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METABOLOME ANALYSIS OF THE YEAST
*Candida albicans* DURING MORPHOGENESIS

By

TING-LI HAN

A thesis submitted in fulfilment of
the requirements for
the degree of
DOCTOR OF PHILOSOPHY

School of Biological Sciences
The University of Auckland
2013
“To raise new questions, new possibilities, to regard old problems from a new angle, requires creative imagination and marks real advance in science.”

Albert Einstein
Summary

*Candida albicans* is a human commensal and opportunistic pathogenic fungus that causes a high mortality rate in severely immunocompromised patients. The ability to change from yeast to filamentous growth and vice-versa, in response to various environmental factors, is considered to be a critical virulence factor of this fungus. Despite the fact that many studies have elucidated the effect of signal transduction pathways and quorum sensing molecules on fungal morphogenesis, the downstream metabolic mechanisms that respond to such signalling molecules and trigger the morphological change at a system level, however, remain unclear. Therefore, the principal aim of this research was to investigate the metabolic reprogramming of *C. albicans* morphogenesis *in vitro* using an emerging post-genomics approach, metabolomics. The specific objectives of this research were: (i) to determine changes in the metabolic pathways of *C. albicans* during the yeast-to-filamentous transition using metabolomics; (ii) to determine the metabolic response of *C. albicans* to quorum sensing molecules involved in morphogenesis; and (iii) gene-knockout mutagenesis of selected metabolic pathways from the central carbon metabolism likely to be associated with morphogenesis.

The chapters of this thesis are a compilation of the work that has been published or submitted for publication during my PhD studies. Chapter one reviews the role of primary metabolism and quorum sensing molecules of *C. albicans* in morphogenesis. The central carbon metabolism and sterol biosynthesis pathways of *C. albicans* were reconstructed *in silico* based on its genome sequence, emphasising the metabolic pathways known to be related to the morphological transition and virulence along with pathways engaged in the biosynthesis of quorum sensing molecules. This initial investigation laid out an important metabolic framework that
guided the interpretation of subsequent metabolomics data and was published as a review article in the journal *Fungal Genetics & Biology*.

To understand the metabolic mechanisms behind *C. albicans*’ morphogenesis, I compared the metabolite profiles of *C. albicans* cells grown under hyphae-inducing conditions to the metabolite profiles of growing yeast cells. This work is described in chapter two and was published in the journal *Metabolomics*. The results revealed a global downregulation of cellular metabolism during the yeast-to-hypha transition. Specifically, seventeen metabolic pathways involved in the central carbon metabolism were significantly downregulated under all three hyphae-inducing conditions. These included metabolic pathways associated with metabolism of amino acids, C5-branched dibasic acid, glutathione, nicotinate/nicotinamide, nitrogen, purine, pyruvate, and acetyl-CoA biosynthesis. These results indicate that downregulation of these central carbon metabolic pathways is likely to be intrinsically involved in the *C. albicans* morphogenetic process. In addition, I demonstrated that filamentous cells contained significant lower concentrations of ATP compared to cells growing in the yeast form. Therefore, these data corroborated the metabolomics results and provided strong evidence that a global downregulation of central carbon metabolism is taking place during the filamentous growth of *C. albicans*.

In order to validate my observations concerning *C. albicans*’ metabolic response to hyphae-inducing conditions, I investigated the metabolic changes of *C. albicans* when hyphal induction was repressed by quorum sensing molecules such as farnesol (chapter 3) and phenylethyl alcohol (chapter 4). These signalling molecules are known to suppress the germ tube formation (initial phase of filamentous growth) of *C. albicans*. Confirming my observations during hyphae-inducing conditions, I demonstrated a general upregulation of metabolic pathways from the central carbon metabolism when filamentous growth was inhibited by either
quorum sensing molecule. By integrating the metabolic profiles from farnesol (chapter 3) and phenylethyl alcohol (chapter 4) experiments with the earlier metabolomics data (chapter 2); I was able to identify seven metabolic pathways that were affected in a consistent fashion in all these studies. Therefore, these seven metabolic pathways are likely to be closely related with the morphogenetic process of *C. albicans*. I also observed a metabolic reprogramming, especially the upregulation of lipid metabolism in response specifically to farnesol and phenylethyl alcohol. This indicates that *C. albicans* may modify its membrane composition in order to minimise the antimicrobial effects of these quorum sensing molecules when they are present at high concentrations. Thus, these results present important new understandings of the metabolic role of quorum sensing in *C. albicans* metabolism.

In order to determine whether or not the quorum sensing molecules were taken up and metabolised by *C. albicans*, I used isotope labelling experiment to trace the metabolic fate of phenylethyl alcohol under hyphae-inducing conditions (chapter 4). The isotope labelling patterns showed that phenylethyl alcohol was taken up by *C. albicans* cells and broken-down intracellularly. Its labelled carbons ended up in the majority of amino acids as well as in lactate and glyoxylate. However, the highest level of carbon labelling was in the pyridine ring of NAD⁺/NADH and NADP⁺/NADPH molecules, indicating that these nucleotides were the major products of phenylethyl alcohol catabolism. Coincidently, two metabolic pathways where these nucleotides play important roles, nitrogen metabolism and nicotinate/nicotinamide metabolism, were among the seven pathways short-listed from the earlier metabolomics results as metabolic pathways likely to be closely involved in *C. albicans* morphogenesis.

Therefore, the final experimental work of this PhD project (chapter 5) was the disruption of a metabolic reaction important to nitrogen and nicotinate/nicotinamide metabolism through gene-knockout mutagenesis and
subsequent evaluation of the morphogenetic ability of the knockout mutants. Thus, I selected two important reactions involved in both the nitrogen metabolism and redox balance of *C. albicans* to be individually deleted using the SAT1 gene disruption method. The selected reactions were those catalysed by NAD⁺-dependent glutamate dehydrogenase (encoded by *GDH2*) and by NADPH-dependent glutamate dehydrogenase (encoded by *GDH3*). The ability of *gdh2/gdh2* and *gdh3/gdh3* mutant strains to undergo morphogenesis was investigated under various hyphae-inducing conditions. The mutants displayed altered morphogenesis and growth rates only when grown on arginine or proline as the sole carbon and nitrogen sources. Both arginine and proline belong to the glutamate family of amino acids. For them to be used as carbon and nitrogen sources they must be first catabolised into glutamate through a series of biochemical reactions, followed by a transamination reaction to form α-ketoglutarate and ammonia, which is catalysed by glutamate dehydrogenase dependent on either NAD⁺ (*GDH2*) or NADPH (*GDH3*). Therefore, the mutants were likely experiencing a change in their redox balance when growing on media supplied with only arginine or proline as carbon and nitrogen sources. The *ghd2/gdh2* mutant strain showed reduced filamentous growth on both proline and arginine, whilst the *ghd3/ghd3* mutant strain showed reduced filamentous growth only when growing on proline. Thus, I demonstrated that some amino acids such as arginine and proline induce morphogenesis in *C. albicans* through nitrogen metabolism, most likely altering the redox balance of the cell. However, to better understand the mechanism of the reduced filamentous growth of these mutants would require a metabolomic study of the mutant strains in comparison to the wild type. Unfortunately these experiments were beyond the scope of this project and could be further pursued in future studies.

In conclusion, I have demonstrated that a metabolomics approach is an effective bottom-up approach to understand the metabolic changes involved with
the morphogenetic process of *C. albicans*. My results not only contribute to the current understanding of fungal morphogenesis and quorum sensing mechanisms, but they also provided the first attempt to connect signal transduction regulation of cellular process with downstream central carbon metabolism (chapter 6).
Acknowledgements

This PhD project would not have been possible without the support of many people. I wish to express my deepest gratitude to my supervisor, Dr. Silas Granato Villas-Bôas, for his invaluable assistance and guidance in my metabolomics work. His endless patience and encouragement have motivated me to carry on pursuing an academic career.

I would also like to extend my sincere gratitude to my co-supervisor, Prof. Richard Cannon. Without his immense knowledge of *C. albicans* biology and molecular biology, this project simply wouldn’t have run so smoothly and successfully.

Special thanks to everyone from the Villas-Boas and Cannon groups, especially Raphael Aggio and Liam Fearnley, for their assistance with metabolomics data analysis, Jenine Upritchard, Dr. Erwin Lamping, Dr. Ann Holmes, and Dr. Kyoko Niimi, for providing invaluable technical support during gene knockout mutagenesis, Dasun Wagachchi and Manya Sabherwal, for assisting with sample preparations, Sergey Tumanov for his help with isotope labelling analysis, Dr. Dung Nguyen and Jeremy Van Houtte, for beautifully maintaining our GC-MS equipment; Tim Liu, Dr. Vidya Washington, and Katie Smart, for proof-reading my manuscripts; and Farhana Pinu, for caring about us like a mother and often bringing delicious food.

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Lastly, I would like to convey my love and gratitude to my beloved family; for their understanding and endless love, especially during times of hardship.
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The metabolic basis of Candida albicans morphogenesis and quorum sensing

| Nature of contribution by PhD candidate | Literature research and writing |
| Extent of contribution by PhD candidate (%) | 80% |

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Metabolome analysis during the morphological transition of Candida albicans

Nature of contribution by PhD candidate: Experimental works, data analysis, and writing.

Extent of contribution by PhD candidate (%): 80%

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The metabolic response of Candida albicans to farnesol under hyphae-inducing conditions.

**Nature of contribution by PhD candidate**

Experimental works, data analysis, and writing.

**Extent of contribution by PhD candidate (%)**

80%

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Metabolic response of Candida albicans to phenylethyl alcohol under hyphae-inducing conditions

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CHAPTER I

The metabolic basis of *Candida albicans* morphogenesis and quorum sensing

Ting-Li Han¹, Richard D. Cannon² and Silas G. Villas-Bôas¹

¹Centre for Microbial Innovation, School of Biological Sciences, The University of Auckland, 3A Symonds Street, Auckland 1142, New Zealand; and ²Department of Oral Sciences, University of Otago, Dunedin, New Zealand.

The content of this chapter has been published

1.1. Abstract

*Candida albicans* is a polymorphic fungus that has the ability to rapidly switch between yeast and filamentous forms. The morphological transition appears to be a critical virulence factor of this fungus. Recent studies have elucidated the signal transduction pathways and quorum sensing molecules that affect the morphological transition of *C. albicans*. The metabolic mechanisms that recognize, and respond to, such signalling molecules and promote the morphological changes at a system level, however, remain unknown. Here we review the metabolic basis of *C. albicans* morphogenesis and we discuss the role of primary metabolic pathways and quorum sensing molecules in the morphogenetic process. We have reconstructed, *in silico*, the central carbon metabolism and sterol biosynthesis of *C. albicans* based on its genome sequence, highlighting the metabolic pathways associated with the dimorphic transition and virulence as well as pathways involved in the biosynthesis of important quorum sensing molecules.
1.2. Introduction

*C. albicans* is a human commensal fungus that can be isolated from approximately 70% of the healthy population (Mavor, *et al.*, 2005). In the majority of cases *C. albicans* is harmless, however, if the person is immunocompromised, it can be an opportunistic pathogen (Wingard, *et al.*, 1979). *C. albicans* is the fourth leading cause of nosocomial bloodstream infections (Pfaller & Diekema, 2007), with an attributable mortality of 37-44% in severely immunocompromised patients (Wisplinghoff, *et al.*, 2004, Leroy, *et al.*, 2009, Moran, *et al.*, 2010).

*C. albicans* and other polymorphic fungi (e.g. *Histoplasma capsulatum*) have the remarkable ability to grow in several distinct morphological forms: yeast, hyphae, and pseudohyphae, according to environmental conditions (Sudbery, *et al.*, 2004, Bastidas & Heitman, 2009). The true hyphae and pseudohyphae (chains of elliptical cells with constrictions at the septa) are often referred to as filamentous forms (Odds, 1988). The ability to switch rapidly from yeast to filamentous growth or vice versa in response to certain environmental cues is considered to be a critical virulence factor for these fungi (Lo, *et al.*, 1997, Mitchell, 1998, Brown & Gow, 1999, Gow, *et al.*, 2002, Rooney & Klein, 2002, Nemecek, *et al.*, 2006). In *C. albicans*, each morphology is believed to confer discrete advantages in the course of infection. The yeast form is important for dissemination through the bloodstream (Bendel, *et al.*, 2003, Saville, *et al.*, 2003), and adheres to endothelial surfaces (Grubb, *et al.*, 2009). The filamentous forms, on the other hand, are more adapted for invasion through the host epithelial tissue (Rooney & Klein, 2002), and also have a higher resistance to phagocytosis due to their morphology. Indeed, an engulfed *C. albicans* yeast cell can destroy a macrophage if filamentous growth is triggered after phagocytosis (Arai, *et al.*, 1977, Lorenz, *et al.*, 2004), and filamentous forms have a higher resistance to neutrophil killing (Smail, *et al.*, 1992, Fradin, *et al.*, 2005). Moreover, experimental
studies support the hypothesis that the morphological transition is an essential virulence factor for *C. albicans*. For instance, a reduced mortality rate has been reported in animal infection models for mice inoculated with *C. albicans* mutants unable to undergo the yeast-to-filamentous transition (Lo, *et al.*, 1997, Gale, *et al.*, 1998). In addition, the induction of hyphal gene expression promoted virulence in a mouse model of systemic candidiasis (Carlisle, *et al.*, 2009).

The morphogenesis of *C. albicans* is predominately determined by environmental signalling. The yeast-to-filamentous transition can be triggered by serum, proline, N-acetylglucosamine, different carbon sources, and other cues as summarised in Table 1.1. However, there is a lack of understanding about how *C. albicans* regulates its morphogenesis in response to these environmental changes. In addition, the synchronised morphogenesis of *C. albicans* cells, and other dimorphic fungi, seems to be coordinated within the cell population by chemical signals (Chen & Fink, 2006, Hogan, 2006b, Hogan, 2006a, Nickerson, *et al.*, 2006, Chen, *et al.*, 2007). Dimorphic fungi are known to produce several signalling metabolites. Farnesol, which suppresses filamentous formation in *C. albicans*, is the best characterized of these molecules (Hornby, *et al.*, 2001), but the mechanism by which farnesol is sensed by *C. albicans* is not yet clear. Tyrosol is another signalling molecule produced by *C. albicans* and it stimulates the yeast-to-hypha conversion (Chen, *et al.*, 2004). Other metabolites such as estradiol (mammalian metabolite) and dibutryl cAMP are also known to affect *C. albicans* morphogenesis (Niimi, *et al.*, 1980, White & Larsen, 1997, Cheng, *et al.*, 2006). Despite many studies showing the effect of small molecules on fungal morphogenesis, the pathways that recognize such signals and their effect on virulence are poorly characterized. Thus, this article reviews our current knowledge of *C. albicans* morphogenesis from a metabolic point of view and relates it to the activity of central carbon metabolism and the biosynthesis of quorum sensing molecules.
### Table 1.1
The effect of environmental cues on the morphogenesis of *C. albicans*

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<tr>
<td>25 °C or lower</td>
<td>↑ yeast formation</td>
<td>(Lee H. Mitchell, 1979)</td>
</tr>
<tr>
<td>37 °C or higher</td>
<td>↑ pseudohypha formation</td>
<td>(Lee H. Mitchell, 1979, Lee, et al., 1999)</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>↑ hypha formation</td>
<td>(Brown Jr, et al., 1999)</td>
</tr>
<tr>
<td></td>
<td>↓ hypha formation (absence of other divalent ions)</td>
<td>(Sabie &amp; Gadd, 1989, Holmes, et al., 1991)</td>
</tr>
<tr>
<td></td>
<td>↑ hypha formation (in presence of Mg²⁺)</td>
<td></td>
</tr>
<tr>
<td>0.2 % of glucose, galactose, fructose or sucrose</td>
<td>↑ hypha formation</td>
<td>(Maidan, et al., 2005a)</td>
</tr>
<tr>
<td>Fructose</td>
<td>↑ hypha formation (in the absence of nitrogen source)</td>
<td>(Vidotto, et al., 1996a)</td>
</tr>
<tr>
<td>Lee’s medium</td>
<td>↑ hypha formation (at 37 °C)</td>
<td>(Lee, et al., 1975)</td>
</tr>
<tr>
<td></td>
<td>↓ hypha formation (at 25 °C)</td>
<td></td>
</tr>
<tr>
<td>Lithium</td>
<td>↓ hypha formation (except in liquid culture and requires at least 15 mM LiCl)</td>
<td>(Martins, et al., 2008)</td>
</tr>
<tr>
<td>Low ammonia medium</td>
<td>↑ hypha formation (at pH 6.7)</td>
<td>(Holmes &amp; Shepherd, 1987, Eisman, et al., 2006)</td>
</tr>
<tr>
<td>N-acetylglucosamine</td>
<td>↑ hypha formation (inhibited when pH &lt; 4.5)</td>
<td>(Sullivan &amp; Shepherd, 1982, Holmes &amp; Shepherd, 1987)</td>
</tr>
<tr>
<td>pH 4.5 (acid pH)</td>
<td>↑ yeast formation (in the presence of glucose)</td>
<td>(Lee H. Mitchell, 1979, Pollack &amp; Hashimoto, 1987)</td>
</tr>
<tr>
<td>pH 6-8</td>
<td>↑ pseudohypha formation (but maintains the yeast form at 25 °C)</td>
<td>(Lee H. Mitchell, 1979) (Pollack &amp; Hashimoto, 1987)</td>
</tr>
<tr>
<td>Proline</td>
<td>↑ hypha formation (not observed when pH &lt; 5.0)</td>
<td>(Dabrowa, et al., 1976, Holmes &amp; Shepherd, 1987)</td>
</tr>
<tr>
<td>Serum</td>
<td>↑ hypha formation</td>
<td>(Reynolds &amp; Braude, 1956, Hilmiglou, et al., 2007)</td>
</tr>
</tbody>
</table>
### Table 1.1
*(continued)*

<table>
<thead>
<tr>
<th>Environmental cues</th>
<th>Effect on morphogenesis</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-oxo-C12-homoserine lactone</td>
<td>↓ hypha formation, ↓ pseudohypha formation</td>
<td>(Hogan, <em>et al.</em>, 2004)</td>
</tr>
<tr>
<td>Dodecanol</td>
<td>↓ hypha formation, ↓ pseudohypha formation</td>
<td>(Hogan, <em>et al.</em>, 2004)</td>
</tr>
<tr>
<td>Human blood without white blood cell</td>
<td>↑ hypha formation</td>
<td>(Fradin, <em>et al.</em>, 2005)</td>
</tr>
<tr>
<td>Parenteral lipid emulsion</td>
<td>↑ germ tube formation</td>
<td>(Swindell, <em>et al.</em>, 2009)</td>
</tr>
</tbody>
</table>

*Note: ↑: inducing, ↓: suppressing*
1.3. Signaling pathways and transcriptional regulators associated with *C. albicans* morphogenesis


GTPases are thought to be the main regulators that activate the two best-studied signaling pathways involved in *C. albicans* morphogenesis: the cAMP-Protein Kinase A (PKA), and the Mitogen-Activated Protein Kinase (MAPK), pathways ([Figure 1.1](#)). The cAMP-PKA pathway involves adenyl cyclase (Cdc35p) (Rocha, *et al.*, 2001) and PKA (Bcy1p, Tpk1p, Tpk2p) (Cloutier, *et al.*, 2003, Cassola, *et al.*, 2004) ([Table 1.2, Figure 1.1](#)). Eventually, the PKA pathway activates an important transcription factor encoded by *EFG1* (Stoldt, *et al.*, 1997) that induces the expression of hyphal-specific
genes (e.g. *HWP1*, *HYR1* and *ALS1*) (Sharkey, *et al.*, 1999, Leng, *et al.*, 2001, Fu, *et al.*, 2002). MAPK pathways typically consist of three kinases; MAPKKK, MAPKK, and MAPK that are sequentially activated one after the other by phosphorylation. There are several different MAPK pathways that have been described and linked to the morphological changes of *C. albicans* (e.g. Cek1p, Hog1p, and Mkc1p MAPK pathways) (Leberer, *et al.*, 1996, Csank, *et al.*, 1998, Navarro-García, *et al.*, 1998, Leberer, *et al.*, 2001, Eisman, *et al.*, 2006, Cheetham, *et al.*, 2007, Alonso-Monge, *et al.*, 2009a). Figure 1.1 illustrates how these signaling pathways and regulatory components are interconnected, and Table 1.2 summarizes how these components lead to modifications in cell wall organization, cell polarity, metabolism, cell cycle, virulence factors, and morphogenesis. Furthermore, transcriptome studies have identified several hyphal-suppressing genes in *C. albicans*. These repressor genes, such as *TUP1*, *SSN6*, *NRG1*, *MIG1*, and *HOG1*, are involved in the regulation of central carbon metabolism (José, *et al.*, 1996, Braun & Johnson, 1997, Murad, *et al.*, 2001, Hwang, *et al.*, 2003) as illustrated in Table 1.3. There are many other genes that are known to be involved in the morphological switch of *C. albicans*. The role of those genes and how they may influence or have their expression changed during morphogenesis are briefly summarized in Table 1.2.
Figure 1.1. Signal transduction pathways and transcriptional regulators that affect the filamentous growth of *C. albicans* and the effect of farnesol on some of these pathways. In response to filamentous-inducing conditions, GTPases (Gpa2p, Ras1p, and Cdc42p) activate two well-characterized signalling pathways; the Cek1p mediated MAP kinase pathway and cAMP-PKA pathway. These lead to the activation of transcriptional regulators Cph1p and Efg1p, respectively, which promote filamentous growth. Nitrogen starvation activates both the MAP Kinase and cAMP-PKA pathways via ammonium permease (Mep2p). Adenylyl cyclase (Cdc35p) not only responds to Ras1p, it is also activated in response to G-proteins (Gpr1p, Gpa2p), which are activated by glucose deficiency and the presence of methionine. Cdc35p also acts as a sensor for CO₂ and peptidoglycans. Oxidative stress and osmotic stress are sensed by a two-component system (Sln1p, Ssk1p), which in turn suppresses Hog1p MAP kinase pathways. At 37 °C, Pi4p5kp synthesises phosphatidylinositol 4,5-bisphosphate (PIP₂) to promote the release of intracellular Ca²⁺. The role of other individual genes are summarised in Table 1.2. Quorum sensing molecule farnesol inhibits filamentous growth by suppressing the expression of *RAS1, SHO1, HST7, CEK1, PDE2*, and *HWPI*, while it up-regulates filamentous-suppressing genes such as *TUP1* and *HOG1*. Blue boxes represent the membrane-anchored proteins, red boxes represent the transcriptional regulators, and purple boxes indicate DNA-binding proteins (Nrg1p, Mig1p, or Rfg1p).
Table 1.2
Genes involved in the morphogenesis of *C. albicans*

<table>
<thead>
<tr>
<th>Genes</th>
<th>Gene functions</th>
<th>Involvement in morphogenesis</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Signaling pathway genes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BCY1</td>
<td>The regulatory subunit of PKA</td>
<td>↑hypha formation</td>
<td>(Cassola, et al., 2004)</td>
</tr>
<tr>
<td>CAP1</td>
<td>Adenylate cyclase-associated protein</td>
<td>↑hypha formation</td>
<td>(Bahn &amp; Sundstrom, 2001)</td>
</tr>
<tr>
<td>CDC42</td>
<td>Rho-type GTPase; a main regulator of cell polarity in fungi with two forms; Cdc42-GTP and Cdc42-GDP. Cdc42-GTP activates Cst20p and promotes septin ring formation</td>
<td>Only CDC42-GTP: ↑hypha formation</td>
<td>(Basiliana, et al., 2003, Su, et al., 2005, Court &amp; Sudbery, 2007)</td>
</tr>
<tr>
<td>CDC24</td>
<td>The GDP-GTP exchange factor for Cdc42p; promotes Cdc42-GTP formation</td>
<td>↑hypha formation</td>
<td>(Basiliana, et al., 2003)</td>
</tr>
<tr>
<td>RGA2,</td>
<td>Cdc42 GTPase-activating proteins; promotes Cdc42-GDP formation</td>
<td>↓hypha formation</td>
<td>(Court &amp; Sudbery, 2007, Wang, 2009)</td>
</tr>
<tr>
<td>BEM3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDC35 or CYR1</td>
<td>Adenyl cyclase, a sensor that responds to CO₂ and bacterial peptidoglycan (PGN)-like molecules.</td>
<td>↑hypha formation ↑Pseudohypha formation</td>
<td>(Rocha, et al., 2001, Klengel, et al., 2005, Xu, et al., 2008)</td>
</tr>
<tr>
<td>CDC53</td>
<td>Encodes cullin, part of ubiquitin-ligase complex SCF</td>
<td>↓hypha formation</td>
<td>(Trunk, et al., 2009)</td>
</tr>
<tr>
<td>CST20</td>
<td>MAPKK kinase of Cek1 pathway</td>
<td>↑hypha formation</td>
<td>(Leberer, et al., 1996)</td>
</tr>
<tr>
<td>HST7</td>
<td>MAPK kinase of Cek1 pathway</td>
<td>↑hypha formation</td>
<td>(Leberer, et al., 1996)</td>
</tr>
<tr>
<td>CEK1</td>
<td>MAP kinase of Cek1 pathway; phosphorylation is prevented by farnesol</td>
<td>↑hypha formation</td>
<td>(Csank, et al., 1998, Román, et al., 2009)</td>
</tr>
<tr>
<td>CPP1</td>
<td>Cek1 phosphatase, Inactivates the Cek1p</td>
<td>↓hypha formation</td>
<td>(Csank, et al., 1997)</td>
</tr>
<tr>
<td>CRK1</td>
<td>Cdc2-related kinase, suppressed by farnesol</td>
<td>↑hypha formation</td>
<td>(Chen, et al., 2000, Sato, et al., 2004)</td>
</tr>
<tr>
<td>GPA2</td>
<td>A G-protein α subunit</td>
<td>↑hypha formation</td>
<td>(Sánchez-Martínez &amp; Pérez-Martín, 2002, Maidan, et al., 2005b)</td>
</tr>
<tr>
<td>MKC1</td>
<td>MAPK kinase; maintains cell wall integrity</td>
<td>↑hypha formation</td>
<td>(Navarro-García, et al., 1998)</td>
</tr>
<tr>
<td>YPD1</td>
<td>A phosphohistidine intermediate protein that transfers a phosphate from Sln1p to Ssk1p</td>
<td>Express in both yeast and hyphal forms</td>
<td>(Payne, et al., 2000)</td>
</tr>
<tr>
<td>SSK1</td>
<td>Response regulator of two component system; suppresses Hog1-mediated MAPK pathways and it is associated with cell wall synthesis and oxidative-stress response</td>
<td>↑hypha formation</td>
<td>(Calera, et al., 2000, Menon, et al., 2006)</td>
</tr>
<tr>
<td>Genes</td>
<td>Gene functions</td>
<td>Involvement in morphogenesis</td>
<td>References</td>
</tr>
<tr>
<td>---------</td>
<td>---------------------------------------------------------------------------------</td>
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<td>-----------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>SSK2</td>
<td>MAPKK Kinase of Hog1 pathway</td>
<td>↓ hypha formation</td>
<td>(Cheetham, et al., 2007)</td>
</tr>
<tr>
<td>PBS2</td>
<td>MAPK Kinase of Hog1 pathway, essential for oxidative-stress response</td>
<td>↓ hypha formation</td>
<td>(Arana, et al., 2005)</td>
</tr>
<tr>
<td>HOG1</td>
<td>High osmolarity glycerol MAP kinase; responds to osmotic stress, temperature upshift, oxidative stress. Represses the activity of Cek1p, regulates respiratory metabolism, and upregulated by farnesol</td>
<td>↓ hypha formation</td>
<td>(José, et al., 1996, Eisman, et al., 2006)</td>
</tr>
<tr>
<td>PDE2</td>
<td>Phosphodiesterase, suppressed by farnesol</td>
<td>↑ hypha formation ↑ pseudohypha formation</td>
<td>(Bahn, et al., 2003, Sato, et al., 2004, Smith, et al., 2004)</td>
</tr>
<tr>
<td>PI4PSK</td>
<td>Phosphatidylinositol-4 phosphate 5-kinase; synthesizes phosphatidylinositol 4,5-bisphosphate (PIP2) in response to high temperature</td>
<td>↑ hypha formation</td>
<td>(Hairfield, et al., 2002)</td>
</tr>
<tr>
<td>RAD53</td>
<td>The kinase involved in mediating DNA damaged–induced hyphal growth</td>
<td>↑ hypha formation</td>
<td>(Shi, et al., 2007)</td>
</tr>
<tr>
<td>RAS1</td>
<td>A member of the GTPase superfamily; responds to nitrogen starvation, glucose and serum. Suppressed by farnesol</td>
<td>↑ hypha formation</td>
<td>(Feng, et al., 1999)</td>
</tr>
<tr>
<td>CDC25</td>
<td>Ras1 guanine exchange factor; responds to glucose and induces cAMP signaling pathway</td>
<td>↑ hypha formation</td>
<td>(Enloe, et al., 2000)</td>
</tr>
<tr>
<td>RHB1</td>
<td>Rheb of Ras superfamily, involves in the nitrogen starvation inducing filamentous growth</td>
<td>↓ hypha formation</td>
<td>(Tsao, et al., 2009)</td>
</tr>
<tr>
<td>TSC2</td>
<td>Homolog of human tuberous sclerosis protein 2, negatively regulates the GTPase activity of Rhb1p</td>
<td>↑ hypha formation</td>
<td>(Tsao, et al., 2009)</td>
</tr>
<tr>
<td>SHO1</td>
<td>An adaptor protein that responds to oxidative stress and cell wall biosynthesis, It activates Cek1 Kinase pathway.</td>
<td>↑ hypha formation</td>
<td>(Román, et al., 2005)</td>
</tr>
<tr>
<td>TEC1</td>
<td>A transcription factor - member of the TEA/ATTS family; regulated by Efg1p</td>
<td>↑ hypha formation</td>
<td>(Schweizer, et al., 2000, Lane, et al., 2001)</td>
</tr>
</tbody>
</table>
### Table 1.2 (continued)

<table>
<thead>
<tr>
<th>Genes</th>
<th>Gene functions</th>
<th>Involvement in morphogenesis</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPK1</td>
<td>The catalytic subunit of PKA</td>
<td>↑hypha formation</td>
<td>(Cloutier, et al., 2003)</td>
</tr>
<tr>
<td>TPK2</td>
<td>The catalytic subunit of PKA</td>
<td>↑hypha formation</td>
<td>(Cloutier, et al., 2003)</td>
</tr>
<tr>
<td>YAK1</td>
<td>Ser/Thr protein kinase; Upstream of Tup1p</td>
<td>↑hypha formation</td>
<td>(Goyard, et al., 2008)</td>
</tr>
<tr>
<td><strong>Transcription factor genes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPH1</td>
<td>Transcription factor that is activated by MAPK pathway; responds to starvation and GlcNAC</td>
<td>↑hypha formation ↑pseudohypha formation</td>
<td>(Lo, et al., 1997)</td>
</tr>
<tr>
<td>CZF1</td>
<td>Putative zinger finger transcription factor; responds to agar-embedded growth conditions</td>
<td>↑hypha formation</td>
<td>(Brown Jr, et al., 1999)</td>
</tr>
<tr>
<td>EFG1</td>
<td>Transcription factor that is activated by PKA pathway; responds to serum, upregulates glycolysis and downregulates TCA cycle</td>
<td>↑hypha formation ↑pseudohypha formation</td>
<td>(Stoldt, et al., 1997, Leng, et al., 2001)</td>
</tr>
<tr>
<td>TUP1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>A general transcriptional repressor; regulates glycolysis and TCA cycle. Upregulated by farnesol</td>
<td>↓hypha formation</td>
<td>(Braun &amp; Johnson, 1997, Cao, et al., 2005, Kebaara, et al., 2008)</td>
</tr>
<tr>
<td>SSN6</td>
<td>A general transcriptional repressor forms a complex with Tup1p</td>
<td>↓pseudohypha formation</td>
<td>(Hwang, et al., 2003)</td>
</tr>
<tr>
<td>MIG1</td>
<td>DNA binding protein (DBP) binds to Tup1p</td>
<td>↓hypha formation</td>
<td>(Murad, et al., 2001)</td>
</tr>
<tr>
<td>NRG1</td>
<td>DBP binds to Tup1p</td>
<td>↓hypha formation ↓Pseudohypha formation</td>
<td>(Braun, et al., 2001, Murad, et al., 2001)</td>
</tr>
<tr>
<td>RGT1</td>
<td>A transcriptional repressor for genes that encode hexose transporters, N-acetylglucosamine transporter (NGT1), and downregulates glycolysis</td>
<td>↓hypha formation</td>
<td>(Sexton, et al., 2007)</td>
</tr>
<tr>
<td>RIM101</td>
<td>pH response regulator, activated by alkaline conditions and regulates both Phr1p and Phr2p. It is required for Aro9 pH dependent expression and required for virulence during systemic infection</td>
<td>↑hypha formation</td>
<td>(Ramon, et al., 1999, Nobile, et al., 2008)</td>
</tr>
</tbody>
</table>
### Table 1.2 (continued)

<table>
<thead>
<tr>
<th>Genes</th>
<th>Gene functions</th>
<th>Involvement in morphogenesis</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>RFG1</td>
<td>Repressor for filamentous growth; DBP binds to Tup1p</td>
<td>↓hypha formation</td>
<td>(Kadosh &amp; Johnson, 2001, Khalaf &amp; Zitomer, 2001)</td>
</tr>
<tr>
<td>GCN4</td>
<td>General amino acid control; regulates both morphogenesis and metabolic responses to amino acid starvation</td>
<td>↑hypha formation</td>
<td>(Tripathi, et al., 2002)</td>
</tr>
<tr>
<td>UME6</td>
<td>A transcriptional regulator; encodes zinc finger DNA-binding protein</td>
<td>↑hypha formation</td>
<td>(Strich, et al., 1994, Banerjee, et al., 2008)</td>
</tr>
<tr>
<td><strong>Receptor genes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDR1, CDR2</td>
<td>Multidrug transporters of the ABC family: an efflux pump that removes estradiol from the cell</td>
<td>↑germ tube is formed in the present of estradiol</td>
<td>(Cheng, et al., 2006)</td>
</tr>
<tr>
<td>GAP1</td>
<td>General amino acid permease; the expression of transporter is induced by GlcNAc and Cph1p.</td>
<td>↑hypha formation</td>
<td>(Biswas, et al., 2003)</td>
</tr>
<tr>
<td>HGT4</td>
<td>Glucose and galactose sensor</td>
<td>↑hypha formation</td>
<td>(Brown, et al., 2006, Brown, et al., 2009)</td>
</tr>
<tr>
<td>GPR1</td>
<td>G protein-coupled receptor; upstream of cAMP-PKA pathway, senses methionine and proline</td>
<td>↑hypha formation</td>
<td>(Maidan, et al., 2005b, Maidan, et al., 2005a)</td>
</tr>
<tr>
<td>HGT4</td>
<td>Glucose sensor; induces the expression of hexose transporters (e.g. HGT12, HXT12 and HGT7)</td>
<td>↓hypha formation</td>
<td>(Brown, et al., 2006)</td>
</tr>
<tr>
<td><strong>Others genes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDC10, CDC11</td>
<td>Non-essential septin genes</td>
<td>cdc10 and cdc11 mutants show abnormal hyphal structure</td>
<td>(Warenda, et al., 2003)</td>
</tr>
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Table 1.2 (continued)

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<th>Genes</th>
<th>Gene functions</th>
<th>Involvement in morphogenesis</th>
<th>References</th>
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<tbody>
<tr>
<td><strong>Metabolic genes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NGT1</td>
<td>N-acetylglucosamine transporter</td>
<td>In a homozygous NGT1 deletant, hyphal growth cannot be induced by GlcNAc</td>
<td>(Alvarez &amp; Konopka, 2007)</td>
</tr>
<tr>
<td>MEP2</td>
<td>Ammonium permease; senses nitrogen starvation. Expression is suppressed by: 10mM of NH₄⁺, overexpression of RHB1, and TSC2 deletion. It activates both MAP kinase pathway and cAMP-PKA pathway</td>
<td>↑pseudohypha formation</td>
<td>(Biswas &amp; Morschhäuser, 2005, Tsao, et al., 2009)</td>
</tr>
<tr>
<td>ALO1</td>
<td>D-arabinono-1,4-lactone oxidase; catalyzes the last step of D-erythrose-4-phosphate aldolase biosynthesis</td>
<td>↑hypha formation</td>
<td>(Huh, et al., 2001)</td>
</tr>
<tr>
<td>ATC1</td>
<td>Cell wall-linked acid trehalase, converts exogenous galactose into glucose.</td>
<td>↑hypha formation ↑pseudohypha formation</td>
<td>(Pedreño, et al., 2007)</td>
</tr>
<tr>
<td>GAL10</td>
<td>UDP-galactose-4-epimerase; converts galactose into glucose</td>
<td>↓hypha formation</td>
<td>(Singh, et al., 2007)</td>
</tr>
<tr>
<td>PDX1</td>
<td>Pyruvate dehydrogenase</td>
<td>↑hypha formation ↑pseudohypha formation</td>
<td>(Vellucci, et al., 2007)</td>
</tr>
<tr>
<td>TSP1</td>
<td>Trehalose-6-phosphate synthase; involved in trehalose biosynthesis.</td>
<td>↑hypha formation</td>
<td>(Zaragoza, et al., 1998)</td>
</tr>
<tr>
<td><strong>Cell wall genes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALS1</td>
<td>Adhesin ; hypha specific gene</td>
<td>↑hypha formation ↑pseudohypha formation</td>
<td>(Fu, et al., 2002)</td>
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### Table 1.2 (continued)

<table>
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<tr>
<th>Genes</th>
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<th>Involvement in morphogenesis</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>INT1</td>
<td>Integrin-like, role in adhesion</td>
<td>↑ hypha formation</td>
<td>(Gale, et al., 1998, Asleson, et al., 2001)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↑ pseudohypha formation</td>
<td></td>
</tr>
<tr>
<td>PHR1</td>
<td>GPI-anchored glycosidase; cell wall structure, expressed at pH 5.5 or alkaline pH</td>
<td>↑ hypha formation</td>
<td>(Ghannoum, et al., 1995, De Bernardis, et al., 1998)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↑ pseudohypha formation</td>
<td></td>
</tr>
<tr>
<td>PHR2</td>
<td>GPI-anchored glycosidase; cell wall structure, expressed at acidic pH</td>
<td>↑ hypha formation</td>
<td>(Mühlschlegel &amp; Fonzi, 1997, De Bernardis, et al., 1998)</td>
</tr>
<tr>
<td>PMT1</td>
<td>Mannosyltransferase</td>
<td>↑ hypha formation</td>
<td>(Timpel, et al., 1998)</td>
</tr>
<tr>
<td>PMT6</td>
<td>Mannosyltransferase, role in adhesion and antifungal resistance</td>
<td>↑ hypha formation</td>
<td>(Timpel, et al., 2000)</td>
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<tr>
<td></td>
<td><strong>Cell cycle-associated genes</strong></td>
<td></td>
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<tr>
<td>CDC28</td>
<td>Cyclin-dependent kinase; phosphorylates Cdc11 p(septin gene) in association with either Ccn1p or Hgc1. Cdc28-Hgc1 negatively regulates Rga2p and also phosphorylates Efg1p</td>
<td>↑ hypha formation</td>
<td>(Sinha, et al., 2007, Wang, et al., 2009)</td>
</tr>
<tr>
<td>HGC1</td>
<td>Hyphal-specific G1 cyclin related protein; maintains the localization of polarity proteins (e.g. actin) on hyphal tip, regulated by cAMP-PKA pathway and Tup1p</td>
<td>↑ hypha formation</td>
<td>(Zheng &amp; Wang, 2004)</td>
</tr>
<tr>
<td>CCNI</td>
<td>G₁ cyclin; role in maintaining hyphal growth</td>
<td>↑ hypha formation</td>
<td>(Loeb, et al., 1999)</td>
</tr>
<tr>
<td>CLN3</td>
<td>G₁ cyclin; is important for yeast budding and negatively regulates the yeast-to-hyphal transition</td>
<td>↓ hypha formation</td>
<td>(Bachewich &amp; Whiteway, 2005, Chapa Y Lazo, et al., 2005)</td>
</tr>
<tr>
<td>CLB2,</td>
<td>B-cyclins that are negative regulators of polarized growth</td>
<td>↓ hypha formation</td>
<td>(Bensen, et al., 2005)</td>
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<tr>
<td>CLB4</td>
<td></td>
<td>↓ pseudohypha formation</td>
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</table>

*Note: ↑: inducing, ↓: suppressing.*

Observations are based on knockout mutagenesis studies

*The expression of these genes is regulated by farnesol.*
Table 1.3
The effect of morphogenesis-related genes, and farnesol, on *Candida albicans* metabolism

<table>
<thead>
<tr>
<th>Metabolism of:</th>
<th>EFG1</th>
<th>GCN4</th>
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<th>TUP1</th>
<th>MIG1</th>
<th>NRG1</th>
<th>RIM101</th>
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<td>Arginine</td>
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<td>Asparagine</td>
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<td>Aspartate</td>
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<tr>
<td>Cysteine</td>
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<tr>
<td>Ergosterol biosynthesis</td>
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<td>Glutamate</td>
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<tr>
<td>Glyoxylate shunt</td>
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<td>Lysine</td>
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<td>Proline</td>
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<td>Serine</td>
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</tbody>
</table>

*Note:* ↑: gene/farnesol upregulates one or more enzymes in a particular biochemical pathway

↓: gene/farnesol downregulates one or more enzymes in a particular biochemical pathway

↓<sup>pH4</sup>: assayed at pH 4, ↑<sup>pH8</sup>: assayed at pH 8
Table 1.3  
(continued)

<table>
<thead>
<tr>
<th>Metabolism of:</th>
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<th>NGT1</th>
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<td>Aspartate</td>
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<td>Ergosterol biosynthesis</td>
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<td>Isoleucine</td>
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1.4. Quorum sensing molecules and *Candida albicans* morphogenesis

The phenomenon of “quorum sensing” is being increasingly recognized as a fundamental aspect of microbial cell-to-cell communication and signalling. Fuqua *et al.* (1994) first coined this term to describe the cooperative behaviour of bacterial cells that can only take place when a certain cell population density threshold is reached. Quorum sensing is often referred to as autoinduction, a process by which individual cells release small diffusible molecules into their environment and these molecules are sensed by all cells in the population (Nealson, 1977, Gray *et al.*, 1994, Hense *et al.*, 2007). When high cell density is reached, these autoinducing molecules accumulate above a certain threshold level, activating and/or repressing certain genes (Fuqua *et al.*, 1994), which in turn induce complex cellular behaviour such as secretion of extracellular enzymes (Rosenberg *et al.*, 1977), bioluminescence (Eberhard, 1972, Eberhard *et al.*, 1981, Fuqua *et al.*, 1994), plasmid transfer (Piper *et al.*, 1993), antibiotic biosynthesis (Bainton *et al.*, 1992), biofilm development (Alem *et al.*, 2006), and morphological switches (Hornby *et al.*, 2001). Despite quorum sensing being well-characterized and evolutionarily-conserved between diverse bacterial species (Gray *et al.*, 1994, Gray & Garey, 2001), only a few quorum sensing like-responses have been reported in eukaryotes, such as *Histoplasma capsulatum* (Kügler *et al.*, 2000), *C. albicans* (Hornby *et al.*, 2001, Chen *et al.*, 2004), and *Saccharomyces cerevisiae* (Chen & Fink, 2006). Curiously, in each of these studies quorum sensing was associated with the morphological switch from yeast to filamentous forms, or vice versa.

Eukaryotic quorum sensing is best studied in *C. albicans*. This fungus has the greatest number of quorum sensing molecules (QSM) identified to date. Tryptophol and phenylethyl alcohol were the first QSMs identified in *C. albicans* (Lingappa *et al.*).
These QSMs are produced by *C. albicans* and inhibit both cell growth and germ tube formation - whether the inhibition of germ tube formation is due to the inhibition of growth remains unclear. In recent years, three additional QSMs have been isolated from *C. albicans*; farnesol, tyrosol and farnesoic acid (Hornby, *et al.*, 2001, Oh, *et al.*, 2001, Chen, *et al.*, 2004, Hornby & Nickerson, 2004), although farnesoic acid has only been reported in one *C. albicans* strain (ATCC 10231, Oh et al. 2001) and has a lower activity than farnesol (Hornby and Nickerson, 2004).

Farnesol is the best characterized QSM in *C. albicans* and it is known to block the yeast-to-filamentous transition at high cell density, as well as under other hyphal-inducing conditions (e.g. serum, proline, and N-acetylglucosamine) (Hornby, *et al.*, 2001); but it is incapable of stopping the elongation of pre-existing hyphae (Mosel, *et al.*, 2005, Navarathna, *et al.*, 2005). Moreover, farnesol is continuously released into the environment during growth, and its accumulation in the medium is roughly proportional to the cell density (Hornby, *et al.*, 2001). It has been proposed that farnesol is secreted by the cells to inhibit germ tube formation in the late stage of biofilm development where there is a high density of interwoven filamentous cells, and, therefore, it promotes the dispersal of yeast cells to colonize new environments (Alem, *et al.*, 2006). After farnesol was discovered in *C. albicans* cultures, several studies have been undertaken to investigate the mechanisms by which QSMs affect morphogenesis. For instance, Sato *et al.* (2004) used RT-PCR to show that farnesol inhibited MAP kinase cascades via the suppression of *HST7* and *CPH1* gene expression (Figure 1.1). Hog1p phosphorylation also increased in the presence of farnesol (Smith, *et al.*, 2004). Cao *et al.* (2005) demonstrated that several morphogenesis-associated genes were downregulated (e.g. *CRK1* and *PDE2*) and some upregulated (e.g. *TUP1*) by the presence of farnesol. It has been suggested that hyphal formation induced by the cAMP-PKA pathway can be repressed by farnesol which suppresses the *RAS1-CDC35* pathway (Davis-Hanna, *et al.*, 2008).
Kebaara et al. (2008) showed that farnesol inhibits hyphal formation by upregulating the global repressor of \textit{TUP1} because it failed to suppress hyphal development in \textit{tup1/tup1} and \textit{nrg1/nrg1} null mutants. Recently, Román et al. (2009) showed that farnesol prevents the activation of Cek1p, which is a part of MAPK cascade. In summary, the studies to date show that farnesol inhibits \textit{C. albicans} morphogenesis by suppressing both MAPK and cAMP-PKA pathways and promoting the expression of hyphal-suppressor genes such as \textit{TUP1} and \textit{HOG1} (Figure 1.1). How yeast cells sense farnesol, however, is still unclear.

Tyrosol is another QSM produced by \textit{C. albicans}. It has been observed that when a dense culture of \textit{C. albicans} is diluted into fresh medium there is long lag-phase before the exponential growth initiates (Chen, \textit{et al.}, 2004). This lag period can be shortened, and germ tube formation can be enhanced, when tyrosol is supplied (Chen, \textit{et al.}, 2004). Alem \textit{et al.} (2006) also suggested that tyrosol stimulated hypha formation in the early and intermediate phases of \textit{C. albicans} biofilm formation. It seems that tyrosol does not induce germ tube formation when \textit{C. albicans} grows under non-filamentous-inducing conditions but rather accelerates the morphological switch from yeast to hyphal growth under favourable environmental conditions (Chen, \textit{et al.}, 2004). In contrast to farnesol, little is known about how tyrosol exerts its effects. A study of gene expression profiles of \textit{C. albicans} cells at different cell densities with and without tyrosol suggested that the acceleration of germ tube formation may simply be due to upregulation of genes associated with DNA replication (e.g. DNA polymerase, chromosome-separation factor) and the cell cycle (e.g. cell cycle checkpoint protein) (Chen, \textit{et al.}, 2004).

Together, these studies demonstrate that the morphogenesis of \textit{C. albicans} is under the positive and negative regulation of QSMs in response to cell density. QSMs such as tryptophol, phenylethyl alcohol, and farnesol suppress hyphal formation when cells grow at high density. The QSM tyrosol accelerates germ tube
formation at low cell density (Figure 1.2). QSMs appear to have other roles in addition to affecting morphogenesis. Farnesol, for example, enhances *C. albicans* resistance to oxidative stress (Westwater, *et al.*, 2005), and tyrosol can act as an antioxidant, protecting *C. albicans* cells during phagocytosis by neutrophils (Cremer, *et al.*, 1999). Moreover, when growing in the human body *C. albicans* often grows in a polymicrobial environment and QSMs appear to be also involved in inter-species competition (Shank & Kolter, 2009). Farnesol not only inhibits filament formation in other *Candida* species (e.g. *Candida dubliniensis* and *Candida tropicalis*) (Henriques, *et al.*, 2007, Zibafar, *et al.*, 2009), it also induces apoptosis in some fungi (e.g. *Saccharomyces cerevisiae*, *Aspergillus nidulans*, and *Penicillium expansum*), and suppresses the growth of *Paracoccidioides brasiliensis* (Semighini, *et al.*, 2006, Fairn, *et al.*, 2007, Derengowski, *et al.*, 2009, Liu, *et al.*, 2009). In contrast, when *C. albicans* is exposed to other QSMs such as 3-OXO-C12 homoserine lactone from *Pseudomonas aeruginosa*, its filamentous growth is suppressed (Hogan, *et al.*, 2004). Interestingly, farnesol seems to be also involved in *C. albicans*-host interactions. Proteomic approaches have shown that farnesol triggers apoptosis in both human oral carcinoma cells and *C. albicans* itself via classic apoptotic pathways (Scheper, *et al.*, 2008, Shirtliff, *et al.*, 2009). It appears that *C. albicans* is more susceptible to farnesol-mediated cell death when log-phase cells grow under nutrient-poor conditions (Langford, *et al.*, 2010). Dècanis *et al.* (2009) demonstrated that farnesol upregulates the expression of toll-like receptor 2, and increases the production of interleukin-6 and β-defensin 2 in the engineered tissue of human oral mucosa. The same authors suggest that farnesol promotes epithelial cell immunity against *C. albicans* (Dècanis, *et al.*, 2009). Moreover, Ghosh *et al.* (2010) showed that farnesol induces the expression of inflammatory cytokines in the macrophage. Therefore, these studies clearly demonstrate that QSMs play complex roles in *C. albicans*-host and *C. albicans*-interspecies interactions.
Figure 1.2. The effect of quorum sensing molecules on the dimorphic transition of *C. albicans*. Tyrosol accelerates hypha formation, while farnesol, tryptophol, and phenylethyl alcohol suppress hypha development.
1.5. *In silico* reconstruction of the central carbon metabolism of *C. albicans* and the biosynthesis of quorum sensing molecules

The central carbon metabolism of most organisms is highly conserved comprising both catabolic and anabolic biochemical reactions. Central carbon metabolism is indispensable for cellular growth and any major cellular event, such as morphogenesis, will certainly be accompanied by significant changes in these important pathways. Therefore, knowledge of the different biochemical pathways involved in the central carbon metabolism of *C. albicans*, as well as how they are affected by the morphogenetic process, is crucial to understand the mechanisms of morphogenesis in this fungus.

*Candida* Genome Database – CGD (http://www.candidagenome.org/), the *Saccharomyces* Genome Database – SGD (http://www.yeastgenome.org/), and the Kyoto Encyclopedia of Genes and Genomes- KEGG (http://www.genome.jp/kegg/) (Figure 1.3).
Figure 1.3. The *in silico* reconstruction of the central carbon metabolism of *C. albicans*. Blue shading indicates the intermediate metabolites of glycolysis and the TCA cycle that act as key precursors for the biosynthesis of amino acids. The intermediates for biosynthesis of individual amino acids are differentiated by different colours and red 3D boxes represent the final amino acid products. Blue 3D boxes indicate quorum sensing molecules derived from aromatic amino acids and the putative genes encoding enzymes are in red text. The few reactions not verified by in vitro studies are indicated by red lines.
1.5.1. Glycolysis

Genes encoding all glycolytic enzymes in *C. albicans* have been identified and are conserved across fungi. Although the mRNA expression of glycolytic genes is not regulated tightly and fluctuates during the yeast-to-hyphal switch (Swoboda, *et al.*, 1994), some differences in the activities of glycolytic enzymes have been correlated with yeast and hyphal growth (Schwartz & Larsh, 1982). For instance, the specific activity of hexokinases in the hyphal form is almost twice than in the yeast form (Schwartz & Larsh, 1982). This might indicate a higher glycolytic flux during hyphal growth. Several glycolytic genes are regulated by signalling pathways associated with morphogenesis. These include enzymes such as hexokinase II (upregulated by Efg1p and downregulated by Ssn6p and Rgt1p) (Doedt, *et al.*, 2004, García-Sánchez, *et al.*, 2005, Sexton, *et al.*, 2007), phosphofructokinase (downregulated by Rgt1p) (Sexton, *et al.*, 2007), glucose-6-phosphate isomerase (upregulated by Efg1p) (Doedt, *et al.*, 2004), fructose-bisphosphate aldolase (upregulated by Efg1p, Gcn4p, Hog1p) (Doedt, *et al.*, 2004, Yin, *et al.*, 2004, Enjalbert, *et al.*, 2006), phosphoglycerate kinase (upregulated by Gcn4p, Hog1p) (Yin, *et al.*, 2004, Enjalbert, *et al.*, 2006), and pyruvate kinase (upregulated by Gcn4p, Hog1p) (Yin, *et al.*, 2004, Enjalbert, *et al.*, 2006). In addition, Shirtliff *et al.* (2009) used a proteomic analysis to demonstrate that several glycolytic enzymes (e.g. glyceraldehyde 3-phosphate dehydrogenase, enolase, phosphoglycerate mutase and pyruvate kinase) were downregulated when *C. albicans* was exposed to farnesol.

1.5.2. Pentose phosphate pathway

In anaerobic respiration, the pentose phosphate pathway is coupled with glycolysis to generate both cytosolic NADPH and ribose. The NADPH provides oxidising energy for biosynthetic reactions (e.g. synthesis of amino acids, fatty acids, and sugar alcohols) while ribose is used in the biosynthesis of nucleotides (e.g. nucleic acids and redox cofactors). The conversion of glucose 6-phosphate to 6-
phosphogluconolactone by a NADP-dependent glucose 6-phosphate dehydrogenase (G6PDH) is the first reaction, and the key-regulatory step, in the pentose phosphate pathway. A putative G6PDH is encoded by the ZWF1 gene in C. albicans and its expression is upregulated by Gcn2p and Gcn4p (Tournu, et al., 2005).

1.5.3. Pyruvate dehydrogenase

In aerobic respiration, pyruvate produced by glycolysis and pentose phosphate pathway is mainly oxidatively decarboxylated to acetyl-CoA by the pyruvate dehydrogenase multi-complex enzyme. This enzyme is highly conserved across species, and the C. albicans genome contains genes with high homology to the S. cerevisiae operon encoding all subunits of pyruvate dehydrogenase. Disruption of one of the pyruvate dehydrogenase subunit genes (PDX1) in C. albicans resulted in a deficiency in filamentous growth (Vellucci, et al., 2007). Thus the availability of acetyl-CoA may be required for the morphogenesis of C. albicans.

1.5.4. TCA cycle

The tricarboxylic acid (TCA) cycle is a central hub of carbon metabolism (Figure 1.3) and, under aerobic conditions, acetyl-CoA formed from pyruvate is oxidized completely to carbon dioxide, water, and chemical energy through this pathway. This process is catalyzed by a series of conserved enzymes. All genes involved in the TCA cycle have been found in the C. albicans genome and all the TCA cycle enzymes have been extracted from C. albicans cells (Rao, et al., 1962). Some TCA cycle enzymes are regulated by signalling pathways. For example, α-ketoglutarate dehydrogenase, succinate dehydrogenase, fumarase, citrate synthase, and malate dehydrogenase are repressed by the Efg1p transcription factor (Doedt, et al., 2004), which appears to be involved in hyphal formation (Stoldt, et al., 1997, Leng, et al., 2001). Citrate synthase is downregulated by Hog1p and cis-aconitase is upregulated by Gcn4p (Enjalbert, et al., 2006). The TCA cycle is, however, amphibolic because the
cycle not only catabolically decarboxylates pyruvate but it also has anabolic functions. Intermediate compounds of the TCA cycle are used as precursors for the biosynthesis of building blocks for the cell (e.g. amino acids and lipids). Therefore, a constant inflow of carbon to supply intermediate compounds for amino acid and lipid biosynthesis is essential for cell growth. An example of a metabolic strategy to maintain the supply of TCA cycle intermediates is the glyoxylate shunt.

1.5.5. Glyoxylate shunt

The glyoxylate shunt assimilates two-carbon molecules such as ethanol and acetate into the TCA cycle; this process not only supplies TCA cycle substrates but also allows microbial growth in environments where only two-carbon compounds are available. The glyoxylate shunt can be considered as a shortcut version of the TCA cycle. The cycle bypasses two decarboxylation steps and directly converts isocitrate to malate using two enzymes: isocitrate lyase (encoded by \textit{ICL1}) and malate synthase (encoded by \textit{MLS1}) (\textbf{Figure 1.3}). The regulation of the glyoxylate shunt is primarily via the activity of isocitrate lyase, which is downregulated by the presence of the quorum sensing molecule farnesol (Enjalbert & Whiteway, 2005) and is usually repressed by glucose in fungi. Although there has been no direct report on the role of the glyoxylate shunt in \textit{C. albicans} morphogenesis, Lorenz & Fink, (2001) showed that the disruption of both \textit{ICL1} and \textit{MLS1} reduced \textit{C. albicans} virulence using a mouse model of intravenous infection.

1.5.6. Amino acid biosynthesis

The biosynthesis of amino acids is achieved through a series of biochemical reactions that can be classified into six distinct pathways. Each amino acid pathway derives its carbon skeleton from a common precursor which is an intermediate compound of glycolysis or the TCA cycle. In \textit{C. albicans}, the glycolytic pathway provides precursors for the biosynthesis of four amino acid families: the histidine,
serine, pyruvate and aromatic families. Only the aspartate and glutamate family precursors are derived from the TCA cycle (Figure 1.3).

1.5.7. Glutamate biosynthesis

Glutamate is synthesized directly from α-ketoglutarate by an NADPH-dependent glutamate dehydrogenase (GDH). This is an important reaction in central carbon metabolism because it serves to assimilate ammonia for the biosynthesis of several other amino acids and at the same time it maintains the redox equilibrium in the cell. The NADH-dependent glutamate dehydrogenase (Gdh2p) favours the catabolic deamination of glutamate to α-ketoglutarate. Therefore, Gdh2p is an important enzyme in the biosynthesis of other amino acids (e.g. aspartate, tyrosine, phenylalanine, etc). Three glutamate dehydrogenase genes have been identified in S. cerevisiae but only GDH2 and GDH3 have orthologues in the C. albicans genome. NADH-dependent GDH2 is transcriptional repressed by Tup1p, Nrg1p and Mig1p (Murad, et al., 2001). NADP-dependent GDH3 is downregulated by Efg1p (Doedt, et al., 2004), and upregulated 2-fold by Rim101p at pH 8 (Bensen, et al., 2004).

1.5.8. Aromatic amino acid and quorum sensing molecule biosynthesis

The discovery that extracellular aromatic alcohols act as quorum sensing molecules during fungal morphogenesis makes aromatic amino acid biosynthesis an important component of the central carbon metabolism regarding morphogenesis. But how the aromatic alcohols are synthesized from aromatic amino acids in C. albicans is still unclear. In contrast, the production of aromatic alcohols in S. cerevisiae has been well characterized. Aromatic alcohols are derived from aromatic amino acid catabolism via the Ehrlich pathway (Felix, 1907). This process in S. cerevisiae involves three consecutive enzymatic steps; a transaminase (encoded by ARO8, ARO9), a decarboxylase (encoded by ARO10), and reduction by alcohol dehydrogenase (encoded by ADH) (Sentheshanmuganathan, 1960, Hazelwood, et al.,
2008). It is proposed that the biosynthesis of aromatic alcohols such as tyrosol, tryptophol, and phenylethyl alcohol in *C. albicans* (Figure 1.2), follows the same biosynthetic pathway as in *S. cerevisiae* (Ghosh, *et al.*, 2008). This study demonstrated that the biosynthesis of aromatic alcohols by *C. albicans* decreased when the *ARO80* gene, which encodes a transcriptional activator known to increase the expression of Aro9p and Aro10p in *S. cerevisiae*, was deleted (Ghosh, *et al.*, 2008).

1.5.9. Global amino acid biosyntheses are regulated by GCN4 and RIM101

There are several morphology-associated genes that also regulate multiple amino acid biosynthetic pathways in response to starvation and pH changes. When amino acids are limiting growth, *C. albicans* switches from yeast to filamentous growth (Tripathi, *et al.*, 2002). This phenomenon is regulated by the transcriptional activator Gcn4p in an *EFG1*-dependent fashion (Tripathi, *et al.*, 2002). The double deletion *gcn4/gcn4* blocks amino acid starvation from inducing morphogenesis, but the *efg1/efg1* knockout mutants fail to express this *GCN4* phenotype (Tripathi, *et al.*, 2002). Gcn4p is not only involved in the dimorphic switch of *C. albicans*, but it also acts as a global regulator of metabolism in response to amino acid starvation (Tripathi, *et al.*, 2002, Tournu, *et al.*, 2005). Tripathi *et al.* (2002) found from DNA microarray analysis that, under amino acid starvation conditions, Gcn4p up-regulates numerous enzymes involved in amino acid biosynthesis, except those responsible for the biosynthesis of cysteine, glycine, alanine, aspartate and glutamine (Table 1.3) (Tournu, *et al.*, 2005). Rim101p is a pH-response regulator that is activated at alkaline pH (Li & Mitchell, 1997). In turn, Rim101p regulates the expression of pH-response regulators (Phr1p and Phr2p) involved in the pH-dependent morphogenesis of *C. albicans* (Ramon, *et al.*, 1999). Bensen *et al.* (2004) compared transcriptional profiles between *rim101/rim101* null-mutants and the wild type strain. They found that Rim101p upregulates arginine, glutamine, and histidine.
biosynthetic enzymes at pH 8, while asparagine and histidine biosynthetic enzymes are downregulated at pH 4, respectively (Table 1.3).

The in silico reconstruction of central carbon metabolic pathways based on the C. albicans genome (Figure 1.3) enables us to integrate post-genomic studies into a metabolic network that helps elucidate the physiological and biochemical mechanisms governing the morphological switch of C. albicans.
1.6. Central carbon metabolism, virulence, and morphogenesis of *C. albicans*

In additional to its role in morphogenesis, central carbon metabolism has increasingly been recognized for its importance in fungal pathogenicity. It has been shown that the deletion of glycolytic transcriptional regulators (*TYE7, GAL4*), a gluconeogenic gene (*FBP1*), glyoxylate cycle genes (*ICL1, MLS1*), or a β-oxidation gene (*FOX2*), attenuates the virulence of *C. albicans* in a murine model of systemic infection (Lorenz & Fink, 2001, Piekarska, et al., 2006, Ramirez & Lorenz, 2007, Askew, et al., 2009). Interestingly, *C. albicans* appears to modulate these carbon assimilatory pathways during infection. When glucose is at similar levels to that found in the bloodstream there is an activation of glycolysis and repression of both the glyoxylate cycle and gluconeogenesis in *C. albicans* (Barelle, et al., 2006, Rodaki, et al., 2009). In contrast, several transcriptomic studies have demonstrated that following the phagocytosis of *C. albicans* by neutrophils or macrophages, there is a downregulation of glycolysis, and an upregulation of the glyoxylate cycle, gluconeogenesis, and β-oxidation (Rubin-Bejerano, et al., 2003, Lorenz, et al., 2004, Fradin, et al., 2005). Since all these metabolic pathways involve the utilization of non-fermentable carbon sources (e.g. amino acids, ethanol, acetate, and fatty acids), it seems likely that *C. albicans* can alter its central carbon metabolism to utilize alternative carbon sources in response to host defences or to changes in the environment, and this metabolic reprogramming is important for the virulence of *C. albicans*. Although the morphological switch from yeast-to-hyphae is observed after *C. albicans* is engulfed by macrophages, Lorenz et al. (2004) suggested that those underling metabolic changes are independent from morphogenesis.

Lorenz et al. (2004) also demonstrated that genes for L-arginine biosynthesis and degradation were upregulated after *C. albicans* cells were engulfed by
macrophages, and other groups have shown that concentrations of CO$_2$ greater than 50 % (v/v) enhanced filamentous growth (Sims, 1986, Mock, et al., 1990, Klengel, et al., 2005, Bahn & Mühlschlegel, 2006). Ghosh et al. (2009) have proposed a mechanism, involving arginine, which connects these two observations. They suggest that arginase (Car1p) converts arginine to urea, which in turn is cleaved into NH$_3$ and CO$_2$ by the amidolyase enzyme (Dur1,2p [sic]). They show that the $dur1,2/dur1,2$ null mutant cannot form germ tubes in macrophages, and that the phenomenon of arginine-induced hyphal formation is not observed in $efg1/efg1$ strains. Therefore, once inside a macrophage, it is proposed that $C. albicans$ produces arginine and arginine degradation produces CO$_2$. The CO$_2$ may then activate adenylyl cyclase (Cdc35p), which in turn activates the cAMP-PKA signalling pathway and Efg1p to induce the yeast-to-hyphal transition (Figure 1.1) and thereby $C. albicans$ escapes from the macrophage (Klengel, et al., 2005).
1.7. The effect of metabolic genes on the utilization of carbon sources

There are several metabolic genes associated with morphogenesis or virulence that seem to affect the ability of *C. albicans* to utilize carbon sources. Firstly, trehalose-6-phosphate synthase (*TPS1*) is a gene involved in the first step of trehalose biosynthesis and it regulates glucose influx (Ernandes, *et al.*, 1998). The *tps1/tps1* null mutant is unable to grow on glucose or fructose as a sole carbon source but this mutant is capable of growing on other carbon sources such as galactose or glycerol (Zaragoza, *et al.*, 1998). In addition, this mutant is impaired in hyphal formation (Zaragoza, *et al.*, 1998). Another gene related to trehalose biosynthesis is the cell wall-linked acid trehalase (*ATC1*) (Pedreño, *et al.*, 2004). The *atc1/atc1* null-mutant is incapable of growing on exogenous trehalose as sole carbon source and the mutant has diminished capacity to form hyphae in various media (e.g. serum, spider medium and Lee’s medium) (Pedreño, *et al.*, 2007).

Galactose metabolism also plays a role in the morphogenesis of *C. albicans*. UDP-galactose-4-epimerase (*GAL10*) is a key enzyme in galactose metabolism that converts UDP-galactose into UDP-glucose. The glucose generated can then be fed into glycolysis as a carbon and energy source. The *gal10/gal10* null-mutant is unable to grow on galactose as sole carbon source (Singh, *et al.*, 2007). Compared to wild type, the mutant also exhibits increased hyphal formation in rich media, Lee’s medium and spider medium (Singh, *et al.*, 2007). Recently, Hgt4p has been reported as a galactose and glucose sensor. Indeed, the *hgt4/hgt4* null-mutant cannot grow on either glucose or galactose in the presence of the respiration inhibitor antimycin A (Brown, *et al.*, 2006, Brown, *et al.*, 2009). The *hgt4/hgt4* mutant is hypofilamentous and less virulent in a mouse model of disseminated candidiasis (Brown, *et al.*, 2006).
In addition, carnitine biosynthesis and acetyl-CoA metabolism can affect the ability of *C. albicans* to utilize different carbon sources. Carnitine is an essential metabolite that acts as a shuttle to transport fatty acid and acetyl group between intracellular compartments. Mutants lacking carnitine synthetic enzymes (e.g. trimethyl-lysine dioxygenase, trimethylaminobutyraldehyde dehydrogenase, and butyrobetaine dioxygenase) or lacking carnitine acetyltransferase, which transfers the acetyl group from acetyl-CoA to carnitine, are unable to utilize non-fermentable carbon sources such as fatty acids, acetate, or ethanol as sole carbon sources (Zhou & Lorenz, 2008, Strijbis, et al., 2009). Moreover, Carman et al. (2008) have shown that a *C. albicans* mutant strain with acetyl-CoA synthase (*ACS2*) deleted was unable to utilize glucose, acetate, or ethanol but the mutant cells were viable when given fatty acids or glycerol. The same authors deleted acetyl-CoA hydrolase (*ACH1*), an enzyme which hydrolysis acetyl-CoA to acetate, but this strain was fully virulent in a mouse model of disseminated candidiasis.
1.8. *In silico* reconstruction of the sterol biosynthetic pathway and farnesol biosynthesis in *Candida albicans*

The sterol biosynthesis pathway generates compounds necessary for the maintenance of cellular structure. These sterols, such as ergosterol in fungi and cholesterol in mammalian cells, are essential for membrane integrity and permeability. The sterol biosynthesis pathway is targeted by several antifungal agents. In addition, sterol biosynthesis is potentially involved in the quorum sensing of dimorphic fungi because this pathway generates farnesol, the most well-characterised quorum sensing molecule involved in *C. albicans* morphogenesis (Hornby et al., 2003, Nickerson, et al., 2006).

Adopting the same approach we used for the central carbon metabolism, we have reconstructed, *in silico*, the *C. albicans* sterol biosynthetic pathway (*Figure 1.4*). The first step in sterol biosynthesis is the condensation of acetyl-CoA and acetoacetyl-CoA into 3-hydroxy-3-methylglutaryl-CoA by hydroxymethylglutaryl-CoA synthase. Seven sequential reactions are then involved in the synthesis of farnesyl pyrophosphate, which is the precursor for the biosynthesis of farnesol. Surprisingly, the enzyme that synthesizes farnesol has not been fully described, but Hornby et al. (2003) claimed to have extracted an enzyme from *C. albicans* possessing this activity. Fungal and mammalian cells share a common sterol biosynthetic pathway from acetyl-CoA through farnesyl pyrophosphate to zymosterol (*Figure 1.4*). From this point there are different pathways in fungi and mammals. 24-C-methyltransferase converts zymosterol into fecosterol leading to the biosynthesis of ergosterol in fungi, whereas 24-dehydrocholesterol reductase directs sterol biosynthesis into the production of cholesterol which serves as a precursor for steroid hormones (e.g.
andro gens and estrogens) in mammalian cells. In general, the biosynthesis of ergosterol is conserved between \textit{C. albicans} and \textit{S. cerevisiae}.

\textbf{Figure 1.4.} The \textit{in silico} reconstruction of the sterol pathway of \textit{C. albicans} and mammalian cells. Farnesol, which suppress germ-tube formation in \textit{C. albicans}, is produced via the sterol pathway, but some enzymes involved in its biosynthesis are still to be identified. Mammals use the same pathway to synthesise estrogens, which promote germ-tube formation in \textit{C. albicans}. 
Interestingly, the presence of estrogens has been reported to have a profound effect on the morphogenesis of dimorphic fungi (White & Larsen, 1997, Cheng, et al., 2006). Estrogen may contribute to the gender bias in human infection by another dimorphic and pathogenic fungus, Paracoccidioides brasiliensis, for which, contrary to C. albicans, the yeast form is the pathogenic state. In healthy individuals, P. brasiliensis infects mainly adult males (Borges-Walmsley, et al., 2002). Estrogen has been shown to suppress the hyphal-to-yeast transition in P. brasiliensis and, thus, it has been proposed that adult women are less prone to infection by P. brasiliensis due to higher levels of estrogen in their bodies compared to males (Borges-Walmsley, et al., 2002).

On the other hand, healthy women often develop recurrent vaginal candidosis, especially during pregnancy (Tarry, et al., 2005). Clinical studies have shown that women are more likely to have symptomatic Candida infections when estrogen levels are high (Tarry, et al., 2005), and estrogen has been shown to promote germ-tube formation in C. albicans (White & Larsen, 1997, Cheng, et al., 2006) which is the first step in the yeast-to-hypha transition (Whiteway & Bachewich, 2007). Low estrogen levels may be one reason why healthy adult males do not commonly develop candidosis.

Therefore estrogen, which seems to promote Candida infection in women, protects them from P. brasiliensis infection because this hormone induces the filamentous growth of both fungi. What is intriguing from the metabolic point of view is the fact that estrogens are synthesized by mammalian cells via the sterol biosynthetic pathway, which is used by fungi to synthesize farnesol (Nickerson, et al., 2006). Thus, the sterol pathway appears to have an important role in both fungal morphogenesis and in pathogenesis.
1.9. The integration of quorum sensing and central carbon metabolism in *C. albicans*

QSMs are products of the central carbon metabolism of *C. albicans*. They function as communication signals and coordinately regulate the growth of *C. albicans* cells, in response to cell density. Their production, however, may simply reflect the flux within the central carbon metabolism pathways and their accumulation and signalling may provide feedback to these pathways to optimise growth under the particular environmental conditions. For example, if carbon and nitrogen sources are available to the cells this may stimulate flux though the pathways for the biosynthesis of QSMs such as farnesol that will repress the filamentous growth of *C. albicans* and, if carbon and/or nitrogen sources become scarce, flux through other pathways involved in the biosynthesis of QSMs that accelerate hypha formation, such as tyrosol, may be activated.

The metabolic hubs of central carbon metabolism are metabolites that take part in more than twenty different pathways and include pyruvate, NADH, NADPH, and ATP (Villas-Bôas, *et al.*, 2007). Thus, central carbon metabolism is highly interconnected and forms a metabolic network that responds very quickly to any environmental change. This is the part of cell metabolism most likely to include metabolic reactions involved in cell responses to QSMs in the environment. Indeed, several observations support the idea that central carbon metabolism provides the carbon sources for the synthesis of QSMs. As discussed above, farnesol, tyrosol, tryptophol, and phenethyl alcohol are derived from glycolytic products. Aromatic QSMs are derived from phosphoenolpyruvate and farnesol is derived from acetyl-CoA. Therefore, if these molecules are synthesised as part of central carbon metabolism, their concentrations are likely to affect the flux distribution in these
primary metabolic pathways by suppressing or inducing specific metabolic reactions. In addition, N-acyl homoserine lactones, which are common QMSs produced by many bacteria (e.g. *Pseudomonas corrugata*, *Erwina carotovora* and others (Schaefer, *et al.*, 1996, Dong, *et al.*, 2001, Licciardello, *et al.*, 2007)), are synthesized directly from methionine, which is derived from oxaloacetic acid in the TCA cycle, and are thus also part of central carbon metabolism. Moreover, the production of QSMs seems to be affected by the availability of their precursors. For instance, the presence of aromatic amino acids increases the level of tyrosol, tryptophol, and phenylethyl alcohol in *C. albicans* (Ghosh, *et al.*, 2008).

Despite the obvious connection between quorum sensing and central carbon metabolism, the role of primary metabolism in the mechanism of quorum sensing has not been investigated. We hypothesize that once extracellular QSMs reach a certain concentration, their passive diffusion across the cell membrane could affect the expression of specific genes or the activity of specific enzymes involved in morphogenesis. Indeed, fluorescently labelled farnesol analogs supplied to *C. albicans* cultures have been found in the cytoplasm of *C. albicans* (Shchepin, *et al.*, 2003). Thus, QSMs could rapidly up- and down-regulate particular metabolic pathways (e.g. aromatic amino acid biosynthesis and sterol biosynthesis), redistributing the metabolic flux of carbon through central carbon metabolism and inducing the morphological switch by altering the biosynthesis of cell wall components. Several transcriptomics studies have shown that the metabolic pathways from central carbon metabolism are highly affected by the presence of quorum sensing molecules (Table 1.3). Therefore, the study on the effect of QSMs on *C. albicans* morphogenesis in the context of central carbon metabolism has the potential to unravel important metabolic mechanisms underlying the morphogenetic process, and thus, should be pursued further.
1.10. Final remarks

The ability to study the behaviour of biological systems *in vivo* under different environmental conditions has increased with recent developments in genomics and post-genomic tools. Gene knockout experiments can demonstrate the involvement of individual genes in biological processes, but often it is unclear how the genes mediate their effects. Proteomic studies can confirm that changes in transcription associated with gene disruption, or environmental changes, result in altered protein expression. The integration of gene knockout, transcriptomic and proteomic studies has elegantly elucidated signalling pathways involved in *C. albicans* morphogenesis. This still, however, only gives a linear view of morphogenesis. This review has used the *C. albicans* genome data to reconstruct the network of metabolic pathways involved in central carbon, sterol, and QSM metabolism. This will provide a framework which can guide the interpretation of metabolomic data and thus generate the fluxome and a more *three-dimensional* appreciation of how QSMs and environmental cues mediate their effects on morphology.

Morphogenesis is considered to be an important virulence factor for *C. albicans* and other dimorphic fungi. Central carbon metabolism and sterol biosynthesis not only supply carbon and lipid sources to generate the building blocks of new cellular structures in response to morphogenesis, but also supply precursors for the biosynthesis of the quorum sensing molecules involved in cell-cell communication and the coordinated dimorphic transition in a population of *C. albicans* cells. Together, the pre-existing genomic sequences and the *in silico* reconstruction of *C. albicans* biochemical pathways should enable a powerful systems biology study of this fungus that would be the cornerstone to assist in the elucidation of the metabolic mechanisms responsible for *C. albicans* morphogenesis and virulence.
1.11. References


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CHAPTER  I


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CHAPTER I


CHAPTER II

Metabolome analysis during the morphological transition of *Candida albicans*

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The content of this chapter has been published

2.1. Abstract

*Candida albicans* is an opportunistic pathogen of humans with significant mortality in severely immunocompromised patients. The ability to switch from yeast to hyphal morphology and vice-versa, in response to various environmental cues, is believed to be a critical virulence factor of this fungus. However, the mechanisms that recognize such environmental signals and trigger the morphological change at a system level are still not clearly understood. Therefore, we have compared the metabolite profiles of *C. albicans* cells growing under different hyphae-inducing conditions to the metabolite profiles of growing yeast cells. Surprisingly our results suggest an overall downregulation of cellular metabolism during the yeast to hyphal morphological transition. Among the metabolic pathways involved in the central carbon metabolism, we have found seventeen that were significantly downregulated in all three hyphae-inducing conditions. This indicates that these central carbon metabolic pathways are likely to be intrinsically involved in the downstream effects of the morphogenetic process.
2.2. Introduction

*Candida albicans* is the major fungal pathogen of humans capable of causing systemic infections in severely immunocompromised patients. The ability to change from yeast to filamentous growth or vice versa, in response to certain environmental cues, enhances *C. albicans* adaptation and survival in the course of infection (Romani, et al., 2003). Despite different morphological forms being found during infection, yeast cells are often associated with dissemination through the bloodstream to colonize internal organs (Bendel, et al., 2003, Saville, et al., 2003), while the filamentous forms are believed to be more suited to penetrate the host mucosa and epidermal surfaces (Rooney & Klein, 2002) as well as to escape from macrophages after phagocytosis (Arai, et al., 1977, Lorenz, et al., 2004).

When the yeast-to-hypha transition is triggered upon phagocytosis of *C. albicans* by neutrophils or macrophages, there is evidence that genes involved in glycolysis are immediately down-regulated while those in the glyoxylate cycle, gluconeogenesis and β-oxidation pathways are upregulated (Rubin-Bejerano, *et al.*, 2003, Lorenz, *et al.*, 2004, Fradin, *et al.*, 2005, Fernández-Arenas, *et al.*, 2007). Since all these upregulated pathways are involved in the catabolism of non-fermentable carbon sources, the *C. albicans* cells appear to undergo carbon starvation when engulfed by macrophages or neutrophils. Other studies show that this upregulation of catabolic pathways not only allows *C. albicans* to survive in an environment with poor carbon sources, it may also affect the morphogenetic process. For example, a key intermediate in the metabolism of non-fermentable carbon sources is acetyl-CoA. When genes encoding enzymes involved in the biosynthesis, or intracellular shuttle, of acetyl-CoA are deleted, such as acetyl CoA synthase (*ACS2*) and carnitine acetyltransferase (*CTN1, CTN2, CAT2*), the resulting mutants are unable to utilize non-fermentable carbon sources such as lactate, acetate, or ethanol as a sole carbon source (Carman, *et al.*, 2008, Zhou & Lorenz, 2008). The mutants lacking *CTN3* and *CAT2* also demonstrate reduced hyphal formation (Prigneau, *et al.*, 2004, Strijbis, *et al.*, 2008). Furthermore, *C. albicans* mutants incapable of metabolizing trehalose, galactose, glycerol, or fructose as sole carbon sources are also unable to form hyphae under hyphal-inducing conditions (Zaragoza, *et al.*, 1998, Pedreño, *et al.*, 2004, Singh, *et al.*, 2007). Despite these studies showing the importance of central carbon metabolism to the morphogenetic process, the over-arching changes in the primary metabolism of *C. albicans* in response to morphogenesis-inducing cues have not been studied.

Metabolomics and fluxomics are the most appropriate approaches to investigate the global changes in the central carbon metabolism of *C. albicans* during morphogenesis. Metabolomics, the latest addition of functional genomics tools,
aims at analyzing all low-molecular weight compounds produced or modified by a biological system (Villas-Bôas, et al., 2005b). Metabolites are not gene products, but their concentrations are affected by the activities of gene products (enzymes) and the availability of precursors (Villas-Bôas, et al., 2007). Because signaling can use existing receptors and enzymes to change fluxes within a rigid metabolic network (Raamsdonk, et al., 2001), the metabolome responds more rapidly to environmental changes than the transcriptome and proteome. For example, when a glucose-limited culture of *Saccharomyces cerevisiae* is perturbed by glucose, a distinct change of glycolytic metabolite levels can be observed within 3 seconds after glucose addition (Visser, et al., 2004), whilst the first significant change in gene expression is visible only after 2 minutes (Kresnowati, et al., 2006). The metabolome is also expected to amplify the changes occurring in the transcriptome and in the proteome (Kell, et al., 2005). Furthermore, the morphogenesis of *C. albicans* is strongly influenced by metabolites such as farnesol, tyrosol, phenylethyl alcohol, tryptophol, estradiol, dodecanol, and different types of carbon and nitrogen sources (Lingappa, et al., 1969, Holmes & Shepherd, 1987, Hornby, et al., 2001, Oh, et al., 2001, Chen, et al., 2004, Hogan, et al., 2004, Maidan, et al., 2005a, Cheng, et al., 2006). Thus, the study of changes in the metabolome in response to environmental signalling has great potential to assist in the elucidation of the fungal morphogenetic process.

Therefore, we have compared the intracellular and extracellular metabolite profiles of *C. albicans* cells growing under different hyphae-inducing conditions to the metabolite profiles of growing yeast cells using GC-MS based metabolomic methods.
2.3. Methods

2.3.1. The rationale behind our experimental design

The metabolite levels of microbial cells will change according to environmental conditions and medium composition. Likewise, *C. albicans* morphogenesis is regulated by environmental conditions and medium composition. Therefore, the challenge for our metabolomics study of *C. albicans* morphogenesis was to differentiate changes in metabolite levels resulting from environmental changes from those changes associated specifically with the morphogenetic process. Thus, we chose to fix the key environmental conditions such as pH, temperature and aeration, and vary the composition of the media to induce morphogenesis. Indeed we used three different hyphae-inducing media and compared each to cells growing in two different media where the yeast form was maintained. Although many changes in the metabolite profile of the cells were related to the different medium compositions, we were able to identify common trends observed in all hyphae-inducing conditions that were independent of the growth media.

2.3.2. Chemicals

Methanol, chloroform, sodium bicarbonate, and sodium hydroxide were obtained from MERCK (Darmstadt, Germany). The internal standard 2,3,3,3-d₄-alanine, the derivatization reagent methylchloroformate (MCF), and pyridine were purchased from Sigma-Aldrich (St. Louis, USA). Anhydrous sodium sulphate was obtained from Fluka (Steinheim, Germany). All chemicals used in this study were of analytical grade.

2.3.3. Fungal strain and culture media

*C. albicans* strain SC5314 (Gillum, *et al.*, 1984) was maintained on YPD containing yeast extract (6 g.L⁻¹), peptone (3 g.L⁻¹), glucose (10 g.L⁻¹), and agar (15
g.L\(^{-1}\)) and incubated at 30 °C. Pre-inocula were prepared in YPD broth medium, with the same composition as the agar plates, but without agar. Five different culture media were used for the metabolomic study: (1) Minimum mineral medium (MM medium) at pH 6.5 containing D-glucose (10 g.L\(^{-1}\)), (NH\(_4\))\(_2\)SO\(_4\) (5 g.L\(^{-1}\)), MgSO\(_4\)⋅7H\(_2\)O (0.5 g.L\(^{-1}\)), KH\(_2\)PO\(_4\) (3 g.L\(^{-1}\)), vitamins and trace metals as previously described (Verduyn, et al., 1992); (2) YPD broth medium at pH 6.5 containing yeast extract (6 g.L\(^{-1}\)), peptone (6 g.L\(^{-1}\)), and glucose (10 g.L\(^{-1}\)); (3) Minimal-proline and N-acetylglucosamine medium (MPA medium), which was the same as the MM medium described above without D-glucose, but supplemented with L-proline (10 mM) and N-acetylglucosamine (2.5 mM) at pH 6.5; (4) Serum medium, which was MM medium without D-glucose, supplemented with bovine serum (10 % v/v) at pH 6.5; and (5) Lee’s medium at pH 6.5 containing D-glucose (12.5 g.L\(^{-1}\)), (NH\(_4\))\(_2\)SO\(_4\) (5 g.L\(^{-1}\)), MgSO\(_4\)⋅7H\(_2\)O (0.2 g.L\(^{-1}\)), KH\(_2\)PO\(_4\) (2.5 g.L\(^{-1}\)), NaCl (5.0 g.L\(^{-1}\)), alanine (0.5 g.L\(^{-1}\)), leucine (1.3 g.L\(^{-1}\)), lysine (1.0 g.L\(^{-1}\)), methionine (0.1 g.L\(^{-1}\)), phenylalanine (0.5 g.L\(^{-1}\)), proline (0.5 g.L\(^{-1}\)), threonine (0.5 g.L\(^{-1}\)), and biotin (0.001 g.L\(^{-1}\)) (Lee, et al., 1975).

2.3.4. Yeast and hyphal growth conditions

*C. albicans* was cultured in 1.4 L of YPD at 30 °C using shake flasks in a rotary shaker overnight. The cells were collected by centrifugation at 2000 g (4 °C) for 5 min and washed in phosphate buffered saline (8 g.L\(^{-1}\) NaCl, 0.2 g.L\(^{-1}\) KCl, 1.44 g.L\(^{-1}\) Na\(_2\)PO\(_4\), 0.24 g.L\(^{-1}\) KH\(_2\)PO\(_4\), at pH 7.5). The cells were resuspended in the five different growth media described above (MM, YPD, MPA, Serum, and Lee’s media) to an initial OD\(_{600}\) of 0.5. The cell suspensions were incubated using shake flasks in a rotary shaker-incubator at 37 °C for 3 h. The morphology of *C. albicans* in each growth medium was monitored using a phase contrast microscope (DMR, Leica).
2.3.5. Sampling and quenching of cell metabolism

Five shake-flasks containing 100 mL of culture from each growth medium were harvested at time zero (right after resuspension), and after 1, 2, and 3 h incubation. For intracellular metabolite analysis, 90 mL of the cultures were rapidly filtered under vacuum (Air Cadet vacuum/pressure station, Thermo), quickly washed with cold saline solution (1-2 °C) and quenched in cold methanol water (50 % v/v) at -30 °C as described by Smart et al. (2010). The remaining 10 mL of microbial cultures were filtered through syringe filters (0.2 μm pore size) to remove the microbial cells, and the filtrate was used for the analysis of extracellular metabolites.

2.3.6. Sample preparation for metabolite analysis

The intracellular metabolites were extracted from the quenched cell pellets using cold methanol water and freeze-thaw cycles following the protocol described by Smart et al. (2010). The Internal standard 2,3,3,3-d4-alanine (0.3 μmol/sample) was added to each sample before extraction. The intracellular metabolite extracts and 1 mL of spent culture medium containing extracellular metabolites were freeze-dried (BenchTop K manifold freeze dryer, VirTis) before chemical derivatization.

2.3.7. Chemical derivatization of metabolites

The freeze-dried samples were derivatised using the methyl chloroformate (MCF) method according to the protocol described by Smart et al. (2010). In summary, the freeze-dried samples were resuspended in 200 μL of sodium hydroxide solution (1 M) and transferred to silanised glass tubes, then mixed with 167 μL of methanol and 34 μL of pyridine. The derivatisation began by adding 20 μL of MCF followed by vigorously mixing for 30 s, and then a further 20 μL of MCF was added followed by vigorously mixing for 30 s. To separate MCF derivatives from the reactive mixture, 400 μL of chloroform was added and vigorously mixed for 10 s followed by the addition of 400 μL of sodium bicarbonate solution (50 mM), and
mixing for an additional 10 s. The aqueous layer was removed and dehydrated with anhydrous sodium sulphate before samples were transferred to GC-MS vials.

2.3.8. Gas chromatography-mass spectrometry (GC-MS) analysis

The MCF derivatives were analysed in an Agilent GC7890 system coupled to a MSD5975 mass selective detector (EI) operating at 70 eV. The column used for all analyses was a ZB-1701 GC capillary column (30 m x 250 μm id x 0.15 μm with 5 m guard column, Phenomenex). The analysis parameters were set according to Smart at al. (2010). Samples were injected under pulsed splitless mode with the injector temperature at 290 °C. The helium gas flow through the GC-column was set at 1.0 mL.min⁻¹. The GC-oven temperature was initially held at 45 °C for 2 min. Then the temperature was raised with a gradient of 9 °C.min⁻¹ until it reached 180 °C and was held for 5 min. Then the temperature ramped at 40 °C.min⁻¹ until it reached 220 °C and was held for 5 min. Then the temperature was ramped at 40 °C.min⁻¹ until it reached 240 °C and was held for 11.5 min. Lastly, the temperature was ramped at 40 °C.min⁻¹ until it reached 280 °C and was held for 2 min. The interface temperature was set to 250 °C and the quadrupole temperature was 200 °C.

2.3.9. Biomass quantification

The cell debris collected after intracellular metabolite extraction was dried by using a microwave (250 W for 20 min) and weighed in order to estimate the biomass (dry weight) content of each sample.

2.3.10. Data mining, data normalization, and data analysis

AMDIS software was used for deconvoluting GC-MS chromatograms and identifying metabolites using an in-house MCF mass spectra library. The identifications were based on both MS spectrum of the derivatised metabolite and its respective chromatographic retention time. The relative abundance of identified
metabolites was determined by ChemStation (Agilent) by using the GC base-peak value of a selected reference ion. These values were normalised by the biomass content in each sample as well as by the abundance of internal standard (2,3,3,3-d₄-alanine). A univariate analysis of variance (ANOVA) was applied to determine whether the relative abundance of each identified metabolite was significantly different between growth conditions. Our Pathway Activity Profiling (PAPi) algorithm (Aggio, et al., 2010) was used to predict and compare the relative activity of different metabolic pathways in C. albicans during the growth conditions tested. This programme connects to the KEGG online database (http://www.kegg.com) and uses the number of metabolites identified from each pathway and their relative abundances to predict which metabolic pathways are likely to be active in the cell. The entire data mining, data normalisation and pathway activity predictions were automated in R software as described by Smart et al. (2010) and Aggio et al. (2010).

2.3.11. Quantification of intracellular ATP pools

The intracellular pool of ATP was determined by using the luminescence ATP detection assay ATPlite™ (PerKinElmer). This assay measures light emission caused by the enzymatic reaction between ATP and luciferin-luciferase. Media without cells were used to determine the assay background signal for each medium. Three technical replicates of 100 μL culture were collected from each flask (n = 5) after 3 h of incubation. The cells were lysed and the total ATP content was quantified according to the manufacturer’s instructions using ATP standard curves obtained from media spiked with ATP standard solutions (one calibration curve for each medium). Light emission was measured using a bioluminescence plate reader (Envision™, PerkinElmer).
2.4. Results

2.4.1. Hyphae-induction in \textit{C. albicans}

To study the metabolomic changes associated with \textit{C. albicans} morphogenesis, we cultivated \textit{C. albicans} in three different hyphae-inducing media (MPA, serum, and Lee’s) for three hours and compared their morphology to cells grown in yeast form in two different media (MM and YPD). Microscopic examination revealed that at least 95% of cells developed hyphae in hyphae-inducing media. Some pseudohyphae (thick hyphae with constrictions at the septa or chains of elongated yeast cells) were also present in Lee’s medium. On the other hand, approximately 100% of cells maintained the yeast form in MM and YPD media after three hours incubation (\textit{Table 2.1} and \textit{Figure 2.1}). We observed a significant increase in the biomass of \textit{C. albicans} cultures growing in serum, Lee’s, and YPD media, whilst the cultures growing in MM, or MPA media showed just a small increase in biomass during three hours of incubation. This was expected since bovine serum, YPD broth, and Lee’s medium, unlike the other media, contained several free amino acids and peptides that are easily metabolised, promoting faster growth (Eisman, \textit{et al.}, 2006).
Table 2.1
Biomass and morphology of *C. albicans* cells cultured in different growth media.

<table>
<thead>
<tr>
<th>Media</th>
<th>Time</th>
<th>1 hour Biomass (mg.mL⁻¹)</th>
<th>1 hour F (%)</th>
<th>2 hour Biomass (mg.mL⁻¹)</th>
<th>2 hour F (%)</th>
<th>3 hour Biomass (mg.mL⁻¹)</th>
<th>3 hour F (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MM</td>
<td>1 hour</td>
<td>0.33</td>
<td>0</td>
<td>0.41</td>
<td>0</td>
<td>0.48</td>
<td>0</td>
</tr>
<tr>
<td>YPD</td>
<td>1 hour</td>
<td>0.32</td>
<td>0</td>
<td>0.62</td>
<td>0</td>
<td>1.18</td>
<td>0</td>
</tr>
<tr>
<td>MPA</td>
<td>1 hour</td>
<td>0.27</td>
<td>17</td>
<td>0.30</td>
<td>77</td>
<td>0.35</td>
<td>95</td>
</tr>
<tr>
<td>Serum</td>
<td>1 hour</td>
<td>0.34</td>
<td>45</td>
<td>0.63</td>
<td>86</td>
<td>1.06</td>
<td>96</td>
</tr>
<tr>
<td>Lee’s</td>
<td>1 hour</td>
<td>0.39</td>
<td>51</td>
<td>0.66</td>
<td>94</td>
<td>1.14</td>
<td>99</td>
</tr>
</tbody>
</table>

*MM* Minimum mineral medium; *YPD* Yeast extract, peptone, dextrose medium; *MPA* Minimum medium without glucose, supplemented with proline and *N*-acetylglucosamine; *F* percentage of filamentous growth determined by counting the number of yeast cells and filaments in 1 mm³ volume.
Figure 2.1. The morphology of *C. albicans* cells incubated under different growth conditions at 37 °C for 3 h. (A) Minimum mineral medium (MM); (B) Yeast peptone dextrose broth medium (YPD); (C) Proline, *N*-acetylglucosamine medium (MPA); (D) Lee’s medium; (E) Serum medium. The Images were taken by Nomarksi contrast microscopy with 100x magnification.
2.4.2. The metabolite profile of *C. albicans* during morphogenesis

We detected over a hundred GC-MS peaks in *C. albicans* intracellular samples, and seventy-five of them were identified using our in-house MCF MS library (Table 2.2). Metabolites identified included a range of intermediates from central carbon metabolism, such as phosphoenolpyruvate, pyruvate, 2-oxoglutarate, oxaloacetate, and others. By comparing the concentration of intracellular metabolites in yeast and hyphal forms, nineteen intracellular metabolites were detected at higher concentrations in hyphae samples from all time points compared to yeast cells (Figure 2.2). For example, aromatic amino acids such as phenylalanine and tyrosine increased their intracellular abundances during the yeast-to-hypha transition. The concentrations of fatty acids such as caprinate and myristate also increased during the morphological transition. The intracellular level of proline also increased in all hyphae-inducing conditions independently of the growth media. Interestingly, TCA cycle intermediates such as malate and succinate were detected at higher intracellular levels in hyphal growth. By examining the intracellular metabolite profiles of yeast and hyphal cells over time, we observed a gradual increase in the global metabolite levels over the three hours of hyphal induction in comparison with yeast cells (Figure 2.2). The higher concentration of intracellular metabolites during hyphal formation indicates that the flux through the respective pathway in which those metabolites play a part must have decreased in comparison to cells growing in the yeast form (Aggio et al. 2010), which directly reflects the altered metabolic states between yeast and hyphal cells. On the other hand, due to the different complexity of the three media used to induce hyphal formation, it was not possible to identify extracellular metabolites that consistently showed significant changes in concentration during morphogenesis in all hyphae-inducing media (data not shown). Therefore, we decided to focus our metabolomics study solely on the profile of intracellular metabolites.
Table 2.2
Intracellular metabolites identified in *C. albicans* cells grown in different growth media.

<table>
<thead>
<tr>
<th>Classification of metabolites</th>
<th>#</th>
<th>Intracellular metabolites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acids and their isoforms</td>
<td>20</td>
<td>Alanine, asparagine, aspartate, cysteine, glutamine, glutamate, glycine histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine, and β-alanine.</td>
</tr>
<tr>
<td>Amino acid derivatives</td>
<td>12</td>
<td>Adenosyl-L-methionine, creatinine, cystathionine, N-acetylglutamate, norleucine, norvaline, trans-4-hydroxyproline, ornithine, S-adenosyl-L-homocysteine, 2-aminoacetate, δ-hydroxylysine, and pyroglutamate.</td>
</tr>
<tr>
<td>TCA cycle intermediates</td>
<td>7</td>
<td>Fumarate, citrate, succinate, cis-aconitate, malate, 2-oxoglutarate, and oxaloacetate.</td>
</tr>
<tr>
<td>Fatty acids</td>
<td>12</td>
<td>Adipate, caprate, caproate, caprylate, D-2-aminoadipate, L-2-amino-adipate, myristate, oleate, pentadecanoate, 14-methylpentadecanoate, stearate, and 3-hydroxyoctanoate.</td>
</tr>
<tr>
<td>Glycolytic intermediates</td>
<td>2</td>
<td>Pyruvate and phosphoenolpyruvate.</td>
</tr>
<tr>
<td>Cofactors and vitamins</td>
<td>3</td>
<td>NADP/NADPH, nicotinate, and 4-amino-n-butyrate.</td>
</tr>
<tr>
<td>Others</td>
<td>19</td>
<td>Benzoate, carbamate, citraconate, citramalate, hydroxybenzoate, glutarate, glycerate, itaconate, lactate, levulinate, malonate, phthalate, 2-isopropylmalate, 2-hydroxybutyrate, 10,13-dimethyltetradecanoate, 2,4-tert-butylphenol, 4-aminobenzoate, 3-methyl-2-oxopentanoate, and 10,12-octadecadienoate.</td>
</tr>
<tr>
<td>Total # of identified metabolites</td>
<td></td>
<td>75</td>
</tr>
</tbody>
</table>
Figure 2.2. Metabolite levels extracted from *C. albicans* cells cultured on different growth media. MM medium ( ); YPD medium ( ); Lee’s medium ( ); MPA medium ( ); and Serum medium ( ). The abundances of metabolites were normalised relative to the internal standard and biomass of each sample, and standard deviations are illustrated by vertical line ranges. Only the metabolites with statistically significant differences (p < 0.05) between both yeast (MM and YPD media) and all hyphal growth (MPA, serum, and Lee’s media) are shown. Note, missing lines mean metabolite levels below detection limits (not-detected).
Predicting metabolic pathway activities in *C. albicans* during the morphological transition

The profile of intracellular metabolites was used to compare the activity of metabolic pathways in *C. albicans* cells during the yeast-to-hypha transition. Surprisingly, we observed that the majority of the metabolic pathways were downregulated during this transition (Figure 2.3). Although a few pathways were upregulated during the first hour of hyphal induction (e.g. fatty acid metabolism), almost all other metabolic pathways related to the metabolites detected in our samples were increasingly downregulated during morphogenesis. To further validate this observation, we quantified the total intracellular pool of ATP in cells from each growth condition. We found a significantly lower concentration of ATP in *C. albicans* cells growing under all hyphae-inducing conditions tested compared to cells growing as yeast (Figure 2.4), which also indicates a global downregulation of cell primary (central carbon) metabolism in hyphal cells.
Figure 2.3. Activities of *C. albicans* metabolic pathways based on intracellular metabolomic data from cells under different hypha-inducing conditions, at various time points, relative to the pathways in yeast cells. The activity scores (AS) for each pathway were calculated using our Pathway Activity Profiling (PAPi) algorithm (Aggio, *et al*., 2010). PAPi calculates an AS for each metabolic pathway listed in KEGG database based on the number of metabolites identified from each pathway and their relative abundances. As a result, the AS represents
the likelihood that a metabolic pathway is active in the cell and, consequently, allows the comparison of metabolic pathway activities using metabolite profile data. \textit{C. albicans} were grown in the following media: \(-\sim\) MM medium; \(\sim\) YPD medium; \(\sim\) Lee’s medium; \(\sim\) MPA medium; and \(\sim\) Serum medium. Related pathways are grouped together according to their cellular metabolism and only pathways with statistically significant differences in activity \((p < 0.05\ \text{by ANOVA})\) are shown.

\textbf{Figure 2.4.} ATP concentrations in \textit{C. albicans} cells incubated under different growth conditions at 37 °C for 3 h. The ATP pool in cells grown under hypha-inducing conditions \((\text{MPA} - \text{minimum proline N-acetylglucosamine medium}; \text{LEE} - \text{Lee’s medium}; \text{Serum} - 10\% \text{ bovine serum medium})\) were all significantly lower than those in cells grown as yeast in minimal mineral medium (MM) and yeast extract, peptone, dextrose broth (YPD) \((P < 0.001)\).
2.4.4. Metabolic changes most likely associated with morphogenesis

Since each hypha-inducing condition involved a different medium that was distinct from the media in which *C. albicans* grew in the yeast form, it is difficult to determine which metabolic pathway was changed due to the morphogenetic process *per se* rather than due to metabolism of different nutrients. Therefore, in order to identify metabolic changes most likely related to morphogenesis, we selected those metabolic pathways that showed significant differences during the yeast-to-hypha transition in all hyphae-inducing media across the three hours of incubation. Seventeen metabolic pathways appeared to have been downregulated in all three hypha-inducing conditions compared to yeast growth (Figure 2.5). Thus, these pathways are likely to be associated with the morphogenesis of *C. albicans* because the transition from yeast to filamentous growth was the only common feature between these three growth conditions. Fourteen of the identified pathways were related to amino acid metabolism. Two pathways, CoA biosynthesis and arginine metabolism, have previously been implicated in the morphogenetic process occurring when *C. albicans* is engulfed by macrophages through transcriptomics and gene-knockout mutagenesis studies (Lorenz & Fink, 2001, Ghosh, *et al.*, 2009). On the other hand, this is the first time that the downregulation of nitrogen, nicotinate, and nicotinamide metabolism has been associated with *C. albicans* morphogenesis.

Nitrogen metabolism not only serves to assimilate and excrete nitrogen–containing compounds, but it also maintains the balance between reduced and oxidized states of NADH, NADPH, and FADH$_2$. Nicotinate and nicotinamide metabolism produces NAD$^+$ through the recycling of compounds containing nicotinamide for the generation of NAD$^+$ and by *de novo* synthesis of NAD$^+$ from tryptophan or aspartate. Subsequently, NAD$^+$ is phosphorylated into NADP$^+$ by NAD$^+$ kinase (Lehninger, 2005). These cofactors can affect the activity of many enzymes in the cell.
Figure 2.5. Metabolic pathways consistently changed in all hyphal growth conditions. Only seventeen metabolic pathways showed significant differences in activity under all hypha-inducing conditions compared with the yeast growth at each of the time points. These metabolic pathways are involved in central carbon metabolism, which highlights the importance of primary metabolism in the regulation of hypha formation. The columns are separated according to growth medium: (A) MM medium (yeast growth); (B) YPD medium (Yeast growth); (C) Lee’s medium (hyphal growth); (D) MPA medium (hyphal growth); and (E) Serum medium (hyphal growth). The rows list the metabolic pathways arranged according to hierarchical clustering analysis. The levels of metabolic pathway activities are indicated by red and green colour intensities. Deeper red colours indicate higher metabolic activities and deeper green colours mean lower metabolic activities. All metabolic activities have been centred in the row direction and scaled to have a mean of zero and a standard deviation of one (Row Z-score). The colour key on the top right is superimposed by a histogram that counts all the metabolic activities.
Integrating the seventeen downregulated metabolic pathways into a metabolic network enabled us to visualise the pathways connecting the central carbon metabolites detected in our samples (Figure 2.6). The alanine asparate and glutamate metabolism, glycine serine and threonine metabolism, and arginine and proline metabolism appear to be the most connected pathways within the network. Furthermore, pyruvate, aspartate and glutamate could be considered as metabolite hubs linking all three pathways together during the morphogenetic process. Coincidently, glutamate and aspartate are also important intermediates in nitrogen metabolism since *C. albicans* can assimilate external ammonia into glutamate and through transamination to form aspartate. Thus, these metabolites act as key intermediates distributing nitrogen sources into different metabolic pathways. Moreover, the network demonstrates that aspartate, nicotinate, and fumarate may act as intermediary metabolites linking nicotinate and nicotinamide metabolism to other pathways. These metabolites could be important precursors for the biosynthesis of NADH and NADPH during *C. albicans* morphogenesis. Therefore, this metabolic network provides a framework that can guide the biological interpretation of metabolite profiles, and provide hypotheses to explain morphogenesis that can be tested in future studies.
Figure 2.6. Metabolic network illustrating how the seventeen morphogenesis-related metabolic pathways (Figure 2.4) are interconnected. Metabolic pathways have been connected via identified metabolites represented by red dots. Blue arrows indicate that the metabolites were detected at a higher level in hyphal growth. Dashed lines indicate multiple hidden steps in that pathway. The names of the pathway are as follow: (1) arginine and proline metabolism, (2) β-alanine metabolism, (3) butanoate metabolism, (4) C5 branched dibasic acid metabolism, (5) cysteine and methionine metabolism, (6) D-alanine metabolism, (7) D-glutamine and D-glutamate metabolism, (8) glutathione metabolism, (9) glycine, serine and threonine metabolism, (10) histidine metabolism, (11) nicotinate and nicotinamide metabolism, (12) nitrogen metabolism, (13) pantothenate and CoA Biosynthesis, (14) pyruvate metabolism, (15) paline, leucine and isoleucine biosynthesis, (16) alanine, aspartate and glutamate metabolism, and (17) purine metabolism. The metabolites labelled by letters are: (a) N-acetylglutamate; (b) S-adensyl-L-homocystein; (c) 2-isopropylmalate; (d) valine; (e) S-3-methyl-2-oxopentanoate; (f) isoleucine; (g) leucine; and (h) S-adenosyl-L-methionine.
2.5. Discussion

The general downregulation of cellular metabolism during the yeast-to-hypha transition was unexpected. Transcriptional upregulation of metabolic pathways seems to be the common trend during biofilm formation (García-Sánchez, et al., 2004, Yeater, et al., 2007), in which the majority of the cellular structures consist of filamentous forms. However, these biofilms were often sampled at 16 to 48 h after hyphal induction, when the cells had become established as a complex community. Besides, Montserrat et al. (2009) demonstrated that yeast cells, hyphae, and biofilms display different cytoplasmic protein profiles. Comparing yeast cells and hyphae, 67 of the 82 identified cytoplasmic proteins were less abundant in the hyphal form (Montserrat, et al., 2009). Our metabolomic results confirm their findings, and we demonstrated that hyphae seem to be less metabolically active than yeast cells.

We have detected a significantly higher concentration of intracellular metabolites per dry weight of hyphal biomass than for yeast biomass (Figure 2.2). Also, growth on serum and Lee’s media resulted in a greater increase in C. albicans biomass (Table 2.1). Therefore, how could a global downregulation of metabolic pathways result in higher levels of intracellular metabolites and still present increased biomass production? Villas-Bôas et al. (2005a) showed that a higher concentration of intracellular metabolites does not always correlate with an increase in biomass. S. cerevisiae cells grown aerobically had a significantly lower concentration of intracellular metabolites and increased biomass production. Furthermore, in this study the C. albicans cultures growing on MPA medium (Table 2.1), also showed downregulation of most metabolic pathways despite showing just a small increase in biomass within three hours just like the control culture growing on MM in the yeast form. Therefore, the increased level of intracellular metabolites
in *C. albicans* seems to be independent of the increase in biomass. Nonetheless, increases in intracellular metabolite levels could indicate a decrease in some biosynthetic activity due to the cells not using some metabolic intermediates, which is in agreement with a global downregulation of the central carbon metabolism. Furthermore, ATP is the key currency of intracellular energy transfer within biological systems, thus its concentration directly reflects the metabolic state of the central carbon metabolism. Decrease in the ATP pool of a cell indicates a decrease in metabolic activity of the central carbon metabolism ([Figure 2.4](#)), which correlates with our metabolomics results and PAPi hypotheses (Aggio, *et al.*, 2010).

We postulate that the prioritization of sugar metabolism in yeast cells is the underlying reason why yeast cells exhibit a higher level of central carbon metabolic activities than hyphae. The metabolism of sugars generates more energy for the cell to perform complex biosynthetic process. Therefore, when energy is available, macromolecules, such as proteins, lipids, and carbohydrates are easily synthesised (Walker, 1998). Indeed, several studies indicate that in *C. albicans* the switch from yeast to hyphal growth is associated with the switch from aerobic to anaerobic respiration (Land, *et al.*, 1975, Aoki & Ito-kuwa, 1982). The cells assimilate diverse alternative carbon sources in absence of glucose (Rubin-Bejerano, *et al.*, 2003, Lorenz, *et al.*, 2004, Fradin, *et al.*, 2005, Fernández-Arenas, *et al.*, 2007), which could be easier to be convert into certain hyphal macromolecules such as the biosynthesis of cell wall chitin directly from external N-acetylglucosamine. Therefore, hyphal growth could, indeed, be less metabolically (energetically) demanding to the fungal cells when growing in carbon or nitrogen poor environment or under anaerobiosis.

Thus, we hypothesise that once the metabolic activity of yeast cells is temporarily disrupted by environmental changes, such as starvation, stress, alkaline pH, depletion in oxygen and high temperatures, the central carbon metabolism of *C. albicans* is globally downregulated. This downregulation of the central carbon
metabolism triggers changes to a growth form that demands less energy. This explains why *C. albicans* undergoes hypha formation when it is starved for several hours and then exposed to poor carbon or nitrogen sources (Holmes & Shepherd, 1987, Holmes & Shepherd, 1988). Moreover, *C. albicans* yeast cells that colonize host mucosa and epithelial surfaces will be in an environment where glucose is scarce, but complex carbohydrates, proteins, and lipids are present. Such cells often form hyphae and penetrate the epithelia.

On the other hand, linking our metabolomics data to pre-existing transcriptomics and proteomics data on morphogenesis of *C. albicans* has presented a challenge. Although there are many transcriptomics and proteomics studies that have examined *C. albicans* morphogenesis, the majority of the studies has utilized mutants with deleted or overexpressed genes involved in signalling cascades or encoding transcriptional regulators (Murad, *et al.*, 2001, Bensen, *et al.*, 2004, Doedt, *et al.*, 2004, Yin, *et al.*, 2004, García-Sánchez, *et al.*, 2005, Tournu, *et al.*, 2005, Enjalbert, *et al.*, 2006, Zacchi, *et al.*, 2010, Stichternoth, *et al.*, 2011). The disruption of any of these genes would interfere not only with morphogenesis, but also with many other essential cellular processes. Recently, Monteoliva *et al.* (2011) investigated the proteome profiles of *C. albicans* yeast and hyphal cells using Lee’s medium at pH 4.3 or 6.6 to obtain yeast or hyphal forms respectively. This means that it is uncertain whether changes in protein levels were the consequence of different pH conditions or morphogenesis. However, they revealed a significantly lower amount of cytosolic proteins in hyphae involved in respiration, the pentose phosphate pathway, and biosynthetic pathways for vitamins, cofactors, purines, nucleotides, glutamate, isoleucine, leucine, and valine. These results are in general agreement with our findings. In addition, the emphasis placed by the authors on the downregulation of redox processes during hypha formation also supports our findings. Here, we have shown that the cellular metabolism of nitrogen, and
nicotinate and nicotinamide were all downregulated while *C. albicans* was growing under hyphal-inducing conditions. These pathways are known to play an important role in the oxidative-reduction processes of the cell.
2.6. **Conclusions**

In this study, we have used metabolomics to present evidence of global metabolic changes in the central carbon metabolism of *C. albicans* during the yeast-to-hypha morphological transition. We have observed a global downregulation of cellular metabolism during hypha formation. These results highlight the importance of the regulation of central carbon metabolism in the morphogenesis of *C. albicans*, as an immediate downstream response to environmental signals. Therefore, the current understanding of morphogenesis can be further elucidated by investigating the key central carbon metabolic pathways identified in this study.
2.7. Acknowledgements

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CHAPTER II


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CHAPTER III

The metabolic response of Candida albicans to farnesol under hyphae-inducing conditions

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The content of this chapter has been published

3.1. Abstract

Farnesol is a quorum-sensing molecule (QSM) produced, and sensed, by the polymorphic fungus, *Candida albicans*. This cell-to-cell communication molecule is known to suppress the hyphal formation of *C. albicans* at high cell density. Despite many studies investigating the signalling mechanisms by which QSMs influence the morphogenesis of *C. albicans*, the downstream metabolic effect of these signalling pathways in response to farnesol-mediated morphogenesis remains obscure. Here we have used metabolomics to investigate the metabolic response of *C. albicans* upon exposure to farnesol under hyphae-inducing conditions. We have found a general upregulation of central carbon metabolic pathways when hyphal formation was suppressed by farnesol evidenced by a considerably larger number of central carbon metabolic intermediates detected under this condition at an overall lower intracellular level. By combining the metabolic profiles from farnesol-exposed cells with previous metabolomics data for *C. albicans* undergoing morphogenesis, we have identified several metabolic pathways that are likely to be associated with the morphogenetic process of *C. albicans* as well as metabolic pathways such as those involved in lipid metabolism that appeared to be specifically affected by farnesol. Therefore, our results provide important new insights into the metabolic role of farnesol on *C. albicans* metabolism.
3.2. Introduction

*Candida albicans* is a commensal, opportunistic, fungal pathogen that is commonly found in the normal human flora. This fungus causes little disease in the healthy population but can result in fatal infections in immunocompromised patients. Indeed *C. albicans* has been attributed as the fourth leading cause of mortality in nosocomial bloodstream infections of severely immunocompromised patients (Pfaller & Diekema, 2007, Moran *et al.*, 2010).

The ability to rapidly switch from budding yeasts to hyphal growth and vice-versa in response to environmental factors is regarded as an important virulence factor for *C. albicans* (Whiteway & Oberholzer, 2004). The morphological change of *C. albicans* is triggered by environmental signalling. The yeast-to-hyphal development can be induced by N-acetylglucosamine, proline, serum, and starvation (Reynolds & Braude, 1956, Holmes & Shepherd, 1987, Maidan *et al.*, 2005, Han *et al.*, 2011). Furthermore, a population of *C. albicans* cells has the capacity to use chemical signals in order to co-ordinate a synchronous morphogenetic process. *C. albicans* is known to produce several quorum sensing molecules (QSMs) that regulate morphogenesis, of which farnesol is the best characterised (Albuquerque & Casadevall, 2012). Farnesol has been shown to stop germ tube formation in the late stage of biofilm development, as well as under other hyphal-inducing conditions (e.g. when grown on N-acetylglucosamine, proline, or serum) (Hornby *et al.*, 2001, Alem *et al.*, 2006) but it is unable to block the elongation of pre-existing hyphae (Mosel *et al.*, 2005, Navarathna *et al.*, 2005).

After farnesol was first isolated from *C. albicans* cultures by Hornby *et al.* (2001), a number of studies have investigated the molecular mechanisms by which QSMs influence *C. albicans* morphogenesis. For example, Sato *et al.* (2004) showed, using RT-PCR analysis, that farnesol suppresses the *HST7* and *CPH1* gene expression.
involved in MAP kinase cascades. Furthermore, a stress-activated protein kinase (Hog1p) was also found to be phosphorylated and accumulate in the nucleus in response to farnesol (Smith, et al., 2004). cDNA microarray analysis showed that some genes related to morphogenesis are down-regulated (e.g. CRK1 and PDE2) while others are up-regulated (e.g. TUP1) in C. albicans biofilms exposed to farnesol (Cao, et al., 2005). Davis-Hanna et al. (2008) suggested that farnesol stops hypha formation by repressing the RAS1-cAMP signalling pathway. Kebaara et al. (2008) demonstrated that farnesol suppressed hyphal growth by upregulating the TUP1 gene (a transcriptional repressor) since it no longer stops germ tube formation in tup1/tup1 and nrg1/nrg1 null mutants. Román et al. (2009) revealed that farnesol prevents the phosphorylation of Cek1p, part of the MAPK signal transduction pathway. And recently, Hall et al. (2011) showed that farnesol inhibited cAMP-PKA cascade via the inactivation of adenylyl cyclase (CYR1p). Together, these studies suggest that farnesol inhibits germ tube formation by repressing both MAPK and cAMP PKA signalling pathways and by stimulating the expression of hyphal-suppressor genes such as TUP1 and HOG1. However, the metabolic effect of farnesol on the central carbon metabolism of C. albicans, which is downstream to signalling pathways, is yet to be studied. Therefore, in this work we have employed a gas chromatography-mass spectrometry (GC-MS) based metabolomics approach to investigate the metabolic response of C. albicans cells to farnesol under hyphae-inducing conditions.
3.3. Methods

3.3.1. Chemical

Methanol, chloroform, sodium bicarbonate, and sodium hydroxide were obtained from MERCK (Darmstadt, Germany). The internal standard 2,3,3,3-d4-alanine, the derivatisation reagent methylchloroformate (MCF), and pyridine were purchased from Sigma-ALdrich (St. Louis, USA). Anhydrous sodium sulphate and farnesol were obtained from Fluka (Steinheim, Germany). All chemicals used in this study were of analytical grade.

3.3.2. Fungal strain and culture media

C. albicans strain SC5314 was maintained on YPD agar medium containing yeast extract (6 g.L⁻¹), peptone (3 g.L⁻¹), glucose (10 g.L⁻¹), and agar (15 g.L⁻¹) ; at 30 °C. C. albicans cells were grown in minimum mineral medium (MM medium) at pH 5.5 containing D-glucose (10 g.L⁻¹), (NH₄)₂SO₄ (5 g.L⁻¹), MgSO₄.7H₂O (0.5 g.L⁻¹), KH₂PO₄ (3 g.L⁻¹), vitamins and trace metals as previously described (Verduyn, et al., 1992).

3.3.3. Culture conditions

C. albicans was cultured in 250 mL of MM medium (pH 5.5) at 30 °C in a rotary shaker overnight. The cells were collected by centrifugation at 2000 g (4 °C) for 5 min and washed in phosphate buffered saline (8 g.L⁻¹ NaCl, 0.2 g.L⁻¹ KCl, 1.44 g.L⁻¹ Na₂PO₄, 0.24 g.L⁻¹ KH₂PO₄, at pH 7.5). The cells were resuspended in 20 shake-flasks containing 100 mL of MM medium at pH 7.5 (to induce filamentous growth) to an initial OD₆₀₀ of 0.2. Five flasks were supplemented with farnesol (1 mM) and another five flasks were used as control. The cell suspensions were incubated in a rotary shaker at 37 °C for 6 h. The morphology of C. albicans in each growth medium was monitored using a phase contrast microscope (DMR, Lecia).
3.3.4. Sampling and quenching of cell metabolism

Five flasks for each growth condition were harvested after 3 and 6 h incubation. A portion (10 mL) of the cultures was filtered (0.2 μm pore size) to remove the *C. albicans* cells, and the filtrate was used for the analysis of extracellular metabolites. The remaining 90 mL of culture were rapidly filtered under vacuum (Air Cadet vacuum/pressure station, Thermo), quickly washed with cold saline solution (1-2 °C) and quenched in cold methanol/water (1:1 v/v) at -30 °C as described by Smart *et al.* (2010).

3.3.5. Sample preparation for metabolite analysis

The intracellular metabolites were extracted from the quenched cell pellets using cold methanol/water and freeze-thaw cycles following the protocol described by Smart *et al.* (2010). The internal standard 2,3,3,3-d₄-alanine (0.3 μmol) was added to each sample before extraction. The intracellular metabolite extracts and 1 mL of spent culture medium containing extracellular metabolites were freeze-dried (BenchTop K manifold freeze dryer, VirTis) before chemical derivatisation.

3.3.6. Chemical derivatisation of metabolites

The freeze-dried samples were derivatised using the methyl chloroformate (MCF) method according to the protocol described by Smart *et al.* (2010). In summary, the freeze-dried samples were resuspended in 200 μL sodium hydroxide (1 M) and transferred to a silanised glass tube, then mixed with 167 μL methanol and 34 μL pyridine. The derivatisation began by adding 20 μL MCF followed by vigorously mixing for 30 s, and then a further 20 μL MCF was added followed by vigorously mixing for 30 s. To separate MCF derivatives from the reactive mixture, 400 μL chloroform was added and vigorously mixed for 10 s followed by the addition of 400 μL sodium bicarbonate (50 mM), and mixing for an additional 10 s. The
aqueous layer was removed and dehydrated with anhydrous sodium sulphate before samples were transferred to GC-MS vials.

3.3.7. Gas chromatography-mass spectrometry (GC-MS) analysis

The MCF derivatives were analysed in an Agilent GC7890 system coupled to a MSD5975 mass selective detector (EI) operating at 70 eV. The column used for all analyses was a ZB-1701 GC capillary column (30 m x 250 μm id x 0.15 μm with 5 m guard column, Phenomenex). The analysis parameters were set according to Smart et al. (2010). Samples were injected under pulsed splitless mode with the injector temperature at 290 °C. The helium gas flow through the GC-column was set at 1 mL min⁻¹. The interface temperature was set to 250 °C and the quadrupole temperature was 200 °C.

3.3.8. Biomass quantification

The cell debris collected after intracellular metabolite extraction was dried using a domestic microwave (250 W for 20 min) and weighed in order to estimate the total biomass content (dry weight) of each sample.

3.3.9. Data mining, data normalization, and data analysis

AMDIS software was used for deconvoluting GC-MS chromatograms and identifying metabolites using our in-house MCF mass spectra library. The identifications were based on both MS spectrum of the derivatised metabolite and its respective chromatographic retention time. The relative abundance of identified metabolites was determined by ChemStation (Agilent) by using the GC base-peak value of a selected reference ion. These values were normalised by the biomass content in each sample as well as by the abundance of internal standard (2,3,3,3-d₄-alanine). A univariate analysis of variance (ANOVA) was applied to determine whether the relative abundance of each identified metabolite was significantly
different between growth conditions. Our Pathway Activity Profiling (PAPi) algorithm (Aggio, et al., 2010) was used to predict and compare the relative activity of different metabolic pathways in *C. albicans* during the growth conditions tested. This programme connects to the KEGG online database (http://www.kegg.com) and uses the number of metabolites identified from each pathway and their relative abundances to predict which metabolic pathway is likely to be active in the cell. The entire data mining, data normalisation and pathway activity predictions were automated in R software as described in Smart et al. (2010) and Aggio et al. (2010). Graphical representations of the results were generated by ggplot2 R packages (Wickham, 2009).
3.4. Results

3.4.1. Suppression of hyphae formation by farnesol

To investigate the metabolomic changes associated with hyphal-suppression by farnesol, we cultivated *C. albicans* in hyphae-inducing conditions supplemented with farnesol for 6 h. Microscopic examination showed that farnesol (1 mM) completely inhibited hyphal formation under the growth condition tested, whilst at least 95% of cells underwent filamentous growth when farnesol was not present (Figure 3.1 and Table 3.1). At the first sampling point (3 h), the biomass concentration was roughly equal in both culture media (with or without farnesol). However, after 6 h incubation, cultures growing in the absence of farnesol had significantly higher biomasses (Table 3.1).
Figure 3.1. The morphology of *C. albicans* cells incubated in the presence or absence of farnesol (1 mM) at 37 °C after 3 or 6 h incubation. MM is minimum mineral medium. The Images were taken by Nomarksi contrast microscopy with 1000x magnification.

Table 3.1. Biomass and morphology of *C. albicans* cells cultured in different growth media.

<table>
<thead>
<tr>
<th>Medium</th>
<th>3 hours</th>
<th>6 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Biomass (mg.mL(^{-1}))</td>
<td>F (%)</td>
</tr>
<tr>
<td>MM</td>
<td>0.39</td>
<td>&gt;95</td>
</tr>
<tr>
<td>MM + Farnesol (1 mM)</td>
<td>0.33</td>
<td>0</td>
</tr>
</tbody>
</table>

MM: minimum mineral medium; F: percentage of filamentous growth determined by counting the number of yeast cells and filaments in 1 mm\(^3\) volume.
3.4.2. The effect of farnesol on the extracellular metabolite profile of *C. albicans*

We detected over 50 GC-MS peaks in extracellular *C. albicans* samples, and 29 of them were identified using our in-house MS library (Table 3.2). The concentrations of 22 metabolites appeared to be significantly different between samples from cells exposed to farnesol and those not exposed to farnesol (Figure 3.2). Interestingly, After 3 h incubation with farnesol, the concentrations of extracellular metabolites secreted into the medium were low when compared to those for cells growing in the absence of farnesol except for caprinate and EDTA (EDTA was part of the medium composition and could be uptaken by the cells). These metabolites consisted of various TCA cycle intermediates (e.g.; fumarate, succinate), amino acids (e.g.; alanine, glutamate, proline, and valine), fatty acids (e.g.; caprinate, oleate), and quorum sensing molecules (e.g.; phenylethyl alcohol).
Table 3.2. *C. albicans* metabolites identified in different culture media.

<table>
<thead>
<tr>
<th>Classification of metabolites</th>
<th>Intra</th>
<th>Extra</th>
<th>Metabolites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acids</td>
<td>19</td>
<td>6</td>
<td>Alanine&lt;sup&gt;a&lt;/sup&gt;, asparagine, aspartate, cysteine, glutamine, glutamate&lt;sup&gt;a&lt;/sup&gt;, glycine&lt;sup&gt;a&lt;/sup&gt;, histidine, isoleucine, leucine, lysine, phenylalanine, proline&lt;sup&gt;a&lt;/sup&gt;, serine, threonine, tryptophan, tyrosine, valine&lt;sup&gt;a&lt;/sup&gt;, and β-alanine&lt;sup&gt;a&lt;/sup&gt;.</td>
</tr>
<tr>
<td>Amino acid derivatives</td>
<td>7</td>
<td>0</td>
<td>Creatinine, cystathionine, N-acetylglutamate, norvaline, ornithine, 2-aminobutyrate, and pyroglutamate.</td>
</tr>
<tr>
<td>TCA cycle intermediates</td>
<td>6</td>
<td>4</td>
<td>Fumarate&lt;sup&gt;a&lt;/sup&gt;, citrate&lt;sup&gt;a&lt;/sup&gt;, succinate&lt;sup&gt;a&lt;/sup&gt;, cis-aconitate, malate, and 2-oxoglutarate&lt;sup&gt;a&lt;/sup&gt;.</td>
</tr>
<tr>
<td>Fatty acids</td>
<td>10</td>
<td>4</td>
<td>Caprinate&lt;sup&gt;a&lt;/sup&gt;, caprylate, Docosanoate, D-2-amino adipate, myristate, oleate&lt;sup&gt;a&lt;/sup&gt;, pentadecanoate, 14-methylpentadecanoate&lt;sup&gt;a&lt;/sup&gt;, stearate&lt;sup&gt;a&lt;/sup&gt;, and 3-hydroxyoctanoate.</td>
</tr>
<tr>
<td>Glycolytic intermediates</td>
<td>2</td>
<td>1</td>
<td>Pyruvate&lt;sup&gt;a&lt;/sup&gt; and phosphoenolpyruvate.</td>
</tr>
<tr>
<td>Cofactors and vitamins</td>
<td>3</td>
<td>1</td>
<td>NADP/NADPH, nicotinate&lt;sup&gt;a&lt;/sup&gt;, and 4-amino-n-butyrate.</td>
</tr>
<tr>
<td>Others</td>
<td>13</td>
<td>8</td>
<td>Benzoate&lt;sup&gt;a&lt;/sup&gt;, cabamate&lt;sup&gt;a&lt;/sup&gt;, citraconate&lt;sup&gt;a&lt;/sup&gt;, citramalate&lt;sup&gt;a&lt;/sup&gt;, eicosanoate, itaconate, lactate&lt;sup&gt;a&lt;/sup&gt;, malonate, 2-isopropylmalate&lt;sup&gt;a&lt;/sup&gt;, 2-hydroxybutyrate, 10, 13-dimethyltetradecanoate&lt;sup&gt;a&lt;/sup&gt;, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-benzene propanoic acid&lt;sup&gt;a&lt;/sup&gt;, and 10,12-octadecadienoate.</td>
</tr>
<tr>
<td>Metabolites only found in extracellular media</td>
<td>0</td>
<td>5</td>
<td>Glyoxylate/glyoxalate&lt;sup&gt;a&lt;/sup&gt;, Phenylethyl Alcohol&lt;sup&gt;a&lt;/sup&gt;, 2,4-bis(1,1-dimethylethyl)-phenol&lt;sup&gt;a&lt;/sup&gt;, EDTA&lt;sup&gt;a&lt;/sup&gt;, and 2-methoxysuccinate&lt;sup&gt;a&lt;/sup&gt;.</td>
</tr>
</tbody>
</table>

*Intra: Number of intracellular metabolites identified; Extra: Number of extracellular metabolites identified; *Metabolites found extracellularly. Glyoxylate/glyoxalate, phenylethyl Alcohol, 2,4-bis(1,1-dimethylethyl)-phenol, EDTA, 2-methoxysuccinate were only found extracellularly.*
Figure 3.2. Relative concentration of extracellular metabolites in the presence and absence of farnesol after 3 and 6 h incubation. Farnesol treated samples (suppression of hyphae) are indicated by blue triangles (△) and non-farnesol treated samples by red round dots (☉). Standard deviations are illustrated by vertical lines. The relative concentrations of identified metabolites have been normalised by internal standard (d₄-alanine) and biomass before the relative concentrations of the corresponding metabolites found in un-inoculated culture medium were subtracted. The line y = 0 distinguishes secretion of metabolites (positive values) from consumption of metabolites from the medium (negative values). Only the metabolites detected with statistically significant ANOVA (p-value < 0.05) are shown.
3.4.3. The effect of farnesol on the intracellular metabolite profile of *C. albicans*

From a total of 60 metabolites identified in intracellular samples of both farnesol-treated and untreated *C. albicans* cells, the concentrations of 34 metabolites were affected significantly by the presence of farnesol (Table 3.2 and Figure 3.3). Most intracellular metabolites were detected at lower levels in samples from cultures supplemented with farnesol. Myristate (a saturated fatty acid) showed the greatest concentration decrease in response to farnesol (> 3 fold). Phenylalanine, isoleucine, glutamate, aspartate, leucine, 2-isopropylmalate, dimethyl-myristate, and methyl-isopalmitate also showed significant decreases in response to farnesol. On the other hand, tryptophan and histidine were detected intracellularly at significant higher concentrations in response to farnesol (Figure 3.3). Other metabolites such as 2-aminobutyrate, ornithine, tyrosine, NADP/NADPH, citramalate and cystathionine, were present at increased concentrations in samples harvested after 3 h incubation in the presence of farnesol, but not after 6 h. Cysteine, however, showed different trends in samples taken at 3h compared to those taken after 6 h incubation in the presence of farnesol (Figure 3.3). After 3 h, cysteine was detected at higher concentrations in samples from cells cultured in the presence of farnesol, while at 6 h the level of cysteine was lower in cells exposed to farnesol.
Figure 3.3. The relative intracellular metabolite concentrations for *C. albicans* cells grown in the presence or absence of farnesol after 3 and 6 h incubation. Metabolite concentrations are given relative to samples from cultures grown without farnesol, using a log$_2$ scale. Deeper red colours (positive values) indicate that metabolite concentrations were increased in response to farnesol while green shades (negative values) indicate decreased concentrations. The colour key on the top right is superimposed with a histogram that counts the relative concentrations of all the metabolites. Only the metabolites for which there was a statistically significant change in concentration (p-value < 0.05) are shown.
3.4.4. Effect of farnesol, and the suppression of hyphal formation, on the metabolic pathways of *C. albicans*

We analysed the concentrations of identified intracellular metabolites of *C. albicans* cells grown in the presence or absence of farnesol with PAPi software (Aggio et al. 2010) to generate a comparative metabolic activity profile (Figure 3.4). The metabolic activities of *C. albicans* cells growing under suppression of hyphae formation by farnesol were compared with those of cells growing without farnesol (filamentous growth). Of the 41 metabolic pathways that showed significant changes in metabolic activity in response to farnesol, 34 were up-regulated (Figure 3.4). In particular, the pathways associated with nitrogen metabolism, acetyl-CoA biosynthesis, fatty acid metabolism (e.g. biosynthesis of unsaturated/saturated fatty acids, fatty acid elongation in mitochondria, and metabolism of linoleic acid), nicotinate and nicotinamide metabolism, and amino acid metabolism (e.g. alanine, β-alanine, aspartate, cysteine, glutamate, histidine, isoleucine, leucine, lysine, methionine, D-alanine, valine), appeared up-regulated after both 3 and 6 h incubation in the presence of farnesol. On the other hand, one pathway that was down-regulated in response to farnesol was tryptophan metabolism, which is coincidently associated with the production of tyrosol, another quorum sensing molecule – but one which stimulates hyphal formation in *C. albicans* (Alem, et al., 2006). Only the biosynthesis of aromatic amino acids such as phenylalanine and tyrosine appear to be downregulated when cells were exposed to farnesol for 3 h, but they were upregulated after 6 h in the presence of farnesol. Interestingly, we observed that the majority of the pathways associated with central carbon metabolism and energy metabolism were significantly upregulated after 6 h of exposure to farnesol. These pathways encompass the TCA cycle, glycolysis/gluconeogenesis, glyoxylate/dicarboxylate metabolism, and oxidative phosphorylation (Figure 3.4).
Figure 3.4. *C. albicans* metabolic pathway activities predicted from intracellular metabolite profiling data. The metabolic pathway activities relative to the samples from cultures grown without farnesol are presented using a log₂ scale. Deeper red colours (positive values) indicate pathways that were up-regulated in response to farnesol while green shades (negative values) indicate a downregulation in metabolic activity. The colour key on the top right is superimposed with a histogram that counts all the pathway activities. Only the metabolic pathways for which there was a statistically significant change in activity (p-value < 0.05) are shown.
3.5. Discussions

We have observed an overall upregulation of metabolic pathways when the hyphal formation of *C. albicans* was suppressed by farnesol (Figure 3.4). This result is in general agreement with our previous study that demonstrated a global downregulation of central carbon metabolism during early hyphal growth of *C. albicans* confirmed by lower ATP formation in filamentous growing cells (Han, *et al.*, 2012). There are several reports showing an increase in mRNA and protein expression in *C. albicans* in response to farnesol (Cho, *et al.*, 2007, Shirtliff, *et al.*, 2009). In general, the upregulated genes and proteins are involved in the TCA cycle, glycolysis, gluconeogenesis, acetyl-CoA pathway, amino acid biosynthesis, nitrogen metabolism, or they are heat shock proteins/chaperones, that protect the cells against environmental and oxidative stress. Although these studies suggest that cells are likely to be more metabolically active in response to farnesol, another study by Deveau *et al.* (2011) found, using Alamar Blue, that farnesol reduced the metabolic activity of *C. albicans* during biofilm formation. However, yeast cells, hyphae and biofilms are in different metabolic states (Montserrat, *et al.*, 2009) and, indeed, proteomic profiles of cells during early hypha formation and biofilm formation appear to be significantly different (Montserrat, *et al.*, 2009). Nonetheless, filamentous cells present a much lower metabolic activity when compared to cells growing as yeast (Han, *et al.*, 2012).

By comparing the metabolic pathways that were affected by farnesol treatment with the 17 metabolic pathways identified in our previous study as being closely associated with the morphogenetic process (Han, *et al.*, 2012), we were able to short-list 9 metabolic pathways that appear to be up-regulated under farnesol exposure (3 h and 6 h) and down-regulated under hyphae-inducing conditions (Figure 3.5). These pathways include the metabolism of alanine, β-alanine, D-
alanine, aspartate, cysteine, C5-branched dibasate, histidine, glutamate, methionine, nicotinate/nicotinamide, nitrogen, and acetyl-CoA biosynthesis. Indeed, Cho et al. (2007) analysed the global transcription profile of *C. albicans* in response to farnesol in *N*-acetylglucosamine and proline medium. They found that genes encoding enzymes involved in amino acid biosynthesis, acetyl-CoA biosynthesis, and nitrogen metabolism were upregulated. Pathways involving the metabolism of butanoate, D-glutamine, D-glutamate, and pyruvate were only found to be differentially expressed when *C. albicans* cells were grown in the presence of farnesol for 3 h and under hyphae-inducing conditions (Figure 3.5). Whereas, metabolism of arginine, proline, glycine, threonine, purine, and biosynthesis of isoleucine, leucine, valine were found to be differentially expressed after 6 h incubation in the presence of farnesol and under hyphae-inducing conditions (Figure 3.5). Therefore, farnesol appears to suppress germ tube formation by upregulating amino acid metabolism, nitrogen metabolism, CoA biosynthesis, and nicotinate/nicotinamide metabolism.
Figure 3.5. Venn diagram to illustrate the metabolic pathways of *C. albicans* that are up-regulated and down-regulated (labelled with superscript *a*) when hyphal growth was suppressed by exposure to farnesol for 3 h or 6 h or under different filamentous growth conditions. Metabolic pathways that respond to farnesol are derived from Figure 3.4. Metabolic pathways that respond to hyphae-inducing conditions are derived from Han *et al.* (2012). The figures in brackets indicate the number of metabolic pathways.
Furthermore, farnesol seems to affect the lipid metabolism and central carbon metabolism independently from morphogenesis. We observed that the majority of the pathways associated with lipid metabolism were significantly upregulated after 3 h incubation with farnesol and detected an upregulation of central carbon metabolism and energy metabolism in response to farnesol exposure for 6 h (Figure 3.4). These metabolic responses are likely to be specific for farnesol since none of these pathways appeared to be differentially expressed under the three hyphae-inducing conditions we tested previously (Han, et al., 2012). Farnesol, at high concentrations, has been reported to induce apoptosis in other fungi, bacteria, mammalian cells, and *C. albicans* itself (Semighini, et al., 2006, Fairn, et al., 2007, Scheper, et al., 2008, Liu, et al., 2009, Shirtliff, et al., 2009) but also protects *C. albicans* from oxidative stress (Westwater, et al., 2005). This indicates some interesting features about the effects of farnesol on the metabolism of *C. albicans*. Not only does *C. albicans* produce the highest level of farnesol among Candida species (Weber, et al., 2008), but it also exhibits the highest level of tolerance to farnesol toxicity (Weber, et al., 2010). This suggests that instead of just being a quorum sensing molecule, there must be other advantages for *C. albicans* to expend energy for the production of farnesol. We speculate that the upregulation of fatty acid metabolism by *C. albicans* in response to farnesol reflects the remodelling of its membrane composition in order to minimize the harmful effects of farnesol at high concentrations. Due to this unique metabolic response, farnesol may benefit *C. albicans* by reducing the growth of other species in the environment and to supress macrophage attack against *C. albicans* by promoting oxidative stress and apoptosis of macrophages (Abe, et al., 2009). On the other hand, our discovery of the upregulation of central carbon metabolism and oxidative phosphorylation by *C. albicans* in response to farnesol is novel. Rozpdowska et al. (2011) suggested that *C. albicans* does not possess an efficient glucose repression of respiration mechanism and Veiga et al. (2000) demonstrated that *C. albicans* can undertake cyanide-
resistant respiration and therefore is often considered as a crabtree-negative yeast. Yamaguchi reported that yeast respiratory activity declined immediately after cells resuspended in a hyphae-inducing medium. Land et al. (1975) showed that electron transfer through oxidative phosphorylation is required to maintain growth in the yeast morphology. The same authors also showed that the yeast form exhibits higher levels of respiration than hyphae, based on the oxygen consumption rate, which is supported by our previous findings where we found a higher pool of ATP in yeast cells compared to filamentous growing cells (Han, et al., 2012). In contrast, Aoki et al. (1982) showed that there is no difference in the respiratory activity between yeast and hyphal forms and by blocking the respiratory chain at complex II via thenoyltrifluoroacetone significantly inhibited the hyphal development of C. albicans (Watanabe, et al., 2006). It is important to note, however, that unlike other studies that induced specific morphological forms by manipulating media components or pH, Aoki et al. (1982) compared the respiratory activity of yeast and hyphal cells by manipulating the temperature. Therefore, temperature induced morphogenesis could have a different mechanism than morphogenesis induced by other factors. Nonetheless, thenoyltrifluoroacetone has been reported as a potent inhibitor of carboxylesterase (Zhang & Fariss, 2002). This drug not only suppresses mitochondrial complex II activity but it may also disturb other esterases such as acetylcholinesterase and lipase (Zhang & Fariss, 2002). In addition, it is possible that the upregulation of central carbon metabolism and oxidative phosphorylation is merely to fulfil the need to increase energy and cofactor production for metabolic activities such as the upregulation of amino acids biosynthesis for rapid biomass increase during mid-exponential growth phase.
3.6. Conclusions

To our knowledge, this is the first metabolomic study focused on the metabolic response of *C. albicans* during suppression of hyphae formation by farnesol. We have shown that *C. albicans* globally upregulates its cellular metabolism in response to farnesol. By combining this metabolite profile with previous hyphae-inducing experiments, a set of metabolic pathways associating with morphogenesis has been identified. Moreover, there is an upregulation of lipid metabolism and subsequent upregulation of central carbon metabolism upon exposure to farnesol independently from morphogenesis. Therefore, this metabolomic study provides new insights not only in our understanding of morphogenetic processes, but equally importantly, on the farnesol-mediated redistribution of metabolic fluxes in the central carbon metabolism.
3.7. Acknowledgements

We thank M. Sabherwal for assisting with sample preparation; R. Aggio for data analysis assistance; and T. Liu for proof reading. This work was supported by Faculty of Science Research Funding, University of Auckland and by the University of Auckland Doctoral Scholarship awarded to Ting-Li Han. The authors declare no conflict of interest.
3.8. References


CHAPTER IV

Metabolic response of *Candida albicans* to phenylethyl alcohol under hyphae-inducing conditions

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The content of this chapter has been submitted for publication
4.1. Abstract

Phenylethyl alcohol is one of the first quorum sensing molecules (QSMs) identified in *C. albicans*. This extracellular signalling molecule inhibits the hyphal formation of *C. albicans* at high cell density. Little is known, however, about the underlying mechanisms by which this QSM regulates the morphological switches of *C. albicans*. Therefore, we have applied metabolomics and isotope labelling experiments to investigate the metabolic changes that occur in *C. albicans* in response to phenylethyl alcohol under defined hyphae-inducing conditions. Our results show a global upregulation of cellular metabolism when hyphal development was suppressed by phenylethyl alcohol. By comparing the metabolic changes in response to phenylethyl alcohol to our previous metabolomic studies, we were able to short-list 7 metabolic pathways from the central carbon metabolism that appear to be associated with *C. albicans* morphogenesis. Furthermore, isotope-labelling data showed that phenylethyl alcohol is indeed taken up and catabolised by the yeast cells. Isotope-labelled carbon atoms were found in the majority of amino acids as well as in lactate and glyoxylate. However, isotope-labelled carbon atoms accumulated mainly in the pyridine ring of NAD+/NADH and NADP+/NADPH molecules, showing that these nucleotides were the main products of phenylethyl alcohol catabolism. Interestingly, two metabolic pathways where these nucleotides play an important role, nitrogen metabolism and nicotinate/nicotinamide metabolism, were also short-listed through our metabolomics analysis as metabolic pathways likely to be closely associated with *C. albicans* morphogenesis.
4.2. Introduction

In *C. albicans*, the switch from yeast to hyphal growth or *vice versa* is determined by environmental signals that trigger signal transduction pathways and change gene expression. The environmental perturbations can arise from modifications in the level of nutrients, or from molecules that are secreted by *C. albicans* in a cell density-dependent fashion known as quorum sensing molecules (QSMs) (Han, *et al.*, 2011). There are a number of QSMs which have been identified in *C. albicans*, including farnesol, farnesoic acid, tyrosol, tryptophol, and phenylethyl alcohol (Lingappa, *et al.*, 1969, Hornby, *et al.*, 2001, Chen, *et al.*, 2004, Hornby & Nickerson, 2004).

Phenylethyl alcohol was one of the first QSMs to be identified in *C. albicans* (Lingappa, *et al.*, 1969). This molecule inhibits both cell growth and germ tube formation of *C. albicans* once its extracellular concentration reaches a certain threshold value. On the other hand, Chen & Fink (2006) demonstrated that phenylethyl alcohol has an opposite effect in *S. cerevisiae*, where it stimulates filamentous growth in response to ammonia starvation and high cell density. These authors also proposed that phenylethyl alcohol is involved in nitrogen and signalling-mediated morphogenesis in *S. cerevisiae*. They suggested that ammonia starvation triggers morphogenesis by regulating the production of phenylethyl alcohol which increases in concentration because reduced ammonium ion concentration alleviates repression of *ARO9, ARO10*, and other genes required for aromatic alcohol production. Phenylethyl alcohol is also thought to promote *S. cerevisiae* morphogenesis by upregulating an essential filamentous gene, *FLO11*, via a PKA pathway-dependent mechanism (Chen & Fink, 2006). In comparison, little is known about the molecular mechanisms by which phenylethyl alcohol regulates the morphological switch of *C. albicans*. What is known, however, is that the
extracellular concentration of phenylethyl alcohol increases in the presence of phenylalanine supplementation in the growth media, low ammonia concentration, under alkaline pH and under anaerobic conditions (Ghosh, et al., 2008). In addition, phenylethyl alcohol production is not affected by alkaline pH when the transcriptional regulators, ARO80 and RIM101 genes, are disrupted (Ghosh, et al., 2008).

Therefore, to further investigate the metabolic response of *C. albicans* to phenylethyl alcohol, we have applied a gas chromatography-mass spectrometry (GC-MS) based metabolomics approach, and isotope labelling experiments, under hyphae-inducing conditions. *C. albicans* is one of the most prevalent pathogenic yeast species in humans causing candidiasis at multiple sites from mucosae to internal organs. Therefore, understanding the metabolic mechanisms behind one of its key virulence traits, morphogenesis, may provide insights for novel therapeutic interventions to prevent *C. albicans* infection.
4.3. Materials and Method

4.3.1. Chemical

Methanol, chloroform, sodium bicarbonate, and sodium hydroxide were obtained from MERCK (Darmstadt, Germany). The internal standard 2,3,3,3-d4-alanine, the derivatisation reagent methylchloroformate (MCF), pyridine, and D-glucose-13C6 were purchased from Sigma-Aldrich (St. Louis, USA). Anhydrous sodium sulphate and phenylethyl alcohol were obtained from Fluka (Steinheim, Germany). All chemicals were of analytical grade.

4.3.2. Fungal strain and culture media

C. albicans strain SC5314 (Gillum, et al., 1984) was maintained on YPD agar medium containing yeast extract (6 g.L\(^{-1}\)), peptone (3 g.L\(^{-1}\)), glucose (10 g.L\(^{-1}\)), and agar (15 g.L\(^{-1}\)); at 30 °C. Pre-inocula were prepared in minimum mineral medium (MM medium) at pH 5.5 containing D-glucose (10 g.L\(^{-1}\)), (NH\(_4\))\(_2\)SO\(_4\) (5 g.L\(^{-1}\)), MgSO\(_4\)\(\cdot\)7H\(_2\)O (0.5 g.L\(^{-1}\)), KH\(_2\)PO\(_4\) (3 g.L\(^{-1}\)), vitamins and trace metals as previously described (Verduyn, et al., 1992). Three different culture media were used for the metabolomic and isotope labelling experiments. (1) Minimum mineral medium (MM medium) at pH 7.5; (2) phenylethyl alcohol medium (PA medium), which consisted of MM medium, supplemented with phenylethyl alcohol (1.5 mM); and (3) phenylethyl alcohol medium with 13C-labelled glucose (PA 13C-glucose medium), which consisted of MM medium supplemented with phenylethyl alcohol (1.5 mM) with glucose (10 g.L\(^{-1}\)) uniformly labelled with 13C instead of 12C-glucose.

4.3.3. Culture conditions

C. albicans was cultured in 250 mL of MM medium (pH 5.5) at 30 °C using shake flasks in a rotary shaker overnight. The cells were collected by centrifugation
at 2000 x g (4 °C) for 5 min and washed in phosphate buffered saline (8 g.L⁻¹ NaCl, 0.2 g.L⁻¹ KCl, 1.44 g.L⁻¹ Na₂PO₄, 0.24 g.L⁻¹ KH₂PO₄, at pH 7.5). The cells were resuspended in the three different growth media described above, at an initial OD₆₀₀ of 0.2. The cells were incubated in a rotary shaker-incubator at 37 °C for 12 h. The morphology of *C. albicans* cells in each growth medium was monitored using a phase contrast microscope (DMR, Lecia).

4.3.4. Sampling and quenching of cell metabolism

Five replicate shake-flask cultures (30 mL) for each growth medium were harvested at late exponential growth phase (determined previously from growth curves obtained under the same experimental conditions). Samples (2 mL) of the microbial cultures were filtered (0.2 μm pore size membrane) to remove the *C. albicans* cells, and the filtrate was used for the analysis of extracellular metabolites. The remaining 28 mL of each culture was rapidly filtered under vacuum (Air Cadet vacuum/pressure station, Thermo), quickly washed with cold phosphate buffered saline solution (1-2 °C) and quenched in cold methanol water (1:1 v/v) at -30 °C as described by Smart *et al.* (2010). The whole sampling procedure took less than 30 s per sample.

4.3.5. Sample preparation for metabolite analysis

The intracellular metabolites were extracted from the quenched cell pellets using cold methanol water and freeze-thaw cycles following the protocol described by Smart *et al.* (2010). The internal standard 2,3,3,3-d₄-alanine (0.3 μmol/sample) was added to each sample before extraction. The intracellular metabolite extracts and 1 mL of spent culture medium containing extracellular metabolites were freeze-dried (BenchTop K manifold freeze dryer, VirTis) before chemical derivatisation.
4.3.6. Chemical derivatisation of metabolites

The freeze-dried samples were derivatised using the methyl chloroformate (MCF) method according to the protocol described by Smart et al. (2010). In summary, the freeze-dried samples were resuspended in 200 μL of sodium hydroxide solution (1 M) and transferred to a silanised glass tube, then mixed with 167 μL of methanol and 34 μL of pyridine. The derivatisation began by adding 20 μL of MCF followed by vigorously mixing for 30 s, and then a further 20 μL of MCF was added followed by vigorously mixing for 30 s. To separate MCF derivatives from the reactive mixture, 400 μL of chloroform was added and vigorously mixed for 10 s followed by the addition of 400 μL of sodium bicarbonate solution (50 mM), and mixing for an additional 10 s. The aqueous layer was removed and dehydrated with anhydrous sodium sulphate before samples were transferred to GC-MS vials.

4.3.7. Gas chromatography-mass spectrometry (GC-MS) analysis

The MCF derivatives were analysed in an Agilent GC7890 system coupled to a MSD5975 mass selective detector (EI) operating at 70 eV. The column used for all analyses was a ZB-1701 GC capillary column (30 m x 250 μm id x 0.15 μm with 5 m guard column, Phenomenex). The analysis parameters were set according to Smart et al. (2010). Samples were injected under pulsed splitless mode with the injector temperature at 290 °C. The helium gas flow through the GC-column was set at 1.0 mL.min⁻¹. The interface temperature was set to 250 °C and the quadrupole temperature was 200 °C.

4.3.8. Biomass quantification

The cell debris collected after intracellular metabolite extraction was dried using a domestic microwave (250 W for 20 min) and weighed in order to measure the total biomass content (dry weight) of each sample.
4.3.9. Data mining, data normalization, and data analysis

AMDIS software was used for deconvoluting GC-MS chromatograms and identifying metabolites using our in-house MCF mass spectra library. The identifications were based on both the MS spectrum of the derivatised metabolite and its respective chromatographic retention time. The relative abundance of identified metabolites was determined by ChemStation (Agilent) by using the GC base-peak value of a selected reference ion. These values were normalised by the biomass content in each sample as well as by the abundance of internal standard (2,3,3,3-d₄-alanine). A univariate analysis of variance (ANOVA) was applied to determine whether the relative abundance of each identified metabolite was significantly different between growth conditions. Our Pathway Activity Profiling (PAPI) algorithm (Aggio, et al., 2010) was used to predict and compare the relative activity of different metabolic pathways in C. albicans during the growth conditions tested. This programme connects to the KEGG online database (http://www.kegg.com) and uses the number of metabolites identified from each pathway and their relative abundances to predict which metabolic pathway is likely to be active in the cell. The entire data mining, data normalisation and pathway activity predictions were automated in R software as described in Smart et al. (2010) and Aggio et al. (2010). Graphical representations of the results were generated by gplots and ggplot2 R packages (Wickham, 2009, Warnes, et al., 2010).

4.3.10. Analysis of isotope labelling distribution in the detected metabolites

Due to the commercial unavailability of isotopically labelled phenylethyl alcohol, we decided to apply inverse isotope labelling whereby all C. albicans metabolites were fully labelled with ¹³C by culturing them in MM medium with ¹³C-U-labelled glucose as the sole carbon source. This way, we searched for ¹²C-enrichment in the metabolite profile originating from the metabolism of ¹²C-
phenylethyl alcohol. First, we identified the $^{13}$C-labelled metabolites based on their chromatographic retention time obtained by GC-MS analysis of metabolite profiles extracted from cells grown under the same conditions but with $^{12}$C-glucose as sole carbon source. The electron-impact fragmentation pattern of each identified MCF derivatized $^{13}$C-labelled metabolite was predicted based on its corresponding $^{12}$C spectrum from our MS library. The degree of labelling was determined by the variation observed in the predicted MS fragmentation pattern of fully $^{13}$C-labelled metabolites.
4.4. Results

4.4.1. Suppression of hyphae formation by phenylethyl alcohol

Phenylethyl alcohol (1.5 mM) inhibited *C. albicans* growth. It reduced both the growth rate and biomass yield (Figure 4.1, Table 4.1) but did not kill cells as the growth curve showed the same dynamics as growth in MM medium without phenylethyl alcohol. As the cells entered stationary phase (t = 12 h), microscopic examination showed that phenylethyl alcohol (1.5 mM) completely inhibited hyphae formation under the growth condition tested, whilst at least 95 % of cells grew as hyphae when phenylethyl alcohol was not present (Figure 4.2).

![Figure 4.1. Growth curves of *C. albicans* cells grown in the presence ( ) or absence ( ) of phenylethyl alcohol.](image-url)
Table 4.1
Biomass and morphology of *C. albicans* cells cultured in different growth media for 12 h.

<table>
<thead>
<tr>
<th>Media (pH 7.4, 37 °C)</th>
<th>Main Carbon source</th>
<th>Biomass (mg/mL)</th>
<th>F (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MM</td>
<td>$^{12}$C-glucose</td>
<td>2.03</td>
<td>&gt;95</td>
</tr>
<tr>
<td>MM + Phenylethyl alcohol</td>
<td>$^{12}$C-glucose</td>
<td>0.82</td>
<td>0</td>
</tr>
<tr>
<td>MM + Phenylethyl alcohol</td>
<td>$^{13}$C-glucose</td>
<td>0.72</td>
<td>0</td>
</tr>
</tbody>
</table>

MM: minimum mineral medium; F: percentage of filamentous growth determined by counting the number of yeast cells and filaments in 1 mm$^3$ volume.

Figure 4.2. The morphology of *C. albicans* cells incubated in the presence and absence of phenylethyl alcohol at 37 °C after 12 h of incubation. (A) Cells grown in Minimum Mineral medium (MM). (B) Cells grown in MM medium and phenylethyl alcohol. The Images were taken by Nomarksi contrast microscopy with 800x magnification.
4.4.2. The extracellular metabolite profile of *C. albicans* under suppression of hyphae formation by phenylethyl alcohol

To monitor how *C. albicans* changes its external environment in response to phenylethyl alcohol, extracellular samples were taken (t = 12 h) from *C. albicans* spent culture medium and were subjected to GC-MS analysis. We detected over 50 metabolites in the spent culture samples and we were able to accurately identify 26 of them using our in-house MS library (Table 4.2). Nineteen metabolites were detected at significantly different levels when comparing MM and phenylethyl alcohol-supplemented MM cultures (Figure 4.3). Of these extracellular metabolites 16 appear to have been secreted by *C. albicans* in response to phenylethyl alcohol supplementation. These metabolites included alanine, β-alanine, benzoate, carbamate, citraconate, citramalate, fumarate, isopalmitate, lactate, nicotinate, proline, succinate, valine, 2-hydroxybutyrate, 2-isopropylmalate, and phenylethyl alcohol itself, the concentration of which increased in the medium. Other compounds such as glyoxylate seem to have been taken up more extensively by *C. albicans* cells growing in the presence of phenylethyl alcohol, because they were detected at significant lower concentration compared to the MM medium with added phenylethyl alcohol.
Table 4.2
Intracellular and extracellular metabolites associated with \textit{C. albicans} growth on different culture media.

<table>
<thead>
<tr>
<th>Classification of metabolites</th>
<th>Intra</th>
<th>Extra</th>
<th>Metabolites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acids</td>
<td>19</td>
<td>5</td>
<td>Alanine&lt;sup&gt;a&lt;/sup&gt;, asparagine, aspartate, cysteine, glutamine, glutamate, glycine&lt;sup&gt;a&lt;/sup&gt;, histidine, isoleucine, leucine, lysine, phenylalanine, proline&lt;sup&gt;a&lt;/sup&gt;, serine, threonine, tryptophan, tyrosine, valine&lt;sup&gt;a&lt;/sup&gt;, and β-alanine&lt;sup&gt;a&lt;/sup&gt;.</td>
</tr>
<tr>
<td>Amino acid derivatives</td>
<td>9</td>
<td>0</td>
<td>Creatinine, cystathionine, D-2-aminoadipate, N-acetylglutamate, norvaline, ornithine, S-adenosyl-L-homocysteine, 2-aminobutyrate, and pyroglutamate.</td>
</tr>
<tr>
<td>TCA cycle intermediates</td>
<td>7</td>
<td>3</td>
<td>Fumarate&lt;sup&gt;a&lt;/sup&gt;, citrate&lt;sup&gt;a&lt;/sup&gt;, succinate&lt;sup&gt;a&lt;/sup&gt;, cis-aconitate, isocitrate, malate, and 2-oxoglutarate&lt;sup&gt;a&lt;/sup&gt;.</td>
</tr>
<tr>
<td>Fatty acids</td>
<td>12</td>
<td>4</td>
<td>Adipate, Caprine&lt;sup&gt;a&lt;/sup&gt;, caprylate, D-2-aminoadipate, lactate&lt;sup&gt;a&lt;/sup&gt;, malonate, myristate, olate, pentadecanoate, 14-methylpentadecanoate&lt;sup&gt;a&lt;/sup&gt;, stearate&lt;sup&gt;a&lt;/sup&gt;, and 3-hydroxyoctanoate.</td>
</tr>
<tr>
<td>Glycolytic intermediates</td>
<td>2</td>
<td>0</td>
<td>Pyruvate and phosphoenolpyruvate.</td>
</tr>
<tr>
<td>Cofactors and vitamins</td>
<td>3</td>
<td>1</td>
<td>NADP/NADPH, nicotinate&lt;sup&gt;a&lt;/sup&gt;, and 4-amino-n-butyrate.</td>
</tr>
<tr>
<td>Others</td>
<td>15</td>
<td>10</td>
<td>Benzoyl&lt;sup&gt;a&lt;/sup&gt;, cabamate&lt;sup&gt;a&lt;/sup&gt;, citraconate&lt;sup&gt;a&lt;/sup&gt;, citramalate&lt;sup&gt;a&lt;/sup&gt;, eicosanoate, glutarate&lt;sup&gt;a&lt;/sup&gt;, itaconate, lactate, malonate, 2-isopropyimaleate&lt;sup&gt;a&lt;/sup&gt;, 2-hydroxybutyrate&lt;sup&gt;a&lt;/sup&gt;, 10,13-dimethyltetradecanoate&lt;sup&gt;a&lt;/sup&gt;, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-benzenepropanoic acid&lt;sup&gt;a&lt;/sup&gt;, 4-aminobenzoate 10,12-octadienoate, and phenylethyl alcohol&lt;sup&gt;a&lt;/sup&gt;.</td>
</tr>
<tr>
<td>Metabolites only found in extracellular media</td>
<td>0</td>
<td>3</td>
<td>Glyoxylate/glyoxalate&lt;sup&gt;a&lt;/sup&gt;, 12-oxoglutarate&lt;sup&gt;a&lt;/sup&gt;, 2,4-bis(1,1-dimethylethyl)-phenol&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total # of identified metabolites</td>
<td>66</td>
<td>26</td>
<td></td>
</tr>
</tbody>
</table>

Intra: Number of intracellular metabolites identified in any samples; Extra: Number of extracellular metabolites identified in any samples; "Metabolites found in any extracellular media."
Figure 4.3. Relative concentrations of extracellular metabolites after 12 h of incubation in the presence or absence of phenylethyl alcohol. Phenylethyl alcohol treatment is represented by blue triangles (▲) and hyphae-inducing conditions (MM) are represented by red circles (●). The relative concentrations of identified metabolites have been normalised by internal standard (d₄-alanine) and biomass before the relative concentrations of the corresponding metabolites found in un-inoculated culture medium were subtracted. Standard deviations are indicated by the vertical line-range. The difference between secretion and consumption of extracellular metabolites is distinguished by the dashed lines (y = 0). Secretion of metabolites is indicated by positive values. Consumption of metabolite is indicated by negative values. Only the metabolites generating with statistically significant ANOVA scores (p-value < 0.05) are shown.
4.4.3. The intracellular metabolite profiles of \textit{C. albicans} under suppression of hyphae formation by phenylethyl alcohol

In order to investigate further how \textit{C. albicans} cells respond to phenylethyl alcohol, we compared the profiles of intracellular metabolites from cells grown in MM medium with and without phenylethyl alcohol. We detected over 100 metabolites and 67 of them were capable accurately identified across samples. Of these, the concentrations of 51 metabolites were significantly affected by the presence of phenylethyl alcohol in the medium (Table 4.2, Figure 4.4). Interestingly, all of the intracellular metabolites were detected at lower concentrations in samples from cultures supplemented with phenylethyl alcohol. These intracellular metabolites include a range of intermediates from the central carbon metabolism such as amino acids, organic acids, fatty acids, and nucleotides. In particular, the concentrations of aromatic amino acids, tyrosine and tryptophan, ornithine, \(\beta\)-Alanine, and lysine were reduced up to 16-fold in response to phenylethyl alcohol. Furthermore, metabolites such as alanine, \(\beta\)-alanine, benzoate, carbamate, citramalate, lactate, nicotinate, proline, succinate, and valine were found at reduced concentrations intracellularly, but increased concentrations extracellularly in cells exposed to phenylethyl alcohol, suggesting that the cells actively secreted those metabolites in response to the phenylethyl alcohol.
Figure 4.4. The ratio of Intracellular metabolite concentrations for cells grown in the presence as opposed to the absence of phenylethyl alcohol after 12 h of incubation. Red circles (●) represent concentrations for samples from cells incubated in the absence of phenylethyl alcohol (hyphae-inducing conditions) - that were set to 0. Blue triangles (▲) indicate metabolite concentrations in cells exposed to phenylethyl alcohol relative to those in cells grown without phenylethyl alcohol. The metabolite levels relative to the hyphal samples have been visualized using a log2 scale. Negative values indicate that the metabolite concentrations were reduced in response to phenylethyl alcohol. Only the metabolites generating statistically significant ANOVA scores (p-value < 0.05) are shown.
4.4.4. Prediction of the metabolic state of *C. albicans* under suppression of hyphal formation by phenylethyl alcohol

Using the profile of intracellular metabolites identified in the samples of *C. albicans* cells growing in the presence or absence of phenylethyl alcohol, we created a comparative metabolic activity profile using PAPi software (Aggio, *et al.*, 2010). Figure 4.5 compares the metabolic activities of *C. albicans* cells under conditions suppressing hyphal formation by phenylethyl alcohol with cells cultured in the absence of the QSM. All of the 48 metabolic pathways that showed significant changes in metabolic activity in response to phenylethyl alcohol were up-regulated. These include a range of metabolic pathways from the metabolism of amino acids, carbon, cofactors, energy, lipids, nucleotides, and secondary metabolites. In particular, ubiquinone biosynthesis, tryptophan metabolism, and D-arginine/D-ornithine metabolism exhibited a marked upregulation in response to phenylethyl alcohol. This is in agreement with our metabolite profile results because two of the main assumptions of the PAPi software is that the higher the flux through a given metabolic pathway the larger the number of detected intermediates from that pathway will be and, most importantly, the lower will be the concentration of those compounds inside the cell. If a pathway is operating at a high flux we expect a high turn-over rate between its intermediates, reducing their intracellular concentration.
Figure 4.5. Activities of *C. albicans* metabolic pathways based on intracellular metabolomic data when cultivating *C. albicans* for 12 h in the presence or absence of phenylethyl alcohol. Red circles (●) represent metabolic activities under hyphae-inducing conditions that were set to 0. Blue triangles (▲) indicate metabolic activities in cells treated with phenylethyl alcohol. The metabolic activities relative to the hyphae-inducing samples have been visualized using log2 scale. Positive values indicate the metabolic pathways had their activity up-regulated in response to phenylethyl alcohol. Only the pathways generating statistically significant ANOVA scores (p-value < 0.05) are shown.
4.4.5. $^{12}$C-labelling distribution through the metabolite profile of *C. albicans* grown in $^{13}$C-U-glucose medium supplemented with $^{12}$C-phenylethyl alcohol

A number of metabolites incorporated at least one carbon from phenylethyl alcohol (*Table 4.3*). These include a number of amino acids (alanine, asparagine, aspartate, cysteine, glutamate, histidine, isoleucine, leucine, lysine, ornithine, phenylalanine, proline, serine, valine, 2-aminobutyrate, D-2-aminoadipate), glyoxylate, and lactate. The majority proportion of threonine and $\beta$-alanine molecules contained at least two $^{12}$C-atoms. However, we detected the pyridine ring from nicotinamide of NAD$^+/\text{NADPH}$ and NAD$^+/\text{NAD}$ almost fully labelled with $^{12}$C in $^{13}$C-cultures supplemented with $^{12}$C-phenylethyl alcohol (*Figure 4.6*). In contrast, intermediates from the TCA cycle (citrate, *cis*-aconitate, $\alpha$-ketoglutarate, fumarate, malate, and succinate), two aromatic amino acids (tryptophan, tyrosine), GABA, $N$-acetylglucosamine, nicotinate, oleate, and pyroglutamate showed no significant $^{12}$C-labelling.
Table 4.3
List of ion clusters used to determine the pattern of isotope labelling in the identified metabolites

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Ion cluster</th>
<th>Carbon Fragment</th>
<th>Mass isotopomer % abundance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>M</td>
</tr>
<tr>
<td>2-aminobutyrate</td>
<td>116</td>
<td>C1-C4</td>
<td>1%</td>
</tr>
<tr>
<td>Alanine</td>
<td>103</td>
<td>C1-C3</td>
<td>1%</td>
</tr>
<tr>
<td>Asparagine</td>
<td>127</td>
<td>C1-C4</td>
<td>0%</td>
</tr>
<tr>
<td>Aspartate</td>
<td>160</td>
<td>C1-C4</td>
<td>2%</td>
</tr>
<tr>
<td>Cis-Aconitate</td>
<td>153</td>
<td>C1-C6</td>
<td>1%</td>
</tr>
<tr>
<td>Citrate</td>
<td>143</td>
<td>C1-C5</td>
<td>3%</td>
</tr>
<tr>
<td>Cysteine</td>
<td>192</td>
<td>C1-C3</td>
<td>1%</td>
</tr>
<tr>
<td>D-2-aminoadipate</td>
<td>188</td>
<td>C1-C6</td>
<td>1%</td>
</tr>
<tr>
<td>Fumarate</td>
<td>113</td>
<td>C1-C4</td>
<td>0%</td>
</tr>
<tr>
<td>GABA</td>
<td>144</td>
<td>C1-C4</td>
<td>0%</td>
</tr>
<tr>
<td>Glutamate</td>
<td>174</td>
<td>C1-C5</td>
<td>8%</td>
</tr>
<tr>
<td>Glutamine</td>
<td>141</td>
<td>C1-C5</td>
<td>0%</td>
</tr>
<tr>
<td>Glycine</td>
<td>88</td>
<td>C1-C2</td>
<td>3%</td>
</tr>
<tr>
<td>Glyoxylate</td>
<td>103</td>
<td>C1-C2</td>
<td>3%</td>
</tr>
<tr>
<td>Histidine</td>
<td>226</td>
<td>C1-C6</td>
<td>0%</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>144</td>
<td>C1-C6</td>
<td>2%</td>
</tr>
<tr>
<td>Lactate</td>
<td>103</td>
<td>C1-C3</td>
<td>13%</td>
</tr>
<tr>
<td>Leucine</td>
<td>144</td>
<td>C1-C6</td>
<td>2%</td>
</tr>
<tr>
<td>Lysine</td>
<td>142</td>
<td>C1-C6</td>
<td>4%</td>
</tr>
<tr>
<td>Malate</td>
<td>113</td>
<td>C1-C4</td>
<td>1%</td>
</tr>
<tr>
<td>N-acetylglucosamine</td>
<td>158</td>
<td>C1-C6</td>
<td>3%</td>
</tr>
<tr>
<td>NADP+/NADPH</td>
<td>138, 94,80</td>
<td>Pyridine ring of nicotinamide</td>
<td>&gt;95%</td>
</tr>
<tr>
<td>NAD+/NADH</td>
<td>171,14, 0,124</td>
<td>Pyridine ring of nicotinamide</td>
<td>&gt;95%</td>
</tr>
<tr>
<td>Nicotinate</td>
<td>106</td>
<td>C1-C6</td>
<td>7%</td>
</tr>
<tr>
<td>Ornithine</td>
<td>128</td>
<td>C1-C5</td>
<td>0%</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>178</td>
<td>C1-C9</td>
<td>0%</td>
</tr>
<tr>
<td>Proline</td>
<td>128</td>
<td>C1-C5</td>
<td>1%</td>
</tr>
<tr>
<td>Pyroglutamate</td>
<td>84</td>
<td>C1-C4</td>
<td>0%</td>
</tr>
<tr>
<td>Serine</td>
<td>100</td>
<td>C1-C3</td>
<td>2%</td>
</tr>
</tbody>
</table>
Table 4.3 (Continued)

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Ion cluster</th>
<th>Carbon Fragment</th>
<th>Mass isotopomer % abundance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>M</td>
</tr>
<tr>
<td>Succinate</td>
<td>115</td>
<td>C1-C4</td>
<td>2%</td>
</tr>
<tr>
<td>Threonine</td>
<td>115</td>
<td>C1-C4</td>
<td>2%</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>130</td>
<td>C1-C9</td>
<td>5%</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>236</td>
<td>C1-C9</td>
<td>5%</td>
</tr>
<tr>
<td>Valine</td>
<td>130</td>
<td>C1-C5</td>
<td>3%</td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>115</td>
<td>C1-C4</td>
<td>1%</td>
</tr>
<tr>
<td>β-alanine</td>
<td>88</td>
<td>C1-C3</td>
<td>6%</td>
</tr>
<tr>
<td>Oleate</td>
<td>296</td>
<td>C1-C18</td>
<td>6%</td>
</tr>
</tbody>
</table>

Ion cluster is a group of ions which belong together, all peaks have the same molecular composition but with different isotopes of the carbon atoms. Carbon fragment describes number of carbon atom in a given ion cluster. M is the main molecular ion peak of a metabolite. M+1 is 1 m/z higher than the M.
Figure 4.6. The incorporation of carbon atoms from phenylethyl alcohol into *C. albicans* metabolites under hyphae-inducing conditions. Stacked column plot indicates the percentage that the number of carbons derived from phenylethyl alcohol contributes to the total ion mass in each identified metabolite (see Table 4.3). Surprisingly, the pyridine ring of NADPH and NADH are completely derived from phenylethyl alcohol.
4.5. Discussion

We have detected a global upregulation of cellular metabolism when \textit{C. albicans} hyphal formation was suppressed by the quorum sensing molecule, phenylethyl alcohol. This result is in accordance with our previous two metabolomic studies of \textit{C. albicans} morphogenesis, in which we also observed a global upregulation of metabolite pathways when hyphal formation was suppressed by farnesol, or a general metabolic downregulation when hyphal growth was promoted by various growth media (Han, \textit{et al.}, 2012a, Han, \textit{et al.}, 2012b).

In an attempt to understand the downstream molecular mechanism of \textit{C. albicans} morphogenesis, we compared the metabolic profiles of \textit{C. albicans} in response to phenylethyl alcohol with the profiles obtained in our previous studies: exposure to farnesol and hyphae-inducing conditions (Han, \textit{et al.}, 2012a, Han, \textit{et al.}, 2012b). This permitted us to short-list 7 metabolic pathways that appear to be altered in all three studies (\textbf{Figure 4.7}). These metabolic pathways encompassed alanine, asparate and glutamate metabolism; \(\beta\)-alanine metabolism; cysteine and methionine metabolism; histidine metabolism; nitrogen metabolism; nicotinate and nicotinamide metabolism; and pantothenate and CoA biosynthesis. Therefore, we believe that these seven metabolic pathways are likely to be closely associated with the morphogenetic process of \textit{C. albicans}.
Figure 4.7. Radar line diagram indicating the up- and down-regulation of *C. albicans* metabolic pathways when hyphal growth was suppressed by farnesol, or phenylethyl alcohol, or induced by various growth conditions. Metabolic pathways responding to phenylethyl alcohol (• • •) are derived from Figure 4.5. Metabolic pathways responding to farnesol (• • •) and hyphae-inducing conditions (• • •) are derived from previous studies (Han, *et al.*, 2012a, Han, *et al.*, 2012b). A small Venn diagram is displayed to illustrate the unique and common metabolic pathways affected by culturing the cells in the presence of farnesol, phenylethyl alcohol or under various hyphae-inducing conditions. a indicates the metabolic pathways that respond in common to all three conditions. b indicates the metabolic pathways that differ from control cells in response to both farnesol and phenylethyl alcohol. The metabolic activities relative to their corresponding controls (set to 0) have been visualized using a log2 scale. A positive value indicates that a metabolic pathway was up-regulated in comparison to the control. A negative value means the metabolic pathway activity was down-regulated when compared to the control. Only the metabolic pathways with statistically significant (p-value < 0.05) changes in activity are shown.
Furthermore, QSMs such as phenylethyl alcohol and farnesol seem to influence the lipid metabolism of *C. albicans* independently from morphological changes (Figure 4.7). We have detected five metabolic pathways related to lipid metabolism that were significantly upregulated only in the presence of phenylethyl alcohol and farnesol. Phenylethyl alcohol at concentrations between 60 and 140 mM is known to exhibit antimicrobial effects (against bacteria (e.g. *E. coli*, *S. aureus*, and *E. faecium*) (Corre, *et al.*, 1990, Lucchini, *et al.*, 1990) and fungi (e.g. *C. albicans*, *S. cerevisiae*, and *K. marxianus*, *C. dubliniensis*) (Fabre, *et al.*, 1998, Martins, *et al.*, 2007). One of its potential antimicrobial mechanisms is proposed to be the result of alterations in membrane functions such as permeabilisation of the cell envelope and leakage of potassium ions (Corre, *et al.*, 1990, Lucchini, *et al.*, 1990). Therefore, the upregulation of fatty acid metabolism in response to the presence of phenylethyl alcohol may support our previous hypothesis (Han, *et al.*, 2012b), in which *C. albicans* changes its membrane composition in order to reduce the antimicrobial effects of certain QSMs at high concentrations such as farnesol and, now, phenylethyl alcohol. In addition, *C. albicans* is also likely to benefit from secreting these toxic quorum sensing molecules through inter-species competition.

Phenylethyl alcohol appears not only to act as a signalling molecule inducing gene expressions (Chen & Fink, 2006), it is also metabolised intracellularly. When we provided labelled cells with unlabelled phenylethyl alcohol we observed that almost all amino acids belonging to the histidine, serine, pyruvate, and glutamate families as well as lactate and glyoxylate, incorporated at least one carbon from phenylethyl alcohol (Figure 4.6 and Table 4.3). However, it was a surprise to find the majority of unlabelled-carbon atoms ending up in NAD⁺/NADH and NADP⁺/NADPH molecules. Therefore, we hypothesized that once uptaken by *C. albicans* cells, phenylethyl alcohol could be cleaved into ethanol and a benzene ring (Figure 4.8). Ethanol would then be easily oxidized into acetaldehyde by alcohol dehydrogenase (*ASD1, ADH7*)
and, then, converted to lactate by aldehyde dehydrogenase (ALD), which explains the isotope-labelling found in lactate. Aerobically, lactate can be oxidised into pyruvate by lactate dehydrogenase (CYB2), or by pyruvate decarboxylase (PDC). Since pyruvate is a metabolic hub in the primary metabolic network, it could then be the source of carbon atoms to be distributed throughout the central carbon metabolism. The reduction of ethanol to acetaldehyde may also explain how some threonine molecules were found to be labelled with two $^{12}$C-atoms, because this amino acid can be synthesised from glycine and acetaldehyde via threonine aldolase (GLY12) in *C. albicans*. However, we cannot explain why we did not detect much isotope-labelling in the TCA cycle intermediates considering that some amino acids from the glutamate family were found to contain $^{12}$C-carbons. We can only speculate that the high carbon flux throughout the central carbon metabolism pathways must have diluted the $^{12}$C-isotopes to concentrations below the detection limits of our GC-MS method, whilst some amino acids would have a lower turnover inside the cell allowing the detection of their $^{12}$C-atoms.
Figure 4.8. The proposed catabolism of phenylethyl alcohol in *C. albicans*. Pathways illustrate how phenylethyl alcohol is hypothesized to be metabolized in *C. albicans* based on isotope-labelling results. The putative genes encoding enzymes are in blue text. The names of genes are as follow: *(ASD1, ADH7)* subunits of alcohol dehydrogenase; *(BNA1)* 3-hydroxyanthranilate dioxygenase; *(NADK)* NAD⁺ kinase, *(PDC)* pyruvate decarboxylase; *(ALD)* aldehyde dehydrogenase; and *(CYB2)* lactate dehydrogenase.
Furthermore, we speculate that the benzene ring from phenylethyl alcohol is incorporated into nucleotide molecules through a metabolic conversion into the pyridine group of NADH and subsequently NADH is phosphorylated to form NADPH (Figure 4.8). Based on the current understanding of NADH biosynthesis, there are three possible metabolic routes by which a benzene ring could potentially contribute to the pyridine ring of NADH: i) via de novo synthesis from aspartate, but aspartate was not found to be greatly labelled; ii) via salvage pathways by recycling of compounds containing nicotinamide, but phenylethyl alcohol does not contain nicotinamide; or iii) via de novo synthesis from tryptophan catabolism. There is no evidence that a benzene ring could be directly converted into pyridine through biochemical reactions. Nevertheless, C. albicans has a dioxygenase that is capable of opening the benzene ring through the incorporation of two atoms of dioxygen and spontaneously rearranging the molecule into a pyridine structure via further biochemical reactions. For example, in the C. albicans tryptophan catabolism pathway, 3-hydroxyanthranilate dioxygenase (BNA1) catalyses the cleavage of the benzene ring from 3-hydroxyanthranilate into 2-amino-3-carboxymuconate semialdehyde (Panozzo, et al., 2002, Jones, et al., 2004). This unstable compound spontaneously cyclises to form a pyridine structure and becomes quinolinate, an intermediate involved in the de novo biosynthesis of NADH from tryptophan. However, we would expect the cells to have some pyridine ring unlabelled due to de novo synthesis; the fully labelled pyridines of cofactors remain ambiguous and require further investigations.

An alternative hypothesis regarding the isotope labelling results is that C. albicans could assimilate $^{12}$C-carbons by fixing exogenous CO$_2$. S. cerevisae has been shown experimentally to fix CO$_2$ into phosphoenolpyruvate by phosphoenolpyruvate carboxykinase (Pck1p) forming oxaloacetate, an intermediate of the TCA cycle (Stoppani, et al., 1958, Oura, et al., 1980). C. albicans often grows
within the host in an environment where the CO₂ level (5 %) is more than 150-fold greater than in the atmosphere (0.033 %) (Klengel, et al., 2005). The ability to utilise additional carbon from CO₂ for biosynthetic purposes during morphogenesis, seems to be a good growth and invasion strategy, considering that an atmosphere containing 5 to 15 % of CO₂ also stimulates germ tube formation in *C. albicans* (Mock, et al., 1990, Bahn & Mühlschlegel, 2006). However, there is no direct evidence that *C. albicans* is capable of fixing CO₂ for cellular growth, even though *C. albicans* does have a putative phosphoenolpyruvate carboxykinase gene (*PCK1*) based on orthologous gene identification from its genome sequence; and we have not found major ¹²C-labelling in TCA cycle intermediates. In addition, it is difficult to speculate how ¹²C from CO₂, a single carbon substrate, could have contributed such a high proportion of the carbon atoms of NADH and NADPH molecules.

By combining the fact that phenylethyl alcohol could be potentially catabolisised mainly into the pyridine structure of NADH/NADPH molecules with the pathways we have previously short-listed as potentially related to the morphogenetic process, we have identified two candidate primary metabolic pathways, nitrogen metabolism and nicotinate/nicotinamide metabolism, which could play a central role in *C. albicans* morphogenesis. These two pathways not only significantly change their activity during various hyphal-inducing conditions as predicted by our metabolomics studies, but they are also responsible for the biosynthesis and replenishment of NADH and NADPH molecules in the cell. Nitrogen metabolism plays a role in maintaining the redox balance between the reduced and oxidized states of NADH and NADPH. Nicotinate and nicotinamide metabolism produces NAD⁺ via reutilizing compounds containing nicotinamide, and by *de novo* synthesis of NAD⁺ from tryptophan or aspartate. Subsequently, NAD⁺ can be phosphorylated into NADP⁺ by NAD⁺ kinase (Lehninger, 2005). It is important to note that NADH and NADPH have a significant influence on central carbon metabolism.
NADH not only acts as an electron carrier mediating energy metabolism, it is also involved in other cellular process such as post-translational modifications that affect the activity of metabolic enzymes. NADPH is a potent reducing agent that drives biochemical reactions toward anabolic metabolism including biosynthesis of lipids, nucleic acids, and amino acids. Therefore, nitrogen metabolism and nicotinate/nicotinamide metabolism have great potential to be the main link between signalling pathways and downstream primary metabolism during *C. albicans* morphogenesis.
4.6. Conclusion

To the best of our knowledge, this is the first investigation of the metabolic response of *C. albicans* to the QSM phenylethyl alcohol. We observed a global upregulation of metabolism when filamentous growth was suppressed by phenylethyl alcohol, and we demonstrated that this QSM is not only secreted by *C. albicans* cells, but it is also actively taken up and catabolised intracellularly. We found strong evidences that phenylethyl alcohol is catabolised mainly into the pyridine ring of NADH and NADPH molecules which could affect the level of these molecules inside the cells as well as their redox state; explaining its marked effect on the central carbon metabolism of *C. albicans* and consequent inhibition of hyphae formation. NADH and NADPH are important electron carriers responsible for the redox balance of the cell. The disruption of the cell’s redox balance would have major effects on primary metabolism. Therefore, our studies suggest that nitrogen metabolism and nicotinate/nicotinamide metabolism play an important role in the morphogenetic process of *C. albicans*, and further investigations are required to better understand how these pathways are connected to the upstream signalling pathways of morphogenetic regulation in *C. albicans*. 
4.7. Acknowledgements

We thank M. Sabherwal for assisting with sample preparation; R. Aggio for data analysis assistance; and T. Liu for proof reading. This work was supported by Faculty of Science Research Funding, University of Auckland and by the University of Auckland Doctoral Scholarship for Ting-Li Han.
4.8. References


CHAPTER V

The effect of deleting *Candida albicans* glutamate dehydrogenase genes *GDH2* and *GHD3* on morphogenesis

This chapter presents preliminary results not submitted for publication
5.1. Abstract

In the pathogenic fungus *Candida albicans* there are two glutamate dehydrogenases: a NAD\(^+\)-dependent glutamate dehydrogenase (encoded by *GDH2*) responsible for the catabolism of glutamate into α-ketoglutarate and ammonium, and a NADPH-dependent glutamate dehydrogenase (encoded by *GDH3*) that catalyses the biosynthesis of glutamate from α-ketoglutarate. These two enzymes are part of the nitrogen and nicotinate/nicotinamide metabolic pathways, which have been shortlisted in my previous studies as potentially playing an important role in *C. albicans* morphogenesis. In this study I created single gene knockout mutants of both dehydrogenases in order to investigate whether or not they affect the morphogenesis of *C. albicans*. The *GDH* genes were deleted using the *SAT1* flipper technique and the phenotypes of the knockout mutants were studied under various hyphae-inducing conditions. I found that the mutants exhibited altered morphogenetic abilities and growth rates when cells were cultured in arginine and proline as the sole carbon and nitrogen sources, but not when grown under other hyphae-inducing conditions. This indicates that certain amino acids such as arginine and proline induce morphogenesis in *C. albicans* through nitrogen metabolism, most likely altering the redox balance of the cell. However, more studies are needed in order to fully characterise the effect of *GDH2* and *GDH3* deletions on *C. albicans* metabolism.
5.2. Introduction

*Candida albicans* is a polymorphic fungus that grows on various human mucosal surfaces. The morphological switch from budding yeast to filamentous forms is often associated with its biological adaptation as an opportunistic pathogen of humans. The yeast-to-hyphae transition has been previously reported to play an important role in the systemic infection of *C. albicans*, by enabling the hyphae to penetrate into endothelial tissue and subsequently seeding of the bloodstream with yeast cells (Rooney & Klein, 2002, Bendel, *et al.*, 2003, Saville, *et al.*, 2003). *C. albicans* yeast cells are also capable of surviving during phagocytosis by developing into the filamentous form and eventually bursting out of the macrophage or neutrophil (Rubin-Bejerano, *et al.*, 2003, Lorenz, *et al.*, 2004). Therefore, understanding cellular mechanisms that drive morphogenesis is crucial in defining its pathogenic properties.

In my previous experiments (Chapters 2-4), I have demonstrated that during the yeast-to-hyphae transition the central carbon metabolism of *C. albicans* is globally downregulated, especially the pathways involved in the metabolism of alanine, **β**-alanine, aspartate, cysteine, histidine, glutamate, methionine, nitrogen, nicotinate/nicotinamide, and the biosynthesis of acetyl CoA (*Figure 2.5* and *Figure 3.5*). Using an isotope labelling experiment to trace the catabolism of phenylethyl alcohol under hyphae-inducing condition (*Figure 4.7*), I have also demonstrated the potential involvement of NADP+/NADPH and NAD+/NADH in phenylethyl alcohol-mediated morphogenesis. This indicates that the redox balance of the fungal cells could be associated with the morphogenetic process. Therefore, I decided to delete the genes encoding NAD⁺-dependent glutamate dehydrogenase (*GDH2*) and NADPH-dependent glutamate dehydrogenase (*GHD3*) in order to assess the role of the cell’s redox balance and nitrogen metabolism in *C. albicans* morphogenesis. These
metabolic enzymes play central roles in nitrogen assimilation and excretion, as well contributing to the maintenance of the redox balance of the cell (Villas-Bôas, et al., 2005). The Gdh2p-catalysed equilibrium favours the deamination of glutamate, in which glutamate is catabolised to α-ketoglutarate and ammonium (Figure 5.1). This reaction also generates an NADH and is considered to be the final step in eukaryotic nitrogen excretion and very important in recycling oxidised NAD⁺. On the other hand, Gdh3p is predominantly involved in the anabolic reaction whereby inorganic ammonium is assimilated into α-ketoglutarate to form glutamate, and in the process oxidises NADPH. Glutamate subsequently serves as an important nitrogen carrier for the biosynthesis of other amino acids via transamination reactions. In addition, dimorphic fungi such as Benjaminiella poitrasii, Mucor racemosus, Schizophyllum commune have been reported to possess different NAD⁺ and NADPH-glutamate dehydrogenase activities in the yeast and filamentous forms (Peters & Sypherd, 1979, Khale, et al., 1992, Amin, et al., 2004).

**Figure 5.1.** The reactions catalysed by glutamate dehydrogenases in *C. albicans*. Large arrows indicate thermodynamically more favourable reactions.
In this study I have used the SAT1 flipper method to delete, individually, both GHD2 and GDH3 genes in *C. albicans*. Conventional approaches to construct *C. albicans* gene disruption mutants often rely on the use of genes encoding nutritional markers (e.g. *URA3*, *ARG4*, *HIS1*, and *LEU2*) for the selection of prototrophic transformants from auxotrophic host strains, by restoring their capacity to synthesise essential nutrients (Wilson, *et al.*, 1999, Noble & Johnson, 2005). The SAT1 flipper method, in contrast, uses disruption cassettes containing two genes: a dominant marker (*CaSAT1*) conferring nourseothricin resistance, enabling selection of successful transformants, and a FLP recombinase gene that induces the excision of the disruption cassette. The crucial advantage of this gene knockout mutagenesis is that it enables us to delete genes in the same (prototrophic) strain as used in my previous metabolomics studies (Chapters 2-4). Moreover, by inserting and subsequently excising the selective marker from the modified strains, it minimises the adverse effects of the selective marker on the metabolic phenotype of the mutants. For example, morphogenesis and virulence of *C. albicans* are profoundly influenced by the expression levels of the *URA3* selective marker (Brand, *et al.*, 2004, Sharkey, *et al.*, 2005).

After deleting both alleles of the *GDH2* and *GDH3* genes, the morphologies and growth characteristics of the mutant and wild type strains were assessed by culturing under various conditions which have been reported to induce filamentous growth in *C. albicans*.
5.3. Materials and Method

5.3.1. *Candida albicans* strains and plasmids

*C. albicans* strain SC5314 (Gillum, *et al.*, 1984) was used as the wild type parental strain from which mutants were generated (Table 5.1). The SAT1 flipper fragment containing the nourseothricin resistance gene was amplified from plasmid pSFS2 (Reuß, *et al.*, 2004).

**Table 5.1.**
*C. albicans* wild-type and mutant strains used in this study

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genotypes</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC5314</td>
<td>Wild-type</td>
<td>(Gillum, <em>et al.</em>, 1984)</td>
</tr>
<tr>
<td>GDH2_M1</td>
<td>SC5314 :: GDH2/gdh2 :: SAT1-FLP-FRT&lt;sup&gt;a&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>GDH2_M2</td>
<td>SC5314 :: GDH2/gdh2 :: FRT&lt;sup&gt;b&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>GDH2_M3</td>
<td>SC5314 :: gdh2/ghd2 :: SAT1-FLP-FRT</td>
<td>This study</td>
</tr>
<tr>
<td>GDH2_M4</td>
<td>SC5314 :: gdh2/ghd2 :: FRT</td>
<td>This study</td>
</tr>
<tr>
<td>GDH3_M1</td>
<td>SC5314 :: GDH3/ghd3 :: SAT1-FLP-FRT</td>
<td>This study</td>
</tr>
<tr>
<td>GDH3_M2</td>
<td>SC5314 :: GDH3/ghd3 :: FRT</td>
<td>This study</td>
</tr>
<tr>
<td>GDH3_M3</td>
<td>SC5314 :: gdh3/ghd3 :: SAT1-FLP-FRT</td>
<td>This study</td>
</tr>
<tr>
<td>GDH3_M4</td>
<td>SC5314 :: gdh3/ghd3 :: FRT</td>
<td>This study</td>
</tr>
</tbody>
</table>

<sup>a</sup>SAT1-FLP-FRT denotes SAT1 flipper cassette

<sup>b</sup>FRT is the abbreviation for FLP recombination target
5.3.2. Chemicals and reagents

All chemicals used in this study were analytical grade sourced from either Sigma-Aldrich (St. Louis, USA) or Merck (Damstadt, Germany). Two types of DNA polymerase reagents were utilised in this study. KOD hot start DNA polymerase from Novgen (Darmstadt, Germany) was used to construct the SAT1 flipper disruption cassette. TaKaRa Ex Taq™ from Takara Bio Inc. (Shiga, Japan) was used for colony PCR amplifications. The 1 Kb plus DNA ladder was obtained from Invitrogen (California, USA). The antibiotic nourseothricin was purchased from WERNER BioAgents (Jena, Germany). The QIAquick Gel Extraction kit was supplied by Qiagen (Valencia, CA) and the Alkali-cation yeast transformation kit was obtained from Qbiogene (Heidelberg, Germany).

5.3.3. Primers designed in this study

Primers were designed using Oligo 6 software (MBI) to have melting temperatures \( T_m \) of approximately 60 °C, with GC contents between 40-60 %, high 5’ end stability, while avoiding secondary structures (no hairpin, self-dimer, and cross dimer structures), and minimum false primer sites. The designed primers were synthesised by either Invitrogen™ (Victoria, Australia) or IDT (Leuven, Belgium). A list of DNA primers used in this study is presented in Table 5.2.
### Table 5.2
DNA primers used in this study

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>GDH2_P1</td>
<td>CCCACCTTTTTTTCTCAC</td>
</tr>
<tr>
<td>GDH2_P2</td>
<td>GATGTAGTGTTGATGTTGC</td>
</tr>
<tr>
<td>GDH2_P3</td>
<td>ATTCCTTGTAAGTTCTCTGTG</td>
</tr>
<tr>
<td>GDH2_P4</td>
<td>TCGCAAGGGAAAAATCAAGT</td>
</tr>
<tr>
<td>SAT1/GDH2_P5</td>
<td>GAACCAACATCAACACTACATCAACAGAAACAGCTATGACCACATG</td>
</tr>
<tr>
<td>SAT1/GDH2_P6</td>
<td>CACAGAGAAACTCAAGGAATCAGTCACGACGTTGAAAAC</td>
</tr>
<tr>
<td>GDH2_C1</td>
<td>TTGGTTGTCTTCTCTTCTTT</td>
</tr>
<tr>
<td>GDH2_C2</td>
<td>GCTTTTGTCCCTTTTAGTA</td>
</tr>
<tr>
<td>GDH2_C3</td>
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<tr>
<td>GDH2_C4</td>
<td>GAGGGTATTTCTCAATTC</td>
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<tr>
<td>GDH2_C5</td>
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<tr>
<td>GDH2_C6</td>
<td>GGCATGAATCTCAACATAAC</td>
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<td>GDH3_P1</td>
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</tr>
<tr>
<td>GDH3_P3</td>
<td>AAAGGTTATAAAGCATTCCC</td>
</tr>
<tr>
<td>GDH3_P4</td>
<td>CTTTCCCTGCACTCTCTCAC</td>
</tr>
<tr>
<td>SAT1/GDH3_P5</td>
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</tr>
<tr>
<td>SAT1/GDH3_P6</td>
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<tr>
<td>GDH3_C1</td>
<td>CTTGCAATAGTTACCAGTAC</td>
</tr>
<tr>
<td>GDH3_C2</td>
<td>GCTTTTGTTCTTTAGTA</td>
</tr>
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<td>GDH3_C3</td>
<td>TTGGCATACGATTAGAGACAC</td>
</tr>
<tr>
<td>GDH3_C4</td>
<td>AAGACAACAAACAAATAACAG</td>
</tr>
<tr>
<td>GDH3_C5</td>
<td>ACGGTTCAGAGTTCAATAAC</td>
</tr>
<tr>
<td>GDH3_C6</td>
<td>GCAATGTGTCAGCCACTTTC</td>
</tr>
</tbody>
</table>
5.3.4. Genomic DNA isolation

The C. albicans strain SC5314 was grown on YPD agar plates at 30 °C. A colony of cells was isolated and suspended in 500 µL phosphate buffer (1.32 g.L⁻¹ Na₂HPO₄, 0.14 g.L⁻¹ NaH₂PO₄ at pH 8). The cells were washed, centrifuged at 2,300 x g for 2 min and the supernatant was discarded. The cell pellets were re-suspended in 270 µL phosphate buffer and 300 µL of SDS lysis buffer (0.06 g.L⁻¹ pH 8.0 Tris-Cl, 16 g.L⁻¹ SDS, and 5.86 g.L⁻¹ NaCl) was added. After adding 300 µL of chloroform-isoamyl alcohol (24:1) and 0.4 g silica bead (0.1 and 2.3 mm, BioSpec), the cells were shaken in a FastPrep® instrument (Qbiogene) at 4.0 m.s⁻¹ for 40 seconds followed by centrifugation at 9,200 x g for 5 min. The supernatant was transferred to a new tube and 360 µL of ammonium acetate (7 M) was added. The SDS layer was separated from aqueous layer by centrifugation at 9,200 x g for 5 min. The aqueous layer was transferred into a new tube and mixed with 310 µL of isopropanol. The mixture was incubated at room temperature for 15 min to precipitate the DNA. After centrifugation at 9,200 x g for 5 min the supernatant was removed and 1 mL of chilled 70% ethanol was used to wash the precipitated DNA. DNA pellets were allowed to air-dry and were then dissolved in 50 µL TE buffer (1.21 g.L⁻¹ Tris-HCl, and 0.29 g.L⁻¹ EDTA).

5.3.5. Construction of SAT1 flipper disruption cassette

Both alleles of target genes were deleted from C. albicans SC5315 using the SAT1 flipper method (Reuß, et al., 2004). Briefly, approximately 0.5 kb DNA upstream (5’ flank) of the open reading frame (ORF) was amplified by genomic PCR using primers P1 and P2 (Table 5.1 and Figure 5.2). Similarly, approximately 0.5 kb DNA downstream (3’ flank) of the open reading frame (ORF) was amplified by genomic PCR using primers P3 and P4. The SAT1 flipper fragment was amplified from plasmid pSFS2 (Reuß, et al., 2004) using primers P5 and P6 that contained 5’
extensions, which were complementary to the 5’ ends of primers P2 and P3, respectively. The details of the PCR reagents and cycling conditions used to amplify the GDH2 gene, GDH3 gene and SAT1 flipper cassette are listed in Table 5.3 and Table 5.4.

Table 5.3
Polymerase chain reaction components used to amplify 5’ flank, 3’ flank, or SAT1 flipper fragments

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 x buffer for KOD polymerase</td>
<td>5 µL</td>
<td>1 X</td>
</tr>
<tr>
<td>MgCl₂ (25 mM)</td>
<td>3 µL</td>
<td>1.5 mM</td>
</tr>
<tr>
<td>Forward and reverse primers (10 µM each)</td>
<td>1.5 µL</td>
<td>0.3 µM</td>
</tr>
<tr>
<td>dNTPs (2 mM each)</td>
<td>5 µL</td>
<td>0.2 mM (each)</td>
</tr>
<tr>
<td>SC5314 DNA (100 ng µL⁻¹) or pSFS2 (10 ng µL⁻¹)</td>
<td>1 µL</td>
<td>2 ng. µL⁻¹ or 0.2 ng. µL⁻¹</td>
</tr>
<tr>
<td>KOD DNA polymerase (1 U.µL⁻¹)</td>
<td>1 µL</td>
<td>0.02 U.µL⁻¹</td>
</tr>
<tr>
<td>PCR-grade water</td>
<td>33 µL</td>
<td></td>
</tr>
<tr>
<td>Total reaction volume</td>
<td>50 µL</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.4
PCR cycling conditions used to amplify 5’ flank, 3’ flank or SAT1 flipper fragments

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature and duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Polymerase activation</td>
<td>95 °C for 2 min</td>
</tr>
<tr>
<td>2. Denaturation</td>
<td>95 °C for 20 s</td>
</tr>
<tr>
<td>3. Annealing</td>
<td>55 °C for 10 s</td>
</tr>
<tr>
<td>4. Extension</td>
<td>70 °C for 10s or 2 min (For pSFS2)</td>
</tr>
<tr>
<td>Repeat steps 2-4</td>
<td>35</td>
</tr>
<tr>
<td>Final extension</td>
<td>70 °C for 2 min</td>
</tr>
</tbody>
</table>
The PCR products were examined by electrophoresis in a 1% agarose gel in 1x TBE (54 g.L\(^{-1}\) Tris-HCl, 27.5 g.L\(^{-1}\) Na\(_2\)B\(_4\)O\(_7\), 2.92 g.L\(^{-1}\) EDTA at pH 8.0) and stained with ethidium bromide (10 mg.mL\(^{-1}\) in water). The PCR fragments were visualized using a Gel Doc\textsuperscript{TM} XR plus system (BIO-RAD) and the sizes of the bands were calculated by comparison to the mobility of a 1 Kb plus DNA ladder (Invitrogen).

To purify the 5’ flank, 3’ flank, and SAT1 flipper fragments for fusion PCR, they were gel purified using a silica-membrane spin column system (QIAquick Gel Extraction kit, Qiagen). The purification process was performed according to the manufacturer’s instructions. In brief, DNA fragments were excised from the agarose gel and 1 volume of gel slice was incubated with 3 volumes of binding buffer at 50 °C for 10 min. After complete dissolution of the gel, 1 volume of isopropanol was added. The sample was added to a Qiagen spin column which was then centrifuged at 9,200 \(\times\) g for 1 min. To wash the DNA fragments, 0.75 mL of washing buffer was added to the spin column followed by centrifugation at 9,200 \(\times\) g for 1 min. To remove residual washing buffer, samples were centrifuged further at 15,700 \(\times\) g for 1 min. To elute DNA fragments from the spin column, 50 µL of eluting buffer was added to the spin column and it was then centrifuged at 15,700 \(\times\) g for 1 min.

Lastly, all three purified DNA fragments were joined together by fusion PCR (Noble & Johnson, 2005) using primers P1 and P4 to complete the construction of SAT1 flipper disruption cassette (Figure 5.2). The specific PCR reagents and PCR conditions used for fusion PCR are listed in Table 5.5 and Table 5.6. The SAT1 flipper disruption cassette was isolated and gel-purified as described previously.
Table 5.5.
Fusion PCR components

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 x buffer for KOD polymerase</td>
<td>10 µL</td>
<td>1 x</td>
</tr>
<tr>
<td>MgCl$_2$ (25 mM)</td>
<td>6 µL</td>
<td>1.5 mM</td>
</tr>
<tr>
<td>P1 and P4 primers (10 µM each)</td>
<td>3 µL</td>
<td>0.3 µM</td>
</tr>
<tr>
<td>dNTP (2 mM each)</td>
<td>10 µL</td>
<td>0.2 mM (each)</td>
</tr>
<tr>
<td>5' flank fragment (50 ng.µL$^{-1}$)</td>
<td>1.5 µL</td>
<td>0.75 ng.µL$^{-1}$</td>
</tr>
<tr>
<td>3' flank fragment (50 ng.µL$^{-1}$)</td>
<td>1.5 µL</td>
<td>0.75 ng.µL$^{-1}$</td>
</tr>
<tr>
<td>SAT1 flipper fragment (50 ng.µL$^{-1}$)</td>
<td>2 µL</td>
<td>1 ng.µL$^{-1}$</td>
</tr>
<tr>
<td>KOD DNA polymerase (1 U.µL$^{-1}$)</td>
<td>2 µL</td>
<td>0.02 U.µL$^{-1}$</td>
</tr>
<tr>
<td>PCR grade water</td>
<td>64 µL</td>
<td></td>
</tr>
<tr>
<td>Total reaction volume</td>
<td>100 µL</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.6.
Fusion PCR cycling condition

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature and duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Polymerase activation</td>
<td>95 °C for 2 min</td>
</tr>
<tr>
<td>2. Denaturation</td>
<td>95 °C for 20 s</td>
</tr>
<tr>
<td>3. Annealing</td>
<td>55 °C for 10 s</td>
</tr>
<tr>
<td>4. Extension</td>
<td>70 °C for 1.5 min</td>
</tr>
<tr>
<td>Repeat steps 2-4</td>
<td>25</td>
</tr>
<tr>
<td>Final extension</td>
<td>70 °C for 2 min</td>
</tr>
</tbody>
</table>
Figure 5.2. The construction of *C. albicans* mutant strains. (A) PCR amplification of DNA fragments (~500 bp) upstream and downstream of target genes using primers P1, P2, P3, and P4. (B) PCR amplification of the SAT1 flipper fragment from plasmid pSFS2 using primers P5 and P6. The SAT1 flipper fragment consists of the maltose-activated promoter (MAL2p), *C. albicans*-adapted FLP gene (*CaFLP*), nourseothricin resistance gene (*CaSAT1*), and FLP recombination target sequence (FRT). (C) Construction of SAT1 flipper disruption cassette by fusion PCR using primers P1 and P4. (D) Chemical transformation of *C. albicans* with disruption cassettes by lithium acetate and gene disruption via homozygous recombination. (E) Verification of the correct integration of the cassette by PCR amplification of the 3' and 5' ends of disrupted alleles using primers C1, C2, C3, and C4. (F) Induction of excision of the SAT1 flipper with maltose via FLP-mediate recombination between FRT sequences. (G) Confirmation of the excision of the SAT1 flipper using PCR with primers C1 and C4. Lastly, primers C5 and C6 were used to confirm disruption of *GDH2* or *GDH3* alleles by colony PCR. The primer sequences are listed in Table 5.1.
5.3.6. *C. albicans* transformation and selection of transformants

*C. albicans* strains were transformed by a combination of heat shock and lithium/caesium acetate provided by Alkali-cation yeast transformation kit (Qbiogene). The transformation protocol was adapted from the kit instructions and the method described by Hernday *et al.* (2010). A colony of *C. albicans* was used to inoculate 10 mL of YPD medium in shake flasks and incubated overnight at 30 °C on a rotary shaker. This culture was then diluted into 30 mL of YPD media to give an initial OD$_{600}$ of 1.0 and incubated at 30 °C on the rotary shaker for 4 h. After centrifugation at 15,700 x g for 5 min the supernatant was discarded and the cell pellet was repeatedly washed in 5 mL of TE buffer and re-suspended in 3 mL of lithium/caesium acetate solution (Qbiogene). The cell suspension was incubated for a further 30 min at 30 °C with shaking. The pellet was then isolated by centrifugation and re-suspended in 300 µL of TE buffer. The following solutions were mixed together: 100 µL of cells in TE buffer, 10 µL of herring sperm DNA (10 mg.mL$^{-1}$), 10 µL of SAT1 flipper disruption cassette (0.1 µg.µL$^{-1}$), and 5 µL of histamine solution (Qbiogene). The mixture was incubated at room temperature for 15 min, followed by adding 1 mL of diluted PEG (0.8 mL of PEG + 0.2 mL of TE/Cation – Kit components) and incubated at room temperature for another 3 h. After this, the cells were exposed to heat shock at 42 °C for 1 h. The cells were isolated by centrifugation and allowed to recover in 5 mL of YPD broth at 30 °C in rotary shaker for 5 h prior to selective growth. Yeast cells were spread on YPD agar plates containing nourseothricin (200 µg.mL$^{-1}$) and incubated at 30 °C for 3 days. Resistant colonies that contained correct 5’ and 3’ junctions of the disrupted alleles were confirmed by colony PCR using primers C1, C2, C3, and C4 (*Figure 5.2* and *Table 5.1*). To perform colony PCR, colonies were picked, suspended in 0.05 mL of nuclease-free water, and incubated at 99 °C for 3 min. A portion of lysed cells was added to the
PCR reaction mixture listed in Table 5.7 and PCR carried out according to the cycling conditions listed in Table 5.8.

Table 5.7
Colony PCR components

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 x buffer for Takara polymerase</td>
<td>1 µL</td>
<td>1 x</td>
</tr>
<tr>
<td>MgCl₂ (25 mM)</td>
<td>0.8 µL</td>
<td>2 mM</td>
</tr>
<tr>
<td>Sense and antisense primers (10 µM each)</td>
<td>0.3 µL</td>
<td>0.3 µM</td>
</tr>
<tr>
<td>dNTP (2.5 mM each)</td>
<td>0.8 µL</td>
<td>0.2 mM (each)</td>
</tr>
<tr>
<td>Lysed cells</td>
<td>3 µL</td>
<td></td>
</tr>
<tr>
<td>Takara Ex Taq DNA polymerase (5 U.µL⁻¹)</td>
<td>0.1 µL</td>
<td>0.05 U.µL⁻¹</td>
</tr>
<tr>
<td>PCR grade water</td>
<td>4 µL</td>
<td></td>
</tr>
<tr>
<td>Total reaction volume</td>
<td>10 µL</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.8
Colony PCR cycling condition

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature and duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Polymerase activation</td>
<td>95 °C for 2 min</td>
</tr>
<tr>
<td>2. Denaturation</td>
<td>95 °C for 10 s</td>
</tr>
<tr>
<td>3. Annealing</td>
<td>55 °C for 10 s</td>
</tr>
<tr>
<td>4. Extension</td>
<td>72 °C for 45 s</td>
</tr>
<tr>
<td>Repeat steps 2-4</td>
<td>35</td>
</tr>
<tr>
<td>Final extension</td>
<td>70 °C for 2 min</td>
</tr>
</tbody>
</table>
The excision of the SAT1 flipper cassette was induced by inoculating 5 mL of YPD broth, containing 2% maltose without nourseothricin, with the nourseothricin-resistant strains and incubating them on a rotary shaker at 30 °C overnight. Approximately 100 cells were plated on YPD agar containing nourseothricin (25 µg.mL⁻¹) and grown at 30 °C for 2 days. Slow growing colonies were patched onto YPD agar plates with and without nourseothricin (200 µg.mL⁻¹) to screen for nourseothricin-sensitive colonies. The excision of the SAT1 flipper cassette was also confirmed by colony PCR using primers C1 and C4. The entire transformation procedure was repeated to delete both copies of GDH alleles. Lastly, a final PCR verification using primers C5 and C6 (Figure 5.2 and Table 5.1) was performed to confirm that no remaining copies of both GDH alleles and SAT1 flipper disruption cassettes were present in C. albicans gdh/gdh homozygous mutants.

5.3.7. Phenotypic tests

5.3.7.1. Preparation of cells for growth assays

C. albicans strains were maintained on YPD agar plates at 30 °C. These cells were used to inoculate 100 mL of YPD broth which was incubated at 30 °C on a rotary shaker overnight. The cells were collected by centrifugation at 805 x g for 5 min and washed in phosphate buffered saline. The cells were re-suspended in the desired growth media to an initial optical density (OD₆₀₀) of 0.1.

5.3.7.2. Morphology of mutant and wild type strains

To study the morphology of mutant and wild type C. albicans strains under various growth conditions, the strains were cultured in eleven different growth media - the compositions of which are listed in Table 5.9. These included the three hyphae-inducing media described in chapter 2 (Han, et al., 2012). The MM¹ medium was the basic minimum mineral medium without any carbon or nitrogen sources. Other hyphae-inducing media were MM¹ medium supplemented with L-arginine (10
mM), L-glutamate (10 mM), L-glutamine (10 mM), L-proline (10 mM), or N-acetylglucosamine (2.5 mM) at pH 6.5. The effect of ammonium (10 mM) or urea (10 mM) as the only nitrogen source on the growth of the different C. albicans strains were also investigated at pH 6.5. The inoculated media were incubated in a rotary shaker at 37 °C for 3 h. The morphology of C. albicans cells in each growth medium was monitored using a phase contrast microscope (DMR, Leica). In order to determine the percentage of yeast and filamentous forms, isolated cells were incubated in 10 mM of NaOH for 10 min and the cluster of filamentous cells was dissociated by sonication for 10 s. The number of yeast and filamentous cells were counted in a Reichert Bright-Line haemocytometer (Hausser Scientific, USA).
Table 5.9
The media used to induce morphogenesis in the *C. albicans* strains

<table>
<thead>
<tr>
<th>Culture media</th>
<th>Media compositions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum mineral medium (MM)</td>
<td>D-glucose (10 g.L⁻¹), (NH₄)₂SO₄ (5 g.L⁻¹), MgSO₄.7H₂O (0.5 g. L⁻¹), KH₂PO₄ (3 g.L⁻¹), vitamins and trace metals at pH 6.5 (Modified from Verduyn et al. 1992).</td>
</tr>
<tr>
<td>Basic mineral medium (MM⁻)</td>
<td>MgSO₄.7H₂O (0.5 g.L⁻¹), KH₂PO₄ (3 g.L⁻¹), vitamins and trace metals at pH 6.5.</td>
</tr>
<tr>
<td>Minimal-proline and N-acetylglucosamine medium (MPA)</td>
<td>MM⁻ medium supplemented with L-proline (10 mM) and N-acetylglucosamine (2.5 mM) at pH 6.5.</td>
</tr>
<tr>
<td>Serum medium</td>
<td>MM⁻ medium supplemented with bovine serum (10 % v/v) at pH 6.5.</td>
</tr>
<tr>
<td>Lee’s medium</td>
<td>(NH₄)₂SO₄ (5 g. L⁻¹), MgSO₄.7H₂O (0.2 g.L⁻¹), KH₂PO₄ (2.5 g.L⁻¹), NaCl (5.0 g.L⁻¹), L-alanine(0.5 g.L⁻¹), L-leucine (1.3 g.L⁻¹), L-lysine (1.0 g.L⁻¹), L-methionine (0.1 g.L⁻¹), L-phenylalanine (0.5 g.L⁻¹), L-proline (0.5 g.L⁻¹), L-threonine (0.5 g.L⁻¹), D-glucose (12.5 g.L⁻¹), and biotin (0.001 g.L⁻¹) at pH 6.5 (Lee, et al., 1975).</td>
</tr>
<tr>
<td>MM⁻ + L-proline</td>
<td>MM⁻ medium supplemented with L-proline (10 mM) at pH 6.5</td>
</tr>
<tr>
<td>MM⁻ + L-arginine</td>
<td>MM⁻ medium supplemented with L-arginine (10 mM) at pH 6.5</td>
</tr>
<tr>
<td>MM⁻ + L-glutamine</td>
<td>MM⁻ medium supplemented with L-glutamine (10 mM) at pH 6.5</td>
</tr>
<tr>
<td>MM⁻ + L-glutamate</td>
<td>MM⁻ medium supplemented with L-glutamate (10 mM) at pH 6.5</td>
</tr>
<tr>
<td>MM⁻ + N-acetylglucosamine</td>
<td>MM⁻ medium supplemented with N-acetylglucosamine (2.5 mM) at pH 6.5</td>
</tr>
<tr>
<td>MM⁻ + D-glucose + ammonium</td>
<td>MM⁻ medium with D-glucose (10 g.L⁻¹), and supplemented with ammonium (10 mM) at pH 6.5</td>
</tr>
<tr>
<td>MM⁻ + D-glucose + urea</td>
<td>MM⁻ medium with D-glucose (10 g.L⁻¹), and supplemented with urea (10 mM) at pH 6.5</td>
</tr>
</tbody>
</table>

MM⁻ is the minimum mineral medium without any carbon and nitrogen sources
5.3.7.3. Growth characterisation and growth rates

C. albicans mutant and wild type strains were cultured in 100 mL of YPD, minimum mineral (MM) or MM' media supplemented with either L-arginine or L-proline. The growth yield of each culture was monitored by dry-weight measurements each hour until the stationary growth phase was reached. The growth rate was calculated by the Equation 5.1, in which $\mu$ refers to the exponential growth rate; $B_0$ denotes biomass measured at the beginning of the exponential growth; $B$ is the biomass measured at the end of exponential growth; $t_o$ is the time (h) at the beginning of exponential growth; and $t$ is the time (h) at the end of exponential growth.

$$\mu = \frac{2.303 \times \log(B - B_0)}{t - t_o}$$

Equation 5.1. The growth rate formula
5.4. Results

5.4.1. Construction of SAT1 flipper disruption cassettes

The SAT1 flipper disruption cassettes for GDH2 and GDH3 gene deletions were successfully synthesised by conventional PCR. The 443 bp upstream and 338 bp downstream of the GDH2 gene were amplified and fused with the 5269 bp SAT1 fragment to form an approximately 6 kb disruption cassette for GDH2 gene deletion (Figure 5.3). Likewise, 527 bp upstream and 446 bp downstream of the GDH3 gene were amplified and fused with the 5269 bp SAT1 fragment to form an approximately 6 kb disruption cassette for GDH3 gene deletion (Figure 5.3).

![Amplification of SAT1 flipper disruption cassettes](image)

**Figure 5.3.** Amplification of SAT1 flipper disruption cassettes. The 5’ flank, 3’ flank and SAT1 fragments were amplified by PCR and the disruption cassettes were generated by joining the three fragments using fusion PCR.
5.4.2. Transformation and PCR verification of GDH2/GDH3 genes knockout mutagenesis

After the first round of transformation, 6 colonies per 1 μg of SAT1 flipper disruption cassette (transformation efficiency) were GDH2 knockout nourseothricin-resistant strains and 8 colonies per 1 μg of SAT1 flipper disruption cassette were GDH3 knockout nourseothricin-resistant strains (Table 5.10). To confirm that the SAT1 flipper disruption cassettes had correctly replaced either the GDH2 or GDH3 gene after double cross-over integrative transformation, the junctions at 5’ and 3’ ends of disrupted alleles were PCR amplified. The successful allele disruptions were validated by using primers C1, C2, C3 and C4 and comparing the correct band sizes as illustrated in Figure 5.4. The expected PCR amplicon sizes for the 5’ check and 3’ check of GHD2 gene were 466 bp and 950 bp. The PCR fragments of GDH2 gene obtained were approximately 500 bp and 1000 bp respectively. Furthermore, the expected PCR amplicon sizes for the 5’ check and 3’ check of GHD3 gene were 607 bp and 751 bp. The PCR fragments of GDH3 gene obtained were approximately 600 bp and 800 bp respectively. Thus, PCR fragments acquired were of the expected sizes confirming correct integrations.

Following successful deletion of the first GDH2 and GDH3 alleles, the SAT1 flipper disruption cassettes were excised out by culturing transformants in medium containing maltose. This induced recombination at the FRT loci and looping out of the nourseothricin resistance gene. The nourseothricin-sensitive derivatives were identified by their smaller colony sizes on YPD agar containing a low concentration of nourseothricin due to their increased drug sensitivity. Figure 5.5 shows that 25 out of 60 colonies were GDH2 knockout nourseothricin-sensitive strains and 80 out of 196 colonies were GDH3 knockout nourseothricin-sensitive strains.

To further confirm that the SAT1 disruption cassettes have been successfully excised from GDH2/gdh2 and GDH3/gdh3 strains the disruption was repeated to
remove the second alleles of each gene, genomic PCR was used with primers C1 and C4 to amplify the region between the upper end of 5’ flank sequence to the lower end of 3’ flank sequence (Figure 5.2). The expected PCR amplicon sizes after the excision of disruption cassettes from GDH2 and GHD3 gene deletions were 1147 bp and 1191 bp respectively. Both PCR amplifications of these regions were observed with approximately 1200 bp. These PCR reactions verified the excision of SAT1 disruption cassettes.

The entire gene disruption procedure was repeated to remove the second alleles of each gene. The transformation efficiency of the second alleles were 9-23 transformants.μg⁻¹ of DNA for GDH2 gene and 12-24 transformants.μg⁻¹ of DNA for GHD3 gene (Table 5.10). The frequencies of removal of SAT1 disruption cassettes were approximately 40%. Lastly, primers C1 and C4 were used again to authenticate that no remaining copy of GHD2 or GHD3 genes were present in the mutant genomes (Figure 5.6).

Table 5.10
The first and second rounds of transformation efficiency

<table>
<thead>
<tr>
<th>Strains</th>
<th># colony</th>
<th>Transformation efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>The first round of transformation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GDH2/gdh2</td>
<td>6</td>
<td>6 transformants.μg⁻¹ of DNA</td>
</tr>
<tr>
<td>GDH3/gdh3</td>
<td>8</td>
<td>8 transformants.μg⁻¹ of DNA</td>
</tr>
<tr>
<td>The second round of transformation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gdh2/gdh2_1</td>
<td>18</td>
<td>18 transformants.μg⁻¹ of DNA</td>
</tr>
<tr>
<td>gdh2/gdh2_2</td>
<td>23</td>
<td>23 transformants.μg⁻¹ of DNA</td>
</tr>
<tr>
<td>gdh2/gdh2_3</td>
<td>9</td>
<td>9 transformants.μg⁻¹ of DNA</td>
</tr>
<tr>
<td>gdh3/gdh3_1</td>
<td>20</td>
<td>20 transformants.μg⁻¹ of DNA</td>
</tr>
<tr>
<td>gdh3/gdh3_2</td>
<td>12</td>
<td>12 transformants.μg⁻¹ of DNA</td>
</tr>
<tr>
<td>gdh3/gdh3_3</td>
<td>24</td>
<td>24 transformants.μg⁻¹ of DNA</td>
</tr>
</tbody>
</table>
Figure 5.4. Correct integration of SAT1 flipper disruption cassettes. The 5’ and 3’ junctions of disrupted GDH2 (A) and GDH3 (B) alleles were verified by genomic PCR amplification. The nourseothricin-resistant strains were tested by growing cells on YPD agar containing 200 µg.mL⁻¹ nourseothricin (30 °C for three days). C, 1, 2 and 3 on the agar plate photos represent parental wild type and three independent transformants respectively. Parental cells were used as the negative control.
Figure 5.5. Screening for nourseothricin-sensitive *C. albicans* transformants after SAT1 flipper disruption cassettes were excised by FLPmediate recombination. (A) YPD agar plate containing GDH2/gdh2 mutant strains. (B) YPD agar plate containing GDH3/gdh3 mutant strains. The larger colonies represent nourseothricin-resistant transformants. The smaller colonies (examples marked by white arrows) correspond to nourseothricin-sensitive transformants in which the SAT1 flipper cassettes had been excised. The cells had been grown on YPD agar containing 25 µg.mL⁻¹ nourseothricin at 30 °C for 2 days.
Figure 5.6. Genomic PCR verification of gene disruption in different *C. albicans* strains constructed in this study using primers C1 and C4. (A) *C. albicans* strains associated with *GDH2* gene knockout. (B) *C. albicans* strains associated with *GDH3* gene knockout. The sizes of DNA fragments (in bp) are indicated on the left and the identity of the fragments are named on the right. *SAT1-FLP-FRT* is the *SAT1*-flipper cassette and *FRT* is the *FLP* recombination sequencing, which remains in the cell after excision of *SAT1* flipper cassette.
5.4.3. Phenotypic characterisations of *C. albicans* gdh2/gdh2 and gdh3/gdh3 mutants

The preliminary phenotypic characterisations of *C. albicans* mutant strains were determinations of morphology and growth rates under various growth conditions that have been previous used to induce morphogenesis. These studies enabled me to gain a better understanding of the effects of *GHD2* and *GHD3* gene deletions on strain fitness and morphogenetic ability.

5.4.3.1. The morphologies of gdh2/gdh2, gdh3/gdh3, and parental strains of *C. albicans* grown on different media.

There was no obvious difference in terms of hyphal development when *C. albicans* wild type (parental) and mutant strains were cultured under the three hyphae-inducing conditions employed in the experiments described in Chapter 2. Both gdh2/gdh2 and gdh3/gdh3 mutants formed filamentous clusters after 3 h incubation in 10% serum, MPA, or Lee’s media, similar to the wild type parental strain (Figure 5.7). Conversely, we observed significant morphological differences between wild type and mutant strains when proline or arginine was provided as sole carbon and nitrogen sources. When grown on proline, both gdh2/gdh2 and gdh3/gdh3 mutant strains failed to form filaments, whilst the wild type developed hyphae (Figure 5.7). On the other hand, during growth on arginine, only the gdh2/gdh2 mutant failed to undergo morphogenesis, whilst both the gdh3/gdh3 and wild type strains completely shifted from yeast to hyphal growth. Consistent results were also observed when I repeated the experiment and monitored the percentages of hypha formation on proline and arginine media over time (Figure 5.8). I observed that the gdh3/gdh3 mutant appeared primed for hyphal formation during growth on arginine, because this mutant shifted from yeast to filamentous growth more rapidly and more extensively than the wild type parental strain. This suggests that *GDH2* and *GHD3* genes have different impacts on the morphogenetic
process of *C. albicans*, which was expected since these two genes encode enzymes that have promote different metabolic equilibria. Although glutamate and glutamine have been previous described to induce morphogenesis in *C. albicans* (Sullivan, *et al.*, 1983, Holmes & Shepherd, 1988), these metabolites did not stimulate germ tube formation and filamentous growth in all strains tested under my culture conditions (*Figure 5.7*).
Figure 5.7. The morphologies of \textit{gdh2/gdh2}, \textit{gdh3/gdh3}, and wild type strains of \textit{C. albicans} grown on various media at 37 °C for 3 h. MM is the minimum mineral medium without any carbon and nitrogen source. MM medium was supplemented with either \textit{N}-acetylglucosamine (NAcGlu), proline, glutamate, glutamine, or arginine. Ammonium (NH\textsubscript{4}\textsuperscript{+}) and urea were supplemented together with 1% glucose in MM medium. The wild type strain is \textit{C. albicans} SC5314. The photos were taken by Nomarksi contrast microscopy with 100x magnification.
Figure 5.8. The percentage of cells forming hyphae when the wild type, \textit{gdh2/gdh2} or \textit{gdh3/gdh3} strains of \textit{C. albicans} were grown on arginine (\textit{A}) and proline (\textit{B}) as sole carbon and nitrogen sources at 37 °C.
5.4.3.2. The growth kinetics of \( \text{gdh2/gdh2}, \text{gdh3/gdh3} \) and wild type strains of \( \text{C. albicans} \)

To characterise the effect of \( \text{GDH2} \) and \( \text{GDH3} \) gene deletions on the growth kinetics of \( \text{C. albicans} \), I monitored the growth of the wild type and mutant strains on YPD, minimum mineral (MM), arginine, and proline media at 37 °C with shaking (200 rpm) (Figure 5.9). We observed no growth difference between wild type and mutant strains on YPD medium. All strains had similar growth rates of approximately \( 0.4 \text{ h}^{-1} \) and reached stationary phase after 6 h with a growth yield of 2.7 g.L\(^{-1}\). When the fungal cells were grown on MM medium, there was also no difference in growth rate (\( 0.40 \text{ h}^{-1} \)) between the wild type and \( \text{gdh2/gdh2} \) strains. The \( \text{gdh3/gdh3} \) mutant, however, showed a 42% reduction in its growth rate (\( 0.23 \text{ h}^{-1} \)) compared to wild type cells. On the other hand, the \( \text{gdh3/gdh3} \) mutant presented similar growth kinetics to the wild type cells when grown on arginine as sole carbon and nitrogen sources, but the \( \text{gdh2/gdh2} \) strain showed a dramatic reduction in its growth rate (\( 0.015 \text{ h}^{-1} \)) and growth yield (\( 0.09 \text{ g.L}^{-1} \)) in comparison to the wild type strain (growth rate = \( 0.092 \text{ h}^{-1} \), biomass = \( 0.30 \text{ g.L}^{-1} \)). This indicates that the \( \text{gdh2/gdh2} \) mutation significantly impaired \( \text{C. albicans} \) growth on arginine as sole carbon and nitrogen source. Similarly, during growth on proline as sole carbon source, both the \( \text{gdh2/gdh2} \) and \( \text{gdh3/gdh3} \) mutant showed lower growth rates (\( 0.093 \text{ h}^{-1}, 0.136 \text{ h}^{-1} \) respectively) and growth yields (\( 0.14 \text{ g.L}^{-1}, 0.24 \text{ g.L}^{-1} \) respectively) compared to the wild type (growth rate = \( 0.206 \text{ h}^{-1} \), biomass = \( 0.31 \text{ g.L}^{-1} \)) (Figure 5.9).
Figure 5.9. The growth curves and growth rates of \textit{gdh2/gdh2}, \textit{gdh3/gdh3}, and wild type strains of \textit{C. albicans} grown on (A) YPD medium, (B) minimum mineral medium, (C) arginine medium, and (D) proline medium. All cultures were incubated at 37 °C with shaking (200 rpm) until the stationary phase was reached. $\mu$ represents the exponential growth rate (h⁻¹).
5.5. Discussion

The only differences in morphologies between both mutants (ghd2/gdh2 and gdh3/gdh3) and wild type strains were observed during growth on arginine or proline as sole carbon and nitrogen sources (Figure 5.7). There was no morphological difference between the mutants and wild type strain when they were grown on relatively rich hyphae-inducing media (serum, MPA, and Lee’s medium; Figure 5.7) as well as on minimal mineral medium supplemented with other compounds such as N-acetylglucosamine, glutamate, and glutamine. Moreover, all mutant strains constructed had similar growth kinetics in rich YPD medium compared to the parental strain (Figure 5.9). I expected to induce a significant alteration in the redox potential of the C. albicans cells when I deleted one of its glutamate dehydrogenases, as previously observed in S. cerevisiae (Villas-Bôas, et al., 2005), and thought this would affect morphogenesis. The deletion of GDH2 or GDH3 genes in C. albicans, however, did not seem to affect its morphogenetic ability greatly. This could mean that the redox potential of the cell is not an important factor in the morphogenesis of C. albicans. However, I did not measure the redox potential of the mutant cells in comparison to the wild type during the different hyphae-inducing conditions tested. C. albicans cells could have the ability to overcome the disequilibrium of the cells’ redox balance by up-regulating alternative pathways capable of recycling the NADH/NADPH pools. This could take place when the mutants are grown in rich media or on particular carbon and nitrogen sources.

Both arginine and proline belong to the glutamate family of amino acids. For them to be used as carbon and nitrogen sources they must be first catabolised into glutamate, followed by a transamination reaction into α-ketoglutarate and ammonia, which is catabolised by glutamate dehydrogenase. Therefore, the deletion of the GDH2 gene will certainly impact on the catabolism of arginine and proline,
considering that Gdh2p is the main enzyme capable of converting glutamate into $\alpha$-ketoglutarate through the reduction of NAD$^+$. As a consequence, the $gdh2/gdh2$ mutant cells would probably alter their metabolism in order to overcome the thermodynamic barrier inherent in using the still present Gdh3p, which is usually an anabolic enzyme with higher affinity for $\alpha$-ketoglutarate than for glutamate. Gdh3p is capable of converting glutamate into $\alpha$-ketoglutarate if the glutamate concentration inside the cells is high (Villas-Bôas, et al., 2005). This need for increasing the concentration of glutamate inside the cell to reverse the direction of the reaction catalysed by Gdh3p towards a catabolic reaction will certainly affect the NAD$^+$/NADH and NADP$^+$/NADPH pools of the mutant cells and consequently alter their redox potential. Therefore, we could speculate that the effect of the $gdh2/gdh2$ mutation can only affect the cell’s redox potential when mutants are grown on arginine and proline as sole carbon and nitrogen sources.

On the other hand, the $gdh3/gdh3$ mutant should easily catabolise both arginine and proline via their conversion into glutamate and deamination into $\alpha$-ketoglutarate, because Gdh2p is still present and has a high affinity for glutamate. Therefore, I would expect the $gdh3/gdh3$ mutant phenotype to be more similar to that of the wild type than the $gdh2/gdh2$ phenotype and this was indeed observed in the morphogenesis assays and growth characterisations. However, the $gdh3/gdh3$ mutant showed significantly attenuated growth and reduced filament formation when growing on proline as sole carbon and nitrogen sources (Figure 5.8, Figure 5.9). This could be explained by the fact that the catabolism of proline involves only NAD$^+$-dependent reactions, including the Gdh2p-catalysed reaction. This is not the case for the catabolism of arginine. Therefore, when proline is the only carbon and nitrogen source, the $gdh3/gdh3$ mutant cells would be probably experiencing a serious shortage of the NAD$^+$ required to catabolise proline, which could inhibit their growth rate as well as alter their redox balance, affecting morphogenesis.
Although glutamate and glutamine are also part of the glutamate family of amino acids, the minimal mineral medium supplemented with either glutamate or glutamine as sole carbon and nitrogen sources did not induce the filamentous growth of either the \textit{gdh2/gdh2}, the \textit{gdh3/gdh3} mutant or the wild type strain. This is surprising considering that the \textit{gdh2/gdh2} mutant would have an impaired ability to catabolise glutamate, which could also affect the redox balance of the mutant. However, both glutamate and glutamine are good nitrogen and carbon sources. Glutamine can be easily deaminated into glutamate, increasing the intracellular glutamate concentrations. Therefore, Gdh3p should easily replace the Gdh2p catabolic role when \textit{gdh2/gdh2} cells were grown on glutamate and glutamine because the glutamate level inside the cells would be significantly higher in both media when compared to cells grown in arginine- or proline-containing media. Thus, the mutant cells would not require extensive alteration of their central metabolism in order to increase intracellular concentrations of glutamate (in the \textit{gdh2/gdh2} mutant) or α-ketoglutarate (in the \textit{gdh3/gdh3} mutant).

Therefore, since the \textit{gdh2/gdh2} mutant cells showed an impaired ability to undergo morphogenesis when growing on arginine- or proline-containing media, and the \textit{gdh3/gdh3} mutant showed reduced morphogenesis when growing on proline; I believe that my hypothesis regarding the redox potential of the cell being associated with the morphogenetic process of \textit{C. albicans} cannot be totally discarded. However, more experiments involving metabolomic analyses and measurements of the redox potential of the mutant cells in comparison with the wild type strain must be carried out in order to validate this hypothesis. Furthermore generating a double knockout of \textit{GHD1} and \textit{GHD3} genes as well as other knockout mutations of genes involved in nitrogen and nicotinamide metabolism, with the potential for altering the cell’s redox balance could also be pursued to better characterise \textit{C. albicans} morphogenesis at a central carbon metabolism level.
5.6. Conclusion

This is the first gene knockout mutagenesis study investigating the effect of \textit{GHD2} and \textit{GHD3} gene deletions on the morphological switch of \textit{C. albicans}. I have observed differential morphogenetic responses between mutant and wild type strains when grown on arginine and proline as the sole carbon and nitrogen sources. I demonstrated that certain amino acids, such as arginine and proline, induce morphogenesis in \textit{C. albicans} through nitrogen metabolism, most likely by altering the redox balance of the cell. Further studies, however, are required to gain a better understanding of this process.
5.7. Acknowledgements

We thank J.E. Uritchard, E. Lamping, and A.R. Homlers for technical assistance with gene knockout mutagenesis. This work was supported by Faculty of Science Research Funding, University of Auckland and by the University of Auckland Doctoral Scholarship for Ting-Li Han.
5.8. References


CHAPTER VI

Overall discussion
6.1. Discussion

This study is the first to use metabolomics and isotope labelling approaches to elucidate how the fungus, *Candida albicans*, changes its cellular metabolism during the yeast-to-hyphal transition and *vice versa*. Although much research to date has been dedicated to examining the signalling transduction and transcription regulators during morphogenesis, the aim of this work was to shed light on the downstream metabolic mechanisms that enable cells to undergo morphological changes as a result of signal transduction. The application of metabolomics was a vital approach in realising this aim by creating comparative metabolic profiles under various hyphae-inducing media and quorum sensing perturbations. The results showed a global downregulation of central carbon metabolism during the early yeast-to-hyphal transition. I also observed a lower intracellular ATP concentration in hyphae than in yeast cells. I was able to reinforce these results by revealing that yeast cells exhibit a higher level of metabolic activities than hyphae when filamentous growth is suppressed by quorum sensing molecules such as farnesol and phenylethyl alcohol. Therefore, my results provided a clear and consistent picture that the morphological changes are correlated with global changes in the central carbon metabolism.

Thus, I hypothesised that once the metabolic activity of yeast cells is temporarily disrupted by environmental changes, such as starvation, stress, alkaline pH, depletion of oxygen or high temperatures, the central carbon metabolism of *C. albicans* is globally downregulated. This downregulation of the central carbon metabolism triggers changes to a growth form that a result of there being less energy available, which is filamentous growth. I also speculated that hyphae exhibit lower metabolic activities due to the preference of utilising alternative carbon sources in contrast with the prioritisation of sugar metabolism in the yeast form. *C. albicans* often lives in the gastrointestinal tract of its mammalian host and on
epithelial surfaces where there is an abundance of different carbon sources and competition with other microorganisms for nutrients is intense. The ability to assimilate alternative carbon and nitrogen sources and enable fast growth during filamentous development, at the same time, with minimal energy expenditure, seems to be an advantageous survival strategy.

My experiments with QSMs also indicated that farnesol and phenylethyl alcohol seem to drive an alteration in lipid biosynthetic pathways in *C. albicans*, most likely affecting cell membrane composition. I postulated that *C. albicans* may change the composition of its cell membranes in order to minimise the antimicrobial effects of farnesol and phenylethyl alcohol. Therefore, this unique metabolic response may enhance *C. albicans*’ fitness in terms of inter- and intra-species competition. By using an isotope labelling approach, phenylethyl alcohol appeared to be catabolised throughout the central carbon metabolism and metabolised mainly into NADPH and NADH through the conversion of its aromatic ring into the pyridine ring of the nucleotides. This discovery pointed out the potential role of the redox balance of the cell in the quorum sensing-mediated morphogenesis of *C. albicans*, because the increase in the NADH/NADPH pool induced by phenylethyl alcohol will affect the redox balance of the cell.

Through combining the results of three separate approaches (hyphae induction, hyphae suppression, and isotope labelling) I was able to identify two metabolic pathways, nitrogen metabolism and nicotinate/nicotinamide metabolism, which are likely to be intrinsically related with the morphogenetic process in *C. albicans* and that have been poorly studied in this context. Therefore, I deleted the *C. albicans* glutamate dehydrogenase genes (*GDH2* and *GHD3*) that participate in the nitrogen assimilation/excretion and regeneration of NADH/NADPH. I observed distinct morphogenetic abilities of the mutants compared to the wild type under growth on proline and arginine as sole carbon and nitrogen sources but this was not
observed when mutants were grown under other hyphae-inducing conditions. Since both proline and arginine change the redox potential and ammonium concentration in the cells upon their entry into TCA cycle via glutamate dehydrogenase, the gene deletion results further strengthened my hypothesis that NADH/NADPH and nitrogen metabolism are likely to be related to filament formation. Nevertheless, further studies are required to fully understand the underlying biochemical process.

The ultimate goal behind this PhD project was to couple metabolomics results with current knowledge of C. albicans morphogenesis at a signal transduction level in order to elucidate the metabolic framework that governs C. albicans morphogenesis. Thus to achieve this aim and facilitate future studies, I have attempted to connect the metabolic pathways I identified as being important in morphogenesis with the signalling pathways previously associated with morphogenesis as well as an in silico construction of the central carbon metabolic network for C. albicans based on my metabolomics data. A diagrammatic representation of this work is shown in Figure 6.1. In brief, the cAMP-PKA and MAP kinase signalling pathways are likely to be associated with nitrogen metabolism since C. albicans is unable to undergo hyphal formation in response to nitrogen starvation upon the disruption of genes upstream of these signalling pathways (e.g. MEP2 and RAS1) (Feng, et al., 1999, Biswas & Morschhäuser, 2005). Although there is a lack of studies demonstrating the effect of signalling pathways on the redox potential of cells, the GDH2 and GDH3 genes, known to change the pool of NADH/NAD+ and NADPH/NADP+, are transcriptionally regulated by Nrg1p, Ssn6p, Efg1p, and Rim101p (Murad, et al., 2001, Bensen, et al., 2004, Doedt, et al., 2004). The influence of other signalling pathways on metabolic pathways is illustrated in Figure 6.1.
Figure 6.1. Preliminary proposal of how metabolic pathways identified in this study as being important in morphogenesis are linked with signalling pathways associated with morphogenesis. (A) Signal transduction pathways and transcriptional regulators affecting the filamentous development of *C. albicans* (derived from Figure 1.1). (B) The metabolic activity of morphogenesis-affected metabolic pathways in response to signal transduction. These
responses are determined based on the following studies: aKlinke et al. (2008); bEnjalbert et al. (2006); cMurad et al. (2001); dDoedt et al. (2004); eRamon et al. (1999); fBensen et al. (2004); and the superscript question marks indicate that the linkage between metabolic and signalling pathways are proposed by my study. (C) A metabolic network created by linking the common metabolites among TCA cycle, glycolysis, amino acid metabolism, and pentose phosphate pathway that had their levels altered during C. albicans morphogenesis. This two-dimensional network is structured regarding to the hierarchical drawing of directed graph, which organises the metabolic network in a top-to-bottom fashion with minimised line crossings and line length (Eades, et al., 1996). The arrowheads indicate the reaction directions and the point of arrowheads are metabolites. The black nodes represent metabolites identified in our metabolomics data. The abbreviations for metabolites (inside black nodes) are listed in Table 6.1.

Table 6.1
The abbreviations for metabolites displayed on 2D metabolic network (Figure 6.1)

<table>
<thead>
<tr>
<th>Abbrev</th>
<th>Name of metabolite</th>
<th>Abbrev</th>
<th>Name of metabolite</th>
</tr>
</thead>
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<tr>
<td>Acn</td>
<td>cis-Aconitate</td>
<td>Mal</td>
<td>Malate</td>
</tr>
<tr>
<td>Ala</td>
<td>Alanine</td>
<td>Met</td>
<td>Methionine</td>
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<td>Asn</td>
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<td>N-Acetyglutamate</td>
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<td>Ornithine</td>
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<td>Oxo</td>
<td>Oxoglutarate</td>
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<td>Citraconate</td>
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<td>Phosphoenolpyruvate</td>
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<td>Phe</td>
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<td>Pro</td>
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<td>Pyruvate</td>
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<td>Isoleucine</td>
<td>SAM</td>
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<td>2-Isopropylmalate</td>
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<tr>
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<td>3MO</td>
<td>3-Methyl-2-oxopentanoate</td>
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<tr>
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<td>Lysine</td>
<td>SAL</td>
<td>5-Aminolevulinate</td>
</tr>
</tbody>
</table>
6.2. Future work

This project has opened up several avenues for future research in order to provide a more comprehensive metabolic view of *C. albicans* morphogenesis. Primers have been designed and purchased in preparation for restoring the deleted *GDH2* and *GDH3* genes in the knockout mutant. If the phenotypes of the mutants can be successfully restored to wild type, this will confirm that only the correct genes have been deleted. To gain a better insight into the NADH/NADPH-mediated morphogenesis, the redox potential of mutant cells, as well as of quorum sensing-treated cells in comparison with the wild type (control) cells should be measured. This can be achieved by determining the intracellular levels of NADH/NAD and NADPH via commercial enzymatic kits. Furthermore, the ability of *C. albicans* to open a benzene ring and subsequently cyclize it into a pyridine ring should also be tested - possibly by incubating an extract of *C. albicans* cells with $^{13}$C-U-labelled-benzene to determine if pyridine or pyridine derivatives labelled with isotopic carbon can be detected. Alternatively, phenylethyl alcohol, fully labelled with $^{13}$C, could be custom synthesized and used to confirm my results from the inverse isotope-labelling experiment.

Additional metabolomics experiments are necessary to elucidate the metabolic effects of *GDH2* and *GDH3* gene deletions on *C. albicans* metabolism. The morphology of mutants can also be tested under diverse environmental parameters (e.g. pH, temperature, and other amino acids as different carbon and nitrogen sources) in order to acquire a more complete picture of mutant phenotypes. The conditions that display differential morphological behaviour between mutant and wild type strains can then be used for other growth characterisations. It is important to determine the biomass yields and growth curves for each strain cultured under the chosen conditions.
CHAPTER VI

Other genes encoding key enzymes involved in the redox balance and in nitrogen metabolism could also be deleted to determine the consequences of their disruption on *C. albicans* morphogenesis. Potential genes to be studied include: NAD⁺ synthase (*NADSYN1*) and NAD⁺ kinase (*NADK*) which participate in the biosynthesis of NAD⁺ and NADP⁺; enzymatic genes involved in pentose phosphate pathways such as glucose-6-phosphate 1-dehydrogenase (*ZWF1*), 6-phosphogluconolactonase (*SOL3*), and phosphogluconate dehydrogenase (*GND1*), the primary functions of which are to regenerate NADPH from NADP⁺. However, the disruption of these genes may be lethal to *C. albicans*. Alternative disruptions could target the genes directly involved in the assimilation of ammonium into glycine (*GCV1* - T subunit of glycine decarboxylase), asparagine (*ASP1* - asparaginase), and glutamine (*GLN* - glutamine synthetase), or simply a double gene knockout of *GDH2* and *GDH3* genes together. These additional gene deletions would help gain a better understanding of the effect of cellular metabolism on fungal morphogenesis.

In conclusion, metabolomics has been demonstrated to be a promising approach for understanding the metabolic changes that occur *in vivo* during the morphogenesis of *C. albicans*. I have shown that *C. albicans* globally reprograms its central carbon metabolism during the early morphological transition and I have identified several key metabolic pathways likely to drive fungal morphogenesis. The results I have obtained consistently indicated the importance of redox potential and nitrogen metabolism on the regulation of hyphal development, which identify these metabolic pathways as potential drug targets. If crucial enzymatic steps specific to fungal cells and essential for these metabolic pathways are found then the inhibition of these enzymes could serve as a possible therapeutic intervention against candidiasis.
6.3. References


