a surface. (2) Biofilm development: an extracellular matrix is produced and the biofilm starts to be a mixture of yeasts and hyphae. (3) Biofilm maturation: extension of the extracellular matrix and increase of hyphal mass. (4) Dispersal and spreading to other sites.

Resistance might be caused by the heterogeneous architecture of the biofilm, reduced access of antifungals due to the density of the extracellular matrix or differential gene expression of, for example, drugs efflux pumps. Biofilm formation can be divided into several stages (**Fig. 4**) (10, 20). Substrate adherence is characterized by the initial settlement...
of *C. albicans* yeasts on a surface and this early attachment requires *C. albicans* adhesins like the Als family. The biofilm develops by the synthesis of an extracellular polysaccharide matrix. During biofilm maturation the extracellular matrix extends and more filaments are observed. In the last phase of biofilm formation it is hypothesized that dispersal of *C. albicans* yeasts cells is hypothesized to take place that can result in colonization elsewhere in the body.

The ability of *C. albicans* to form biofilms might be related with early stages of sexual reproduction, because pheromones produced by rare opaque cells in a population can signal the white majority of opposite mating type to form a biofilm (26). Within a biofilm, chemotropic signaling between rare a/a and α/α member of the population is facilitated.

### Antifungal drugs

“In humans, systemic fungal diseases were considered a rarity until the mid-20th century, when advancements in medicine produced antimicrobial drugs, indwelling venous catheters, and immunosuppressive therapies.”

Arturo Casadevall

*Fungal Genetics and Biology, 2005*

There are several factors that determine the outcome of the competition between *C. albicans* and its host. On the side of the fungus we see a diversity of traits that determine maintenance and/or virulence of the organism, whereas on the side of the host the immune system and especially the phagocytic cells of the innate immune system are essential in withholding *C. albicans* from invading the body (see below). Besides the natural human defenses against infections, antifungal drugs are one extra determinant that can influence the outcome in favor of the host. One of the major difficulties in the process of antifungal drug development are the possibility of side effects, because both the fungal pathogen and the mammalian host are eukaryotes and share many cellular processes. The following classes of antifungals are currently available: the polyenes interact with ergosterol in the plasma membrane, forming a pore, while the imidazoles inhibit biosynthesis of ergosterol, which is an essential constituent of the plasma membrane. The newer class of echinocandins inhibits biosynthesis of the cell wall (*Table 1*). The classification of antifungal differentiates between fungicidal and fungistatic drugs and the most stringent definitions identify fungistatic drugs as those that inhibit growth, whereas fungicidal drugs kill fungal pathogens.

Development of antifungal resistance is a major concern because most of the available antifungals target similar cellular processes and therefore putative fungal adaptation would result in a dramatic decrease in suitable antifungals. Resistance could occur through transcriptional changes of for example eflux pumps or target genes, or immediate target protein alterations. As has been discussed above, biofilms confer resistance to antifungals, although the mechanism through which that occurs has not been elucidated.
Table 1. Currently available antifungals

<table>
<thead>
<tr>
<th>Drug</th>
<th>Usage</th>
<th>Antifungal activity</th>
<th>Side effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphotericin B</td>
<td>Widely used for systemic fungal infections</td>
<td>Interact with ergosterol in fungal membranes (fungicidal)</td>
<td>Severe side effects: interaction with cholesterol</td>
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<tr>
<td>(Polyenes)</td>
<td></td>
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<tr>
<td>Voriconazole</td>
<td>Broadspectrum antifungals</td>
<td>Inhibition of fungal ergosterol biosynthesis (fungicidal)</td>
<td>Severe side effects: inhibition of steroid synthesis</td>
</tr>
<tr>
<td>(Azoles)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluconazole</td>
<td>Invasive cand., neutropenic patients</td>
<td>Specific inhibitors of β(1,3)-D-glucan synthase (fungicidal)</td>
<td>Low incidence in side effects compared to Amp B</td>
</tr>
<tr>
<td>(Echinocandins)</td>
<td></td>
<td></td>
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<tr>
<td>Flucytosine</td>
<td>Mostly used in combination therapy</td>
<td>Inhibits DNA and RNA synthesis (fungicidal)</td>
<td>Antiproliferative actions on bone marrow and GI</td>
</tr>
<tr>
<td>(Antimetabolites)</td>
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**Immunity against C. albicans**

“Within the teleological context of the host-parasite relationship, Candida may seek to induce a protective host immune response that permits its own survival.”

Luigina Romani

Current Opinion in Microbiology, 1999

To cause a systemic infection, *C. albicans* must leave its commensal niche in the gastrointestinal tract, which requires transmigration of the epithelial layer of the gut and spreading through the bloodstream. Macrophages and neutrophils of the innate immune system play an essential role in detecting these trespassers, which is illustrated by the fact that neutropenia is a predisposing factor for the establishment of a systemic *C. albicans* infection (76).

**Role of the innate immune system**

Macrophages and neutrophils are the first line of defense against *C. albicans* infection. Both neutropenia and macrophage defects lead to systemic and superficial *C. albicans* infections (76). Although macrophages and neutrophils can both recognize and phagocytose *C. albicans*, the outcome of this encounter in ex-vivo models is very different for the two cell types. While phagocytosis by neutrophils results in killing of the fungus, phagocytosis by macrophages initiates hyphae formation and eventual escape of the fungus from the phagolysosome (34, 131). The differences in outcome between phagocytosis of *C. albicans* by macrophages and neutrophils are poorly understood. *C. albicans* has a rigid cell wall that acts as a protective barrier and is essential for survival of the organism. The innate immune system readily identifies this cell wall as “non-self,” as the outer surface of the yeast consists mainly of carbohydrates such as β-glucans and mannan, structural molecules that are absent from mammalian cells (Fig. 5). Recognition of these conserved
carbohydrate structures is mediated by mammalian pattern-recognition receptors (PRRs) expressed by the phagocytic cells of the innate immune system. Binding of the PPRs to accessible β-glucan, mannan and other cell wall components initiates phagocytosis and subsequent fungal killing by the respiratory burst and the production of cytokines and chemokines. Two groups of PRRs are important in \textit{C. albicans} immunity: the Toll-like receptors (TLRs) and the C-type lectin receptors (CLRs). Two well-studied TLRs, TLR2 and TLR4 on macrophages and neutrophils, recognize \textit{C. albicans}. Their activation leads to initiation of a pro-inflammatory (Th1) or regulatory T cell response (Th2). Other contributors to antifungal activity of phagocytes are the family of C-type lectin receptors, of which Dectin-1 especially is involved in the initiation of phagocytosis, the respiratory burst and downstream signaling.

\textbf{Figure 5: Fungal cell wall composition}
Electron microscopy picture and schematic representation of the \textit{C. albicans} cell wall. Cell wall components are: mannoproteins (35-40%; round), β(1,6)-Glucan (20%; grey lines), β(1,3)-Glucan (40%; black lines) and Chitin (1-2%; dashed line).

\textbf{Role of the adaptive immune system}
Recognition of \textit{C. albicans} by the antigen-presenting cells of the immune system (macrophages, neutrophils and dendritic cells) leads to the induction of cytokine production and the initiation of either the pro-inflammatory (Th1) or regulatory T cell response (Th2). The Th1 response can involve two pathways: MyD88-mediated induction of the transcription factor NF-kB-dependent release of pro-inflammatory cytokines or transcription factor IRF3-dependent release of type I interferons, which induce the secondary production of Th1-type cytokines such as IFNγ. Activation of the Th1 adaptive immunity results in phagocytosis-mediated defenses. The other outcome of \textit{C. albicans} recognition is the phagocytosis-independent Th2 response in which pro-inflammatory cytokines are down regulated. Persistent colonization of various tissues by \textit{C. albicans} as a commensal organism requires suppression of host Th1 adaptive immunity, which the fungus accomplishes by activation of the Th2 response through TLR2-mediated IL-10 release (109, 151).

\textbf{Inside the phagolysosome}
After recognition of \textit{C. albicans} antigens, macrophages and neutrophils initiate phagocytosis and the fungus becomes engulfed by the plasma membrane of the phagocyte leading to its internalization. In both phagocytes, granules start fusing with the fungus-
containing phagosome, thereby releasing their lytic content to attack the pathogen. Although granule-associated proteins are generally similar between macrophages and neutrophils, also some distinctive differences have been reported (148). After phagocytosis, both phagocytes produce an antimicrobial respiratory burst generated by different enzymes. Macrophages activate nitric oxide synthase, which leads to the synthesis of nitric oxide (NO) and has cytostatic or cytotoxic activity against microbes (95). Neutrophils express NADPH oxidase, an enzyme that generates $H_2O_2$ and other reactive oxygen species (ROS) by oxidation of NADPH (130). Myeloperoxidase (MPO) is released from the granules during phagocytosis and interacts with $H_2O_2$ and a halide, presumably chloride, to generate hypochlorous acid (HOCl), which is a potent antibacterial substance (74, 75). Neutrophils and macrophages from patients with chronic granulomatous disease (CGD), in which NADPH oxidase activity is defective, exhibited minimal fungicidal activity against *C. neoformans* (101). The antimicrobial activity of neutrophils is fiercer compared to macrophages, but the latter have the capacity to kill a larger diversity of microbes compared with neutrophils. Another important difference is that, in contrast to mature neutrophils, macrophages retain the ability to synthesize proteins, including a variety of cytokines that enhance the inflammatory response (148).

**Figure 6. Transcriptional and morphological response of *C. albicans* upon phagocytosis.**

Figure adapted from references 15 and 34. Phagocytosis by macrophages initiates induction of hyphal-associated genes and transcription profiling indicates that the fungus experiences a moderate oxidative stress and nitrogen starvation. Hyphal formation eventually leads to puncturing of the plasma membrane and release of the fungus. Phagocytosis by neutrophils on the other hand inhibits growth in general and specifically hyphae formation. Transcriptional profiling shows oxidative stress and carbohydrate and nitrogen starvation.

Phagocytosis of *C. albicans* by macrophages *in vitro* stimulates hyphae formation of the fungus that eventually leads to escape by puncturing of the plasma membrane. It is not known whether *C. albicans* retrieves nutrients inside the phagolysosome, but one would argue that the biomass formation required for hyphal development would only be feasible when sufficient nutrients are available. Neutrophils on the other hand are able to prevent hyphal induction thereby ensuring longer exposure to antifungal activity by trapping the pathogen in the phagolysosome. Transcriptional profiling of *C. albicans* during phagocytosis by macrophages revealed that inside the phagolysosome, the fungus only experiences a moderate oxidative stress and a moderate nitrogen starvation. Phagocytosis by neutrophils leads to more extensive oxidative stress and carbohydrate and nitrogen starvation, leading to induction of fatty acid $\beta$-oxidation, glyoxylate cycle, gluconeogenesis and amino acid synthesis (Fig. 6) (34, 90).
Oxidative stress defense

At the risk of inducing unpleasant flashbacks to college chemistry courses, let us consider the class of chemical reaction upon which our bodies rely for energy production. Reduction-oxidation (or redox) reactions are at the core of our metabolic machinery.

Joe M. McCord
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One of the most noticeable transcriptional reactions of C. albicans to phagocytosis is the upregulation of oxidative stress defense genes, probably a direct response to the antifungal respiratory burst of ROS and RNS produced by the phagocyte. Additionally, one of the characteristics of aerobic metabolism is the production of endogenous ROS in different places in the cell: the respiratory chain in the mitochondria and during β-oxidation of fatty acids in peroxisomes. Yeasts are excellent model systems to study oxidative stress because of the availability of mutants and oxidative agents. One of the overall findings of the extensive studies on this subject is that many of the oxidative stress defense systems in the cell play overlapping roles.

Formation of ROS and RNS

ROS are a variety of molecules derived from molecular oxygen and also comprise free radicals: species with one or more unpaired electrons. Molecular oxygen contains two unpaired electrons in its outer shell (a bi-radical), but is not very reactive due to the fact that both electrons have the same spin. When one of the electrons is excited, however, the resulting singlet oxygen becomes a powerful oxidant. Oxygen as an oxidant is central to energy metabolism, as it serves as the terminal electron acceptor (of four electrons) for cytochrome c oxidase in the respiratory chain. However, using this reactive molecule also poses the risk of ROS production due to incomplete reduction. Reduction of oxygen by one electron yields the superoxide anion (O$_2^-$), a relatively stable intermediate. If two electrons are transferred, the resulting product is hydrogen peroxide (H$_2$O$_2$). In the presence of reduced metals, partial reduction of hydrogen peroxide generates the hydroxyl radical (·OH), one of the strongest oxidants in nature. This reaction is called the Fenton reaction when iron is involved. ROS are so harmful because they can lead to protein, lipid and DNA damage that is often irreversible. Protein damage by ROS includes formation of carbonyl groups on the side chains of certain amino acids and oxidation of methionine to methionine sulphoxide (145). RNS derived from nitric oxide (NO) are able to promote modification of thiol groups to yield S-nitrosothiols (40). Nitric oxide can react with superoxide anion to form peroxynitrate (ONOO$^-$), a potent derivate that can lead to nitration of tyrosine residues. Oxidative stress can also be generated by addition of xenobiotics that accept an electron from a respiratory carrier and transfer it to molecular oxygen, stimulating superoxide formation. Examples of such xenobiotics that are often used in yeast studies are the herbicide paraquat (1,1’-dimethyl-4,4’-bipyridinium) or the polycyclic aromatic ketone menadione (2-methylnaphthalene-1,4-dione). Biological metabolic processes in which ROS are produced will be discussed in the organelle-specific paragraphs (see below).
ROS-decomposing pathways

To protect themselves against the extracellular ROS attack and the inevitable metabolic production of ROS, cells have developed various strategies, which involve small antioxidant molecules and/or enzymatic degradation. The superoxide anion can be converted to hydrogen peroxide by a reaction with water catalyzed by superoxide dismutases (Sods). Sods require redox active metal ions for their activity and are divided in two different classes: CuZnSods and MnSods (25). Three classes of enzymes are the main decomposers of H$_2$O$_2$: cytochrome c peroxidases (Ccp), catalases (Cta) and glutathione peroxidases (Gpx) (Fig. 7).

Ccp is a haem-containing enzyme that takes reducing equivalents from cytochrome c to reduce hydrogen peroxide to water (102). The reaction mechanism of Cta is thought to be similar to Ccp, as catalase is also a haem-containing enzyme that doesn’t require cofactors (174). Gpxs on the other hand are dependent on cofactor glutathione (γ-glutamylcysteinylglycine), which needs to be in the reduced state. The enzymatic reduction of glutathione is performed by glutathione reductase (Glr), a reaction that is dependent on the reducing power of NADPH. There are two classes of Gpxs: the classical multimeric soluble Gpxs that act on organic hydroperoxides and the monomeric membrane-associated phospholipid hydroxyperoxide Gpxs (Phgpxs) that play an essential role in the repair of lipid hydroperoxides in membranes, one of the major consequences of oxidative stress. Other glutathione-dependent enzymes are glutaredoxins, implicated in detoxification of superoxide and hydroperoxide (94) and glutathione S-transferases that conjugate glutathione to exogenous and endogenous electrophilic compounds, which results in detoxification and excretion (23, 47). Several of these glutathione-dependent enzymes play an important role in repair of oxidized proteins. A second complete enzymatic antioxidant system is the thioredoxin system that is analogous to the glutathione system. The thioredoxin and glutathione systems often play overlapping roles in oxidative stress defense (42, 144). Besides the enzymatic protection conveyed by different classes of
oxidoreductases described above, small molecules like reduced glutathione, ascorbate or vitamin E can also directly function as antioxidants in the cell (30).

**Production of NADPH**

The reduction of GSSG to GSH by Glr1 requires NADPH (Fig. 7) and therefore the complete glutathione system in the cell is indirectly dependent on the availability of this reducing equivalent. The pentose phosphate pathway (PPP), the anabolic alternative of glycolysis, is the main source of NADPH in the cell. The flow of glucose-6-phosphate through glycolysis or PPP is determined by the NADP+/NADPH ratio, as high levels of NADPH inhibit the first enzyme of the PPP, glucose-6-phosphate dehydrogenase (Zwf1), and high levels of NADP+ result in an increased flow through the PPP (7, 86).

The oxidative branch of the PPP converts glucose-6-phosphate to ribulose-5-phosphate in three enzymatic steps: glucose-6-phosphate dehydrogenase (Zwf1), gluconolactonase (Sol3) and 6-phosphogluconase dehydrogenase (Gnd1). One molecule of NADPH is produced during the reactions catalyzed by each Zwf1 and Gnd1 (Fig. 8). The non-oxidative PPP consists of reactions performed by epimerase, transketolase, transaldolase and isomerase that serve to rearrange ribulose-5-phosphate into the glycolytic and gluconeogenic intermediates. These rearrangements also produce ribose-5-phosphate, a precursor for nucleotide synthesis. A defective pentose phosphate pathway directly results in a dysfunctional regulation of the redox state of the cell because glutathione reductase (Glr1) and thioredoxin reductase (Trt1) lack the required reducing equivalents in the absence of sufficient NADPH (reviewed for *S. cerevisiae* by 42). The relationship between the pentose phosphate pathway and oxidative stress is illustrated by the symptoms of human G6PDH deficiency and phenotypes of PPP null mutants of *S. cerevisiae*. Human G6PDH deficiency results in haemolytic anemia, which is caused by reduced NADPH levels resulting in a shortage of reduced glutathione in red blood cells (17). Deletion mutants of pentose phosphate pathway genes of *S. cerevisiae* showed an elevated sensitivity to oxidative stress agents (70) and cells without a functional pentose phosphate pathway loose viability due to loss of antioxidant function (104).

![Figure 8. Generation of NADPH in the pentose phosphate pathway.](image)

The oxidative branch of the pentose phosphate pathway converts glucose-6-phosphate to ribulose-5-phosphate in three enzymatic steps: glucose-6-phosphate dehydrogenase (Zwf1), gluconolactonase (Sol3) and 6-phosphogluconate dehydrogenase (Gnd1). NADPH is produced during both dehydrogenase reactions. The non-oxidative branch of the pentose phosphate pathway consists of a series of enzymatic conversions (epimerase, transketolase, transaldolase and isomerase) that eventually lead to the production of glycolysis intermediates glyceraldehydes-3-phosphate and/or fructose-6-phosphate.
General Introduction

Other minor sources of NADPH in the cell are NADP⁺-dependent isocitrate dehydrogenase (Idp) and malic enzyme. The *S. cerevisiae* genome was shown to encode three NADP⁺-dependent isocitrate dehydrogenases that are targeted to three different locations: Idp1 to the mitochondria, Idp2 to cytosol and Idp3 to the peroxisomes (9, 46, 89). The *C. albicans* genome however encodes only two isocitrate dehydrogenases, that appear to be orthologs of the mitochondrial Idp1 and cytosolic Idp2 (our observations). Besides its function in oxidative stress defense, the other important function of NADPH is to provide reducing power for anabolic reactions like lipid and nucleic acid synthesis. The function of the NADPH-producing malic enzyme (malate to pyruvate) is to provide NADPH for lipid synthesis (reviewed by 126). As far as we know of, no biosynthesis defects have been reported associated with PPP or isocitrate dehydrogenase deficiencies.

**Extracellular protection against oxidative attack**

The respiratory burst produced by phagocytes consists of a multitude of reactive species like superoxide, hydrogen peroxide, nitric oxide and peroxynitrate and results in an oxidative attack that is met extracellularly by *C. albicans*. The fungus expresses secreted superoxide dismutases (Sod) that convert superoxide into the less damaging hydrogen peroxide. One of these Sods is the CuZn-containing Sod5, a hyphal-induced GPI-anchored protein (34) that is essential for virulence in the mouse model of infection (97). However, macrophage survival of the *SOD5* null mutant *in vitro* was comparable to the wild type survival rate, suggesting that the role of Sod5 might relate to another aspect of virulence in the mouse model of infection (97). Hyr1 (or Gpx1) is a predicted GPI-anchored protein, which is induced in hyphae compared to yeast and during interaction with macrophages (6). The moonlighting protein thioredoxin Tsa1 is localized to cell wall in hyphae and important for extracellular oxidative stress (160).

**ROS-defense in the cytosol**

As ROS and RNS can easily cross biological membranes, the cytosol and organelles also encounter exogenously produced oxygen and nitrogen species. The cytosol is thought to cope with the majority of ROS decomposition, simply because it contains the biggest volume. *C. albicans* and *C. neoformans* mutants of the cytosolic Sod1 were shown to have virulence defects when tested for macrophage survival and in mouse models (24, 65, 66). The glutathione system is known to function both in the cytosol and in the mitochondria and Glr1 has been shown to localize to both compartments in *S. cerevisiae* and *S. pombe* (115, 144). While Glr1 of *S. cerevisiae* was shown to be required for protection against oxidative stress (43), *S. pombe* Glr1 is absolutely required for growth (84). Disruption of glutathione synthesis in a *C. albicans* γ-glutamylcysteine synthetase (*GCS1*) knockout strain resulted in complete glutathione auxotrophy on minimal glucose medium and an increase of apoptotic markers (5). The growth defect of *S. pombe* and *C. albicans* glutathione mutants could be caused by damage of oxidation-sensitive iron-sulphur cluster proteins as has been reported for *S. pombe* aconitase (144).

**Mitochondria: production of ROS during respiration**

The respiratory chain in mitochondria is the major source of intracellular ROS production because of premature electron leakage to oxygen and subsequent O₂ generation. *C. albicans* has two alternative oxidases that can (temporarily) decrease ROS production by the
respiratory chain by reduction of proton translocation (59, 60). The main antioxidant enzyme in the mitochondrial compartment is the haem-containing enzyme cytochrome c peroxidases (CcP1). As described above, Glr1 also localizes to mitochondria (115) and is essential in prevention of destabilization of iron-sulphur clusters in *S. pombe* (144).

**Hydrogen peroxide production in peroxisomes**

Peroxisomes are single membrane-bound organelles that are named for their production of peroxides. In yeasts like *C. albicans* and *S. cerevisiae*, β-oxidation of fatty acids is completely peroxisomal (78). By contrast, mammalian fatty acid oxidation is predominantly mitochondrial, while peroxisomes are involved in shortening of very long chain fatty acids (reviewed by 169). One round of β-oxidation entails shortening of the fatty acid chain by 2 carbons and the production of one acetyl-CoA, which is accomplished in four enzymatic reactions. H$_2$O$_2$ is one of the byproducts of the first peroxisomal β-oxidation enzyme, acyl-CoA oxidase, as that enzyme transfers two electrons via the electron acceptor FAD to O$_2$, forming H$_2$O$_2$ in the peroxisomal matrix. Other peroxisomal oxidases that produce H$_2$O$_2$ as a by-product are D-amino oxidase, which converts D-amino acids to the respective imino acids and is present in peroxisomes of most organism and alcohol oxidase of the methylotrophic yeast *Candida boidinii* (53, 54). The scavenging enzyme catalase (Cta1) can decompose reactive H$_2$O$_2$ and is well known for its association with peroxisomes. The protein Cta1 itself is protected against inactivation by its toxic substrate by an interaction with NADPH (73). *S. cerevisiae* has two catalases that are localized to the cytosol (Ctt1) and peroxisomes (Cta1), but *C. albicans* has a single catalase that localizes to the peroxisomes (119). *C. albicans CTA1* was shown to be induced after extracellular oxidative stress and phagocytosis by neutrophils in an ex-vivo model (28, 29). The protein was shown to play a role in virulence as tested in the mouse model of infection (107, 172), however how this relates to the peroxisomal localization has not been addressed. The *C. neoformans* genome encodes four catalases, however a quadruple knockout was not attenuated in virulence (39). Another oxidative stress defense enzyme that associates with peroxisomes is peroxisomal membrane protein 20 (Pmp20), which has thiol-specific antioxidant activity. Pmp20 is a membrane protein in *S. cerevisiae*, but its human and mouse orthologs do not have transmembrane domains (173). Pmp20 is the major membrane protein in methanol-induced peroxisomes in *C. boidinii* (38), where it contributes to oxidative stress defense (54).

**Subcellular compartmentalization in eukaryotes**

The evolution of complex cells could have been divided into two major phases. First, the emergence of the eukaryotic cell possessing a nucleus, endomembrane and cytoskeleton. Second, the symbiotic origin of mitochondria resulting in the appearance of the first energetically efficient aerobic protozoan.

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While the bacterial cell consists of a single compartment, the division of metabolic pathways over different intracellular organelles is one of the major characteristics of
eukaryotic cells. The creation of different compartments within one cell facilitates the housing of specialized pathways in optimized sub-environments. Compartmentalization was initiated by an early fusion event of an alpha-proteobacterium with a pre-eukaryotic cell that resulted in a successful symbiotic union. The internalized proteobacterium grew out to be the nowadays mitochondria and the presence of mitochondrial DNA is a remnant of an independent lifestyle in the past (96). The evolutionary origin of other organelles is less clear. Although peroxisomes can divide, they do not contain DNA and recent data indicate that these organelles originate from the ER (reviewed by 155). Compartmentalization of cellular processes in membrane-surrounded organelles necessitates specific targeting of enzymes to these compartments and transport of metabolites over membranes. The shape and subcellular localization of mitochondria and peroxisomes in *C. albicans* grown on rich glucose medium are depicted in Figure 9.

**Figure 9. Mitochondria and peroxisomes in *C. albicans* grown on rich glucose medium.**
The peroxisomal protein Thiolase was labeled with GFP and incubated with the fluorescent dye Mitotracker (Invitrogen) to strain the mitochondria. Left panel: Phase contrast picture of the Thiolase-GFP strain grown on rich glucose medium. Middle panel: Mitochondrial straining by Mitotracker shows tubular connected structures. Right panel: Thiolase-GFP signal showing punctated fluorescence representing peroxisomes. Some mitochondrial straining is still visible.

**Mitochondria**
Mitochondria are indispensable for energy metabolism in all eukaryotic cells as these organelles harbor the tricarboxylic acid (TCA) cycle and the respiratory chain. Together these two pathways enable generation of adenosine triphosphate (ATP), the major energy storage and transfer molecule in the cell. The mitochondrial matrix is surrounded by a double membrane that consists of the inner mitochondrial membrane, the intermembrane space and the outer membrane. Mitochondrial proteins are synthesized in the cytosol and contain a N-terminal mitochondrial targeting sequence (MTS) that is recognized by the mitochondrial import machinery and is often cleaved of after import. The MTS is not strictly defined, but consists of several positively charged and non-polar (hydrophobic) residues. These amino acids form an amphiphilic α-helix in which positively charged residues cluster on one side of the helix, while uncharged hydrophobic residues cluster on the opposite side. Transport of mitochondrial proteins over both membranes occurs in an unfolded, linear state (37, 114). The TCA cycle proteins are localized in the mitochondrial matrix. The overall reaction of the TCA cycle is the linkage of acetyl-CoA (a C2 compound) to oxaloacetate (a C4 compound) and the production of CO₂ and NADH that feeds protons into the respiratory chain. The protein complexes that form the respiratory chain are imbedded in the inner mitochondrial
membrane and make use of the membrane for the build-up of a proton gradient that is essential for ATP generation during oxidative phosphorylation.

**Peroxisomes**

In contrast with mitochondria, peroxisomes are single membrane bound and do not contain DNA. Localization of biochemical pathways to peroxisomes differs between organisms, but the single pathway that is peroxisomal in almost all eukaryotic organisms is the \( \beta \)-oxidation of fatty acids. Other biochemical pathways that are peroxisomal in many organisms are ether phospholipid biosynthesis and the breakdown of D-amino acids (121, 169). Peroxisomal number and size can be greatly increased in yeasts in the presence of fatty acids or other compound that require a higher rate of peroxisomal metabolism, which is illustrated by the expression of the peroxisomal enzyme thiolase fused to GFP on rich glucose or rich oleate medium (Fig. 10).

![Thiolase-GFP](Rich glucose)

**Figure 10. Induction of peroxisomes on rich oleate medium compared to rich glucose medium.** Fluorescence and phase-contrast pictures of the Thiolase-GFP strain grown on Rich glucose medium (left panels) or rich oleate medium (right panels).

Peroxisomal matrix proteins contain a C-terminal peroxisomal targeting signal 1 (PTS1) or a N-terminal PTS2. The PTS1 sequence is recognized by receptor Pex5 and was originally defined as S/A/C-K/R/H-L\textsubscript{COOH} (154). However, with the identification of more PTS1 proteins it became clear that the PTS1 is not as strict as originally defined but may vary depending on the organism or protein context (2, 14). The majority of peroxisomal proteins are targeted to peroxisomes via the PTS1-import route, but a few PTS2 proteins are known that are imported via the PTS2 receptor Pex7. The PTS2 is located near the N-terminus and is defined by the consensus sequence R[L/V/I]X\(_5\)[H/Q][L/A] where X is any amino acid (135, 149, 153, 159). The PTS1 and PTS2-containing proteins are synthesized in the cytosol and are bound by the cycling receptors Pex5 and Pex7, respectively, forming a receptor-cargo complex that associates with the docking complex on the peroxisomal membrane. After association with the docking complex, the cargo protein is translocated over the membrane and the receptors Pex5 and Pex7 disassociate from the membrane and can be used for another round of transport. Around 12 proteins called peroxins play important roles in the receptor cycle of Pex5 and Pex7, but the exact mechanism of import of peroxisomal matrix proteins remains to be clarified. It is intriguing that, in contrast with mitochondrial proteins, peroxisomal proteins are translocated over the membrane in a completely folded conformation and even protein complexes and gold-particles can be translocated from cytosol to peroxisomal matrix (168). Peroxisomes are essential for the utilization of fatty acids by eukaryotes, as is illustrated by yeast peroxin (pex) mutants that are unable to grow on
fatty acids as sole carbon source due to non-functional peroxisomes. Human disorders that are associated with peroxisomal dysfunction are divided into two major groups: the peroxisome biogenesis disorders (PBD’s) and the single peroxisomal enzyme deficiencies (PED’s). General loss of peroxisomal function is observed in PBD’s due to a non-functional import system for either peroxisomal membrane or matrix proteins that leads to increased levels of very long chain fatty acids (VLCFA’s), bile acids intermediates and phytanic acid and a decrease in plasmalogen levels in the body. Patients that suffer from PBD’s typically display a delay in development, facial abnormalities, hypotonia and liver disease and, in the majority of cases, die prematurely. One example of such a PBD is rhizomelic chondrodysplasia punctata (RCDP) that is caused by a defect in peroxisomal import of PTS2 proteins. RCDP can be caused by mutation of PTS2-receptor Pex7 or a single-enzyme deficiency in one of the following PTS2 proteins involved in phospholipid biosynthesis: dihydroxyacetonephosphate acyltransferase deficiency (DHAPAT; RCDP type 2) or alkyl dihydroxyacetonephosphate synthase (alkyl-DHAP synthase; RCDP type 3) (50, 52).

PED’s are caused by the inactivation or mislocalization of a single peroxisomal enzymes and can be divided in the following classes: ether phospholipid biosynthesis, fatty acid β-oxidation, peroxisomal α-oxidation, glyoxylate detoxification, and H₂O₂ metabolism (170).

The β-oxidation of fatty acids is completely peroxisomal in yeasts like C. albicans and S. cerevisiae, which is in contrast with the partially mitochondrial β-oxidation in mammals (78). It is not exactly clear how fatty acids are imported into peroxisomes, but they probably enter as acyl-CoA esters through the ATP binding cassette (ABC) transporter and associated acyl-CoA synthetase (reviewed by 167). In yeast it has been shown that short fatty acids can pass the membrane and are activated in the matrix (49). After import the acyl-CoA’s enter the β-oxidation pathway that consists of four enzymatic steps catalyzed by acyl-CoA oxidase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl thiolase. One round of β-oxidation results in shortening of the acyl-CoA chain by 2 carbons, resulting in the production of one molecule of acetyl-CoA for each round of β-oxidation. Acetyl-CoA subsequently needs to be transported to the mitochondria to be used in the TCA cycle (see below). The glyoxylate cycle is a pathway that enables assembly of C4 units by linkage of acetyl-CoA (C2) to glyoxylate (C2). This pathway allows growth of the organism on fatty acids and C2 compounds such as ethanol and acetate as sole carbon source. The key enzymes of the glyoxylate cycle, isocitrate lyase (Icl1) and malate synthase (Mls1), are localized to peroxisomes in C. albicans (119) (see below). Another metabolic process that takes place in the peroxisomes of most eukaryotes (except plants) is the oxidative deamination of D-isomers of neutral and polar amino acids by D-amino acid oxidase (DAO). The products of the FAD-mediated DAO reaction are imino acids, ammonia and H₂O₂. DAO plays a catabolic role in yeasts, as the ability to oxidize a large number of D-amino acids allows growth during limitation of classical carbon sources (reviewed by 121).

The transport of metabolic intermediates over the peroxisomal membrane has been a matter of research for several decades and still remains controversial. The peroxisomal membrane was shown to be permeable in vivo to low molecular weight, hydrophobic solutes such as glycolate and urate, but not to more bulky cofactors like NADH or acetyl-CoA (3, 161). A number of groups have reported porin-like proteins in peroxisomes of...
mammals, plants and yeast (reviewed by 129) but also a number of specific transporters have been identified (reviewed by 167).

**C. albicans central carbon metabolism**

“...the discovery of the vitamin status of carnitine in one species of insect led to our understanding of the process whereby fat supplies energy to the organism”.  
George Wolf  

**Nutrient acquisition during the commensal and pathogenic lifecycles**

As described above, growth must also be considered a virulence factor. There is not much information available on nutrient acquisition of *C. albicans* during the different stages of the commensal and/or pathogenic lifecycle. We can reason however that the normal commensal niche of *C. albicans* in the gut is rich in nutrients and that especially fatty acids might be available in high concentrations. However, because of the presence of many other microorganisms in the gut, acquisition of the necessary nutrients might be very competitive in this environment. In the course of the establishment of a systemic infection, *C. albicans* passes through the glucose-rich blood stream and starts to invade organs like liver, kidneys and spleen. Superficial *C. albicans* infections require growth on mucosal surfaces or the skin. Making use of the model systems described above, the behavior of *C. albicans* under many of these conditions has been tested. The release of secreted lipases, phospholipases, esterases and proteinases might play an important role in nutrient acquisition during infection, as is illustrated by the decreased virulence of strains affected in any of these enzymes. The glyoxylate cycle is a metabolic pathway unique to plants, bacteria and some fungi and was shown to be important for the virulence of a different pathogenic organisms, including *Mycobacterium tuberculosis* (99) and *C. albicans* (91). Another metabolic observation was the downregulation of *C. albicans* genes involved in glycolysis and the upregulation of fatty acid oxidation genes during phagocytosis of the fungus by macrophages (90). The virulence deficiency of *C. albicans* glyoxylate cycle mutants together with the upregulation of fatty acid oxidation genes seems to point at fatty acids as the main carbon source during phagocytosis and systemic infection. However, we showed that β-oxidation of fatty acids does not contribute to virulence of *C. albicans* by testing a *fox2* mutant that is unable to oxidize fatty acids in the mouse model of infection (120). Therefore the exact role of the glyoxylate cycle and the contribution of central carbon metabolism in *C. albicans* pathogenesis remain unclear.

Independent of the carbon source that cells grow on, the C2 unit acetyl-CoA is the central metabolite that is the substrate or product of various metabolic pathways. Acetyl-CoA is essential for the production of ATP, as it is used to replenish the mitochondrial TCA cycle with C2 units and therefore acetyl-CoA supply in the mitochondrial matrix is absolutely essential for growth. However, growth on carbon sources other than glucose lead to the production of acetyl-CoA in other cellular compartments and therefore requires transport of acetyl units over (the) organellar membrane(s). Below we summarize the current knowledge on acetyl transport and related pathways in *C. albicans*. 
General Introduction

**Glucose metabolism**

Glucose is taken up by the cell and phosphorylated by hexokinase (Hxk1), a reaction that requires ATP, to trap the molecule in the cell. The formed glucose-6-phosphate can enter the catabolic glycolysis where it is degraded to 2 molecules of pyruvate, which results in a net gain of 2 ATP and 2 NADH. Pyruvate can be transported over the mitochondrial membrane and is converted to acetyl-CoA in the mitochondrial matrix by the pyruvate dehydrogenase complex (Pda). Therefore, during growth on glucose acetyl-CoA is directly synthesized in the mitochondrial matrix and no transport of acetyl-CoA over the mitochondrial membrane is required (Fig. 11).

**Fatty acid β-oxidation and the glyoxylate cycle**

The β-oxidation of fatty acids is solely peroxisomal in *C. albicans* as can be deduced from the observation that a *C. albicans* mutant of peroxisomal multifunctional enzyme has lost all β-oxidation activity (119). After import into the peroxisome, acyl-CoA is oxidized completely to acetyl-CoA units in four enzymatic steps performed by three different enzymes. The *C. albicans* genome encodes multiple isozymes for some of the enzymatic steps of β-oxidation (two acyl-CoA oxidases, one multifunctional enzyme and three 3-ketoacyl-CoA thiases), a complexity that resembles the human β-oxidation pathway (three acyl-CoA oxidases, two multifunctional enzymes and three 3-ketoacyl-CoA thiases) but not that of *S. cerevisiae* (one acyl-CoA oxidase, one multifunctional enzyme and one 3-ketoacyl-CoA thiolase). The increased number β-oxidation isozymes in *C. albicans* might indicate that this fungus has undergone an adaptation towards a more sophisticated fatty acid metabolism compared to *S. cerevisiae*, allowing it to handle a wider variety of fatty acid substrates. For yeasts to grow on fatty acids as sole carbon source, acetyl-CoA needs to be transported to the mitochondrial TCA cycle for ATP generation and also fed into the glyoxylate cycle to generate C4 units for gluconeogenesis (Fig. 11). This pathway is unique to plants and microorganisms as *E. coli* and yeasts and is essential for growth on non-fermentable carbon sources like fatty acids, ethanol or acetate as sole carbon source. Disruption or mutation of one of the key enzymes of the glyoxylate cycle, either isocitrate lyase (Icl1) or malate synthase (Mls1), leads to a complete growth defect on non-fermentable carbon sources (45, 91, 99). The glyoxylate cycle essentially is a shunt of the TCA cycle that acts on citrate, thereby excluding the decarboxylation steps of the TCA cycle catalyzed by isocitrate dehydrogenase (Idh1) and α-ketoglutarate dehydrogenase (α-Kgdh). In *C. albicans* Icl1 and Mls1 are compartmentalized in peroxisomes (119), as is the case in most organisms, with the exception of *S. cerevisiae* where Icl1 is cytosolic and Mls1 is localized to the peroxisomes or cytosol depending on the growth conditions (79, 98). The *C. albicans* genome encodes two aconitases (Aco), the TCA/glyoxylate cycle enzyme that converts citrate to isocitrate. Both *C. albicans* Aco1 and Aco2 have a predicted MTS, but Aco1 additionally has a putative PTS1 (-SKYCOOH). We determined the subcellular distribution of the aconitases experimentally and found a dual localization. The majority of the signal detected with an α-Aco1 antibody associated with the mitochondria and some associated with the peroxisomes. The presence of Aco1 in peroxisomes implies transport of citrate from mitochondria to peroxisomes where it feeds into the glyoxylate cycle. The peroxisomal presence of the Fe-S cluster-containing Aco1 would be very remarkable, because this class of proteins has never been found in the oxidative environment of the
peroxisomal matrix. Mitochondrial aconitase was shown to be inactivated in the absence of sufficient oxidative stress defense (144).

**Carnitine-dependent transport of acetyl units**

Because of its amphiphilic nature, acetyl-CoA cannot cross the biological membranes without the aid of a carrier, carnitine (L-3-hydroxy-4-N,N,N-trimethylaminobutyrate). Carnitine acetyl-transferases reversibly link carnitine to the acetyl unit of acetyl-CoA forming acetyl-carnitine that can cross the membrane. The *C. albicans* and *S. cerevisiae* genomes encode three putative carnitine acetyl-transferases: Cat2, Yat1 and Yat2. Cat2 is the major carnitine acetyl-transferase that has a dual localization to peroxisomes and mitochondria in both yeasts (Chapter 2, reference 27). In *S. cerevisiae* two pathways for acetyl-CoA transport from peroxisomes to mitochondria were identified: one depending on the enzyme carnitine acetyl-transferase (Cat2) and the other on peroxisomal citrate synthase (Cit2). These pathways function in parallel in *S. cerevisiae* and only a cat2Δ/cit2Δ double knockout looses the ability to grow on fatty acids (162). In *C. albicans* however, we have shown that peroxisomes lack Cit and are completely dependent on Cat2 for the transport of acetyl units (Chapter 2). Peroxisomal Cat2 generates acetyl-carnitine inside the organelle which can subsequently be transported across the membrane. Upon arrival in the mitochondria, the acetyl units are released from acetyl-carnitine by the reverse reaction catalyzed by mitochondrial Cat2 (**Fig. 11**). Although this metabolic scheme indicates that peroxisomal and mitochondrial Cat2 are equally important for acetyl unit transport from peroxisomes to mitochondria during growth on fatty acids, this does not seem to be the case (Chapter 3).

While a strain that lacks the peroxisomal Cat2 is still able to β-oxidize fatty acids, it cannot grow on this carbon source. We hypothesize that the growth defect of this mutant strain is due to a non-functional glyoxylate cycle cased by a peroxisomal import defect of (iso)citrate (Chapter 3). Furthermore the wild type β-oxidation activity (based on CO2 production measured in whole cells) suggests that acetyl units are still exported from the peroxisomal matrix and enter the mitochondrial TCA cycle, even in the absence of peroxisomal Cat2. We hypothesize that this might be accomplished by peroxisomal thioesterases that convert acetyl-CoA to acetate that might be able to cross the peroxisomal membrane. The *C. albicans* genome encodes four thioesterases that are predicted to be peroxisomal, a situation that again is more comparable to humans (three thioesterases) than to *S. cerevisiae* (a single thioesterase). During growth on ethanol or acetate, acetyl-CoA is produced in the cytosol and needs to be transported to the peroxisomal glyoxylate cycle and the mitochondrial TCA cycle. We have shown that growth on ethanol and acetate is dependent on the mitochondrial Cat2, but not strictly on the peroxisomal Cat2. This again suggests that acetate might be capable of crossing the peroxisomal membrane but not the mitochondrial (inner) membrane (Chapter 3).

Yat1 and Yat2 localize to the cytosol in *C. albicans* (Addendum Chapter 3, reference 35), while *S. cerevisiae* Yat1 localizes to the outer mitochondrial membrane (140) and the localization of Yat2 is unknown. In *S. cerevisiae* disruption of either CAT2, YAT1 or YAT2 in the cit2Δ background has the same effect: all double knockouts cannot grow on oleate, ethanol or acetate (**Table 2**) (152). By contrast, the growth phenotypes of *C. albicans*...
Figure 11. Central carbon metabolism in *C. albicans*

YAT1 and YAT2 disruption strains are quite different: a yat1Δ strain is unable to grow on ethanol or acetate, but a yat2Δ strain does not display a growth defect (Table 2) (124, 176). We hypothesize that the phenotypic difference between the C. albicans yat1Δ and yat2Δ strain is correlated with the much higher expression of YAT1 compared to YAT2 during growth on oleate and acetate (176). During growth on ethanol or acetate as sole carbon source, acetyl-CoA is produced in the cytosol by the enzymatic activities of alcohol dehydrogenase (Adh), acetaldehyde dehydrogenase (Ald) and acetyl-CoA synthase (Acs). The cytosolic acetyl units need to be transported to mitochondria and peroxisomes for energy and C4 synthesis, respectively. Because of their homology to carnitine acetyltransferases and the observed phenotypes of the disruption strains, the Yats were hypothesized to function as carnitine acetyl-transferases on cytosolic acetyl-CoA derived from ethanol and acetate (Fig. 11). We attempted to investigate this hypothesis, but so far could not find convincing evidence in support of a role for C. albicans Yat1 or Yat2 as carnitine acetyl-transferases (Addendum to Chapter 3).

Table 2. Growth phenotypes of S. cerevisiae and C. albicans disruption and mutant strains

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Glucose</th>
<th>Ethanol</th>
<th>Acetate</th>
<th>Oleate</th>
<th>Reference</th>
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<tbody>
<tr>
<td>S. cerevisiae</td>
<td>cat2Δ</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(152, 162)</td>
</tr>
<tr>
<td></td>
<td>cit2Δ</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(152)</td>
</tr>
<tr>
<td></td>
<td>cit2Δ/cat2Δ</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(152)</td>
</tr>
<tr>
<td></td>
<td>cit2Δ/yat1Δ</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(152)</td>
</tr>
<tr>
<td></td>
<td>cit2Δ/yat2Δ</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(152)</td>
</tr>
<tr>
<td>C. albicans</td>
<td>cat2Δ/Δ</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Chapter 2, 3 and (176)</td>
</tr>
<tr>
<td></td>
<td>perCAT2</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Chapter 3</td>
</tr>
<tr>
<td></td>
<td>mitCAT2</td>
<td>+</td>
<td>+/-</td>
<td>+/-</td>
<td>-</td>
<td>Chapter 3</td>
</tr>
<tr>
<td></td>
<td>yat1Δ/Δ</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>(176)</td>
</tr>
<tr>
<td></td>
<td>yat2Δ/Δ</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(124, 176)</td>
</tr>
<tr>
<td></td>
<td>tmldΔ/Δ</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Chapter 4</td>
</tr>
</tbody>
</table>

$ Growth medium with additional carnitine

Note: S. cerevisiae is unable to synthesize carnitine endogenously and therefore a cit2Δ mutant is unable to grow on non-fermentable carbon sources unless carnitine is added, which allows the Cat2-dependent pathway to function.

Carnitine transporters

Acetyl-carnitine can be transported over the peroxisomal membrane in yeasts, but peroxisomal acetyl-carnitine transporter protein(s) remain thus far unknown. However, acetyl-carnitine and acyl-carnitine transport over the mitochondrial membranes has been well characterized in humans, where peroxisomal β-oxidation acts on very long chain fatty acids and shortened long chain acyl units are transported to the mitochondrial matrix as acyl-carnitine for further oxidation. Long chain fatty acids taken up by the cells are first activated in the cytosol to acyl-CoAs. The enzyme carnitine palmitoyltransferase I (CPT I) associated with the outer mitochondrial membrane then links carnitine to the acyl unit of acyl-CoA. Next, acyl-carnitine is transported over the outer mitochondrial membrane by diffusion or an unknown transporter and over the inner
mitochondrial membrane via the specific carrier carnitine-acylcarnitine translocase (CACT). CPT II in the mitochondrial matrix links the acyl unit to intramitochondrial CoA, forming acyl-CoA that can enter mitochondrial fatty acid β-oxidation. CACT is also able to transport acetyl-carnitine (reviewed by 132). The S. cerevisiae gene Yor100c (CRC1) was identified as the ortholog of human CACT (116, 162) and the human carrier was shown to functionally complement a S. cerevisiae cit2∆/crc1∆ mutant (68). The likely C. albicans CACT/CRC1 ortholog is orf19.2599. Besides carnitine-mediated transport between organelles, carnitine can also be taken up from extracellularly. S. cerevisiae Agp2, a transporter that is a member of the amino acid permease family, was shown to localize to the plasma membrane where it is responsible for the uptake of carnitine from the extracellular medium (162). We identified the C. albicans orf19.4679 as the most likely Agp2 ortholog, but a disruption mutant was still able to import carnitine. The screen performed by Van Roermund et al. (162) aimed at isolation of S. cerevisiae mutants affected in acetyl unit transport from peroxisomes to mitochondria failed to identify a peroxisomal acyl/acetyl-carnitine carrier. Therefore identification of the carriers involved in carnitine-mediated transport over the peroxisomal membrane remains a challenge for the future.

Carnitine biosynthesis
The sole dependence of C. albicans on carnitine acetyl-transferases during utilization of non-fermentable carbon sources, led us to the hypothesis that C. albicans would be able to synthesize carnitine de novo. The carnitine biosynthesis pathway was chemically characterized in the 1970s in experiments with labeled substrates and subsequent detection of carnitine, carnitine biosynthesis intermediates and byproducts in Neurospora crassa and rat (13, 51, 64, 71, 156). The precursor of carnitine biosynthesis is the trimethylated amino acid lysine (6-N-trimethyllysine; TML), a product of lysosomal degradation of trimethylated proteins. TML is converted into carnitine in four enzymatic steps, encoded by TML dioxygenase (TMLD), 3-hydroxy-TML aldolase (HTMLA), 4-trimethylaminobutyraldehyde dehydrogenase (TMABADH) and γ-butyrobetaine dioxygenase (BBD). In the 1990s the human genes encoding TML, TMABADH and BBD were identified (163-165), but the nature of the HTMLA gene and protein remained elusive (reviewed by 166). We have identified a complete carnitine biosynthesis pathway in C. albicans, including the previously unknown HTMLA (Chapter 4). The protein that performs the aldolytic cleavage of HTML has high homology with threonine aldolases, and we discuss the chemical similarities between both reactions in detail. We show that carnitine biosynthesis mutants have the same growth defect on non-fermentable carbon sources as carnitine acetyl-transferase mutants. To our knowledge, we are the first to identify all genes encoding the four enzymatic steps of the carnitine biosynthesis pathway in any organism. These data suggest that in C. albicans carnitine has become an essential metabolite during growth on non-fermentable carbon sources. By contrast, S. cerevisiae can employ alternative pathways to transport acetyl units and is unable to synthesize carnitine (152, 162).
Scope of this thesis

This thesis focuses on two major subjects of *C. albicans* biology: central carbon metabolism and transport of acetyl units between compartments is discussed in Chapters 2, 3 and 4 and oxidative stress defense is the subject of Chapters 5, 6 and 7. Special attention is paid to the compartmentalization of proteins and metabolic pathways and the requirement for transport of metabolites between organelles.
General Introduction

References


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