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Physiology, Regulation, and Pathogenesis of Nitrogen Metabolism in the Opportunistic Fungal Pathogen *Candida albicans*

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Physiology, Regulation, and Pathogenesis of Nitrogen Metabolism in the Opportunistic
Fungal Pathogen *Candida albicans*

By

Suman Ghosh

A Dissertation

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Physiology, Regulation, and Pathogenesis of Nitrogen Metabolism in the Opportunistic
Fungal Pathogen *Candida albicans*

Suman Ghosh

University of Nebraska, 2009

Advisor: Kenneth W. Nickerson

ABSTRACT

Candida albicans is an opportunistic polymorphic fungus that causes clinically important disease candidiasis to humans. Being polymorphic *C. albicans* can grow in yeast, hyphae, or pseudohyphae forms and the switch from one form to another is required for virulence. The morphological transitions from one phase to another are carefully orchestrated events which are regulated by several signal transduction pathways. Several environmental factors determine the morphology of the fungus *C. albicans*. For example growth at lower temperature ~30 °C, in preferred nitrogen sources cause the fungus to grow as yeasts while at higher temperature ~37 °C and in poor nitrogen sources, in the presence of serum, N-acetyl glucosamine, or high CO₂, *C. albicans* cells grow as hyphae. Under several clinically relevant circumstances, including biofilms, *C. albicans* cells encounter poor or low nitrogen conditions. In this project utilization of different nitrogen sources by *C. albicans* was evaluated and their roles in pathogenesis were studied. The aromatic amino acids are metabolized when the *C. albicans* cells grow under poor nitrogen conditions, and the resulting carbon skeletons are secreted outside the cell. They are well known as fusel oils or aromatic alcohols. The

aromatic alcohol biosynthesis is enhanced under anaerobic conditions compared to aerobic conditions, by the presence of precursor amino acids (phenylalanine, tyrosine, or tryptophan), and in alkaline conditions compared to acidic conditions, but it is reduced greatly in the presence of ammonia. Also, aromatic alcohol yield is dependent on the transcription regulators Aro80p and Rim101p. In another project the role of arginine metabolism in the yeast to hypha morphological switch was studied. When *C. albicans* cells enter the bloodstream, they first encounter macrophages and are engulfed by them. But in four to six hours *C. albicans* cells form hyphae, penetrate and kill the macrophages, and get out in the bloodstream again. In this series of events at the initial phase, right after engulfment, *C. albicans* up-regulates arginine biosynthesis. We found that arginine biosynthesis is critical for the fungus because it is metabolized and produces CO₂ inside the cell, a signal important for the yeast to hypha switch. *C. albicans* mutants that either failed to make arginine (arginine auxotrophs) or could not metabolize arginine to CO₂ (urea amidolyase mutants) were defective in making germ tubes inside the macrophages. However, wild type *C. albicans* and *C. albicans* auxotrophic for other amino acids than arginine can kill macrophages within four to six hours after phagocytosis. So, another project studied if macrophages can induce appropriate cytokines within that short time span before being killed by *C. albicans*. We found that after engulfing *C. albicans*, macrophages induce cytokines within one hour. Chief among them were IL-6, IL-23, and TGF- β , important for the development of the Th-17 subset of T cells. Finally, two major components of *C. albicans*, the quorum sensing molecule farnesol and the cell wall component zymosan, together induced TLR2, a pattern recognition receptor, and both were responsible for the induction of IL-6, IL-23, and

TGF- β . Farnesol was ca. 100 times more effective than farnesoic acid at inducing these cytokines. Overall, this body of work has taken a major step towards elucidating farnesol's mode of action as a virulence factor and a lipid signaling molecule while at the same time highlighting the magnitude of the gaps remaining our knowledge.

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TABLE OF CONTENTS

| | |
|---|------------|
| ACKNOWLEDGEMENTS..... | ii |
| TABLE OF CONTENTS..... | iv |
| LIST OF FIGURES..... | vii |
| LIST OF TABLES..... | x |
| ABBREVIATIONS..... | xi |
| | |
| CHAPTER 1: Introduction..... | 1 |
| | |
| CHAPTER 2: Regulation of aromatic alcohol production in <i>Candida albicans</i>..... | 26 |
| Abstract..... | 27 |
| Introduction..... | 28 |
| Methods..... | 29 |
| Results..... | 32 |
| Discussion..... | 38 |
| References..... | 41 |
| Figure legends..... | 49 |
| Figures..... | 52 |
| | |
| CHAPTER 3: The Regulation of Aro80p in <i>Candida albicans</i>..... | 57 |
| Abstract..... | 58 |

| | |
|---------------------|----|
| Introduction..... | 59 |
| Methods..... | 60 |
| Results..... | 62 |
| Discussion..... | 65 |
| References..... | 67 |
| Figure legends..... | 70 |
| Figures..... | 71 |

| | |
|---|-----------|
| CHAPTER 4: Arginine induced germ tube formation in <i>Candida albicans</i> is essential for escape from murine macrophage RAW264.7 Line..... | 73 |
| Abstract..... | 74 |
| Introduction..... | 75 |
| Methods..... | 77 |
| Results..... | 80 |
| Discussion..... | 86 |
| References..... | 92 |
| Figure legends..... | 100 |
| Figures..... | 104 |

| | |
|---|------------|
| CHAPTER 5: <i>Candida albicans</i> Activates Th17-Inducing Cytokines in Murine Macrophage RAW264.7 Line..... | 114 |
| Abstract..... | 115 |
| Introduction..... | 116 |

| | |
|---|------------|
| Methods..... | 118 |
| Results..... | 123 |
| Discussion..... | 127 |
| References..... | 131 |
| Figure legends..... | 138 |
| Figures..... | 140 |
| | |
| CHAPTER 6: Future Directions..... | 143 |
| 6.1. The role of aromatic alcohols in morphogenesis in <i>C. albicans</i> | 144 |
| 6.2. A hypothesis on recruitment of Tup1p with Gcn4p derepression results in morphogenesis in <i>C. albicans</i> | 150 |
| 6.3. Early phase arginine biosynthesis inside the macrophage is regulated by non-sense mediated mRNA decay (NMD) pathway in <i>C. albicans</i> | 151 |
| 6.4. Arginine induced biofilm formation is mediated by nitrogen catabolite repression in <i>C. albicans</i> | 153 |

LIST OF FIGURES

| | |
|---|----|
| Figure 1-1. Model for Nitrogen Catabolite Repression..... | 24 |
| A. Nitrogen Catabolite Repression (NCR) | |
| B. Model of reciprocal regulation of GATA factor gene expression and GATA factor regulation of NCR-sensitive gene expression per se | |
| Figure 1-2. Summary of the major control mechanisms regulating Gcn4p levels in the cell and transcription of target genes subject to GAAC..... | 25 |
| Figure 2-1. Regulation of the production of aromatic alcohols from aromatic amino acids..... | 52 |
| Figure 2-2. GC/MS analysis of ethyl acetate extracts from cell free supernatants of <i>C. albicans</i> | 53 |
| Figure 2-3. Effects of environmental conditions on production of aromatic alcohols.... | 54 |
| Figure 2-4. Effects of Aro80p on production of aromatic alcohols..... | 55 |
| Figure 2-5. Effects of pH on production of aromatic alcohols..... | 56 |
| A. GC/MS analysis of <i>C. albicans</i> at 30°C | |
| B. Northern blot analysis of RNA prepared from mid-log phase cells grown at 30°C | |
| Figure 3-1. Effects of nitrogen catabolite repression (NCR) on <i>ARO80</i> | 71 |
| A. Growth of <i>aro80/aro80</i> mutant on aromatic amino acids | |
| B. Northern blot analysis of RNA prepared from SC5314 mid-log phase cells grown in GPP (P), GPR (R), or GPA (A) at 30°C | |
| Figure 3-2. Effects of pH on production of aromatic alcohols in <i>aro80/aro80</i> strains.... | 72 |
| A. GC/MS analysis of <i>C. albicans</i> at 30°C | |

| | |
|--|-----|
| B. Northern blot analysis of RNA prepared from mid-log phase cells grown at 30°C | |
| Figure 4-1. Urea amidolyase mutants..... | 104 |
| A. Growth of <i>dur1,2/dur1,2</i> mutant on urea | |
| B. Germ tube formation by GlcNAc, arginine, urea and CO ₂ in <i>dur1,2/dur1,2</i> mutants | |
| Figure 4-2. Germ tube formation by GlcNAc, arginine, urea, and CO ₂ in non-filamentous mutants..... | 105 |
| Figure 4-3. Suggested pathway for arginine induced germ tube formation..... | 106 |
| Figure 4-4. Interaction of <i>C. albicans</i> with macrophages..... | 107 |
| A. <i>C. albicans</i> A72 (<i>DUR1,2/DUR1,2</i> , parental strain) (first row), KWN6 (<i>dur1,2/dur1,2</i>) (second row) and KWN8 (<i>dur1,2/dur1,2::DUR1,2/DUR1,2</i>) (third row)..... | 107 |
| B. SC5314 (<i>URA3/URA3</i> , wild type) (first row), CAF2-1 (<i>ura3/URA3</i>) (second row) and CAI4 (<i>ura3/ura3, iro1/iro1</i>) (third row)..... | 108 |
| C. Auxotrophic mutants..... | 109 |
| D. <i>gcn4</i> mutants..... | 110 |
| Figure 4-S1. Germ tube formation in KWN6 (<i>dur1,2/dur1,2</i>) as induced by 5% CO ₂ | 111 |
| Figure 4-S2. Interaction of <i>C. albicans</i> with macrophages..... | 112 |
| Figure 4-S3. Interaction of <i>C. albicans</i> with macrophages..... | 113 |
| Figure 5-1. RAW264.7 cells express IL-6 (A), TGF-β (B), IL-23 p40 (C) and IL-23 p19 (D) in response to challenge by <i>C. albicans</i> | 140 |
| Figure 5-2. RAW264.7 cytokine expression in response to the challenge by yeast cell components..... | 141 |
| Figure 5-3. Expression of TLRs in RAW264.7 cells..... | 142 |
| Figure 6-1. Effects of aromatic alcohols in morphogenesis in wild type <i>C. albicans</i> | 163 |

| | |
|--|-----|
| Figure 6-2. Effects of tyrosol in morphogenesis in non-filamentous mutants..... | 164 |
| Figure 6-3. Farnesol tyrosol competition in morphogenesis in <i>C. albicans</i> | 165 |
| Figure 6-4. Derepression of Gcn4p by aromatic alcohols in <i>C. albicans</i> | 166 |
| Figure 6-5. Model of aromatic amino acid metabolism and morphogenesis in <i>C.</i> <i>albicans</i> | 167 |
| Figure 6-6. Effect of arginine in wrinkled type colony formation in <i>C. albicans</i> | 168 |
| Figure 6-7. Effect of nitrogen sources on biofilm formation <i>in vitro</i> by wild type <i>C.</i> <i>albicans</i> | 169 |

LIST OF TABLES

Table 4-1: Germ tube formation in wild type and mutant *C. albicans*.....99

Table 6-1: Mutant strains that formed wrinkled colonies on YPD.....162

Table 6-2: Mutant strains that formed smooth colonies on GPR.....162

ABBREVIATIONS

AIDS: Auto immune deficiency syndrome

cAMP/PKA: cAMP dependent protein kinase A

GAAC: General amino acid control response

GlcNAc: N-acetyl glucosamine

GPA: Glucose ammonium sulfate phosphate media

GPP: Glucose proline phosphate media

GPR: Glucose arginine phosphate media

GTF: Germ tube formation

GTK: Germ tube kinetics

IFN- γ : Interferon gamma

IL: Interleukin

MAPK: Mitogen activated protein kinase

NCR: Nitrogen catabolite repression

PEA: Phenethyl alcohol

PRR: Pattern recognition receptor

TGF- β : Transforming growth factor-beta

Th: T helper

TLR: Toll like receptor

TOH: Tyrosol

TrpOH: Tryptophol

YPD: Yeast extract, tryptone, dextrose media

CHAPTER 1

Introduction

Candida albicans is a polymorphic opportunistic pathogen that is a part of normal microbial flora of mouth, gastrointestinal and genitourinary tract, and to a lesser extent the skin, of most humans as a commensal. Patients with compromised immune systems such as those with AIDS, those undergoing cancer chemotherapy or organ and bone marrow transplants, those born prematurely, and those whose normal flora have been eliminated by antibiotics are all at high risk for a lethal infection called candidiasis. Etiologically candidiasis can be oropharyngeal/esophageal (OPC), genital/vulvovaginal (VVC), or invasive candidiasis / candidemia. In the latter case the fungus *C. albicans* enters the blood causing bloodstream infection. Invasive candidiasis or candidemia is fourth most common cause of nosocomial bloodstream infections among hospitalized patients in the United States. A survey conducted at CDC reported incidence of candidemia is 8 cases per 100,000 in the general population. Higher incidences occur among neonates and African-Americans. There are number of drugs currently available for the treatment of candidiasis. Fluconazole or voriconazole (azole drugs) are the drugs of choice, but there are other drugs such as echinocandins (caspofungin), amphotericin B, which are used alone or in combination in cases of candidiasis. *C. albicans* becomes resistant to these drugs in case of repeated use. Development of new drugs and new targets therefore is imperative for successful control of the disease candidiasis.

The fungal pathogen *C. albicans* shows considerable plasticity in morphology. It can grow in yeast form or hyphal form or intermediate as pseudohyphae. Yeast cells are oval single cells and they divide by budding (axial or bipolar pattern), give rise to two asymmetrical mother and daughter cells. After START there is transition from G1 to S phase of cell cycle. Emergence of a bud is associated with replication of DNA and

spindle pole body duplication. After mitosis (M), yeast cells separate by cytokinesis, giving rise to a mother and a small daughter cell. Daughter cells enter the next cell cycle slightly after the mother when they reach the same cell size as their mothers. The budding mechanism of *C. albicans* yeast cells and is similar to *S. cerevisiae* (27). Like a true fungus *C. albicans* can also form germ tubes. Germ tube forms after induction of initial bud site selection, and extends to an elongated filament separated by septae (2).

Pseudohyphae are an intermediate state but the mechanism of pseudohyphae formation more closely resembles budding. Pseudohyphae are elongated cells connected in chains that resemble hyphae, but individually similar to yeast. They basically have delayed isotropic growth after bud emergence and they divide as unipolar budding pattern (2, 27). In certain conditions *C. albicans* can also form chlamydospore, which is a distinct larger cellular form (42).

Key virulence factors leading to mucosal or systemic candidiasis are: morphogenesis – yeast to hyphae switching; phenotypic switching, e.g. white – opaque switching; epithelial adhesion; production of extracellular enzymes, e.g. phospholipase B and aspartyl proteases; and production of farnesol (33-35). Each and every factor is being studied to identify the mechanisms that are unique to *C. albicans*. The yeast to hypha switch has been very well studied (1, 4, 29, 46). It is a carefully coordinated event which is regulated by multiple factors and several signal transduction pathways. The environmental triggers for hyphal development include growth at 37°C, the presence of serum or *N*-acetylglucosamine (GlcNAc), non-acid pH, high CO₂, and nitrogen starvation (1, 4, 29, 46). These environmental stimuli act by turning on one or more signal transduction pathways that stimulate hyphal specific genes. These pathways include the

CPH1-mediated MAPK pathway and the *EFG1*-mediated cAMP dependent protein kinase A (PKA) pathway, which has two isoforms of PKA, Tpk1p and Tpk2p, with differential effects on hyphal morphogenesis. Two other hyphal regulators, Rim101p and Czf1p, may function through Efg1p or act in parallel with Efg1p while another transcription factor *TEC1* is regulated by either or both Efg1p and Cph1p. The MAPK cascade includes Cst20p (MAPKKK), Hst7p (MAPKK), Cek1p (MAPK), and the downstream transcription factor Cph1p, which is a homolog of the *S. cerevisiae* transcription factor Ste12p. *C. albicans* also has negative regulators of the hyphal transition. Chief among these is Tup1p which acts in concert with Rfg1p, Nrg1p, or Rbf1p (1, 4, 29, 46). The downstream targets of these environmental sensing pathways include the hyphal wall protein Hwp1p, adhesins (the ALS family), and extracellular hydrolytic enzymes (secreted aspartyl proteases, phospholipases) (1, 4, 29, 46).

Nitrogen metabolism in *C. albicans* is complex and linked to the yeast – hypha morphological switch (3, 8, 44). Like most other microorganisms, *C. albicans* prefers some nitrogen sources over others. Ammonia and glutamine are preferred by *C. albicans* over other nitrogen sources such as proline, histidine, arginine, and urea (32). This phenomenon is called nitrogen catabolite repression (NCR) and has been demonstrated in numerous fungi (32). In response to activation of NCR genes, fungi initiate morphological changes, express virulence factors, or initiate sexual and asexual sporulation (32). For example the virulence of *Aspergillus fumigatus*, a pulmonary pathogen, is dependent on its ability to respond to limited nitrogen sources (14). Similarly, *Cryptococcus neoformans* haploid MAT α cells switch from yeast to hyphae and develop fruiting bodies under limited nitrogen sources, and this phenomenon is

inhibited by ammonia (47). Another prime example of inhibition of NCR is induction of rice blast disease by *Magnaporthe grisea* under poor nitrogen conditions (10). Diploid *Saccharomyces cerevisiae* also differentiate to pseudohyphae when grown on poor nitrogen sources. A mutation in *SHR3*, a gene required for amino acid uptake, also enhances the pseudohyphal phenotype in this organism (11). *C. albicans* grows as budding yeasts in the presence of ammonia as a nitrogen source but as hyphae when grown in the presence of poor nitrogen sources (3). When ammonia is absent, or present in only low concentrations, *C. albicans* cells express *MEP1* and *MEP2* genes which encode ammonium transporters. Mep2p, but not Mep1p, activates both the Cph1p-dependent MAP kinase and the cAMP-dependent protein kinase A pathways, and thereby inducing morphogenesis in poor nitrogen conditions (3). *MEP2* is under the control of two NCR positive regulators *GLN3* and *GATI*. *GLN3* mutants do not form filaments in poor nitrogen sources (8). Addition of ammonium ions also represses proline uptake (16), peptide transporters (37), and inhibits expression of secreted aspartyl proteinases (20, 31, 45).

The mechanism for genetic regulation of NCR is well established in *S. cerevisiae*. When excess or preferred nitrogen sources are available, the cells activate Ure2p, which represses the positive regulators of NCR, *GLN3* and *GATI*, also known as GATA transcription factors. In the absence of excess or preferred nitrogen sources, and when the cells are grown in the presence of poor nitrogen sources, Ure2p is repressed activating the GATA factors *GLN3* and *GATI*. These GATA transcription factors then bind to the promoter regions of NCR sensitive GATA factor regulated genes and activate them (6). They bind to a conserved region containing a GAT(A/T)(A/G) motif, hence named

GATA factors. There are several NCR sensitive GATA factor regulated genes that serves different purpose in cell. This global control mechanism prevents expression of genes for utilization of secondary nitrogen sources as long as preferred nitrogen sources are already available. For example, in the presence of ammonia, most of the amino acid permeases are shut off, but in the presence of poor nitrogen sources the transporters of arginine, proline, urea, allantoin, GABA, are activated (6). In *Aspergillus nidulans* over 100 genes are regulated by NCR and at least 30 genes are nitrogen regulated in case of *S. cerevisiae* (7, 38). Although we have general idea about nitrogen regulation in *C. albicans*, we do not have detailed molecular and genetic knowledge about the nitrogen regulation, specifically what genes are being regulated by NCR? Since nitrogen regulation is related to morphogenesis and thus to virulence, it is of enormous importance to understand the system in order to identify a novel mechanism and to develop new anti-fungal drugs.

The nitrogen starvation response by a cell is a distinctly different response than NCR. The NCR response is basically when the cells are shifted from a preferred nitrogen source to a non-preferred one or when the preferred nitrogen source is used up and the cells then shift to utilize non-preferred nitrogen sources. In contrast, when a cell is starved for a nitrogen source or a particular amino acid, the response is known as the general amino acid control (GAAC) response (15). Fig. 1-2 is taken from a review article by Hinnebusch 2005 that shows GAAC in the yeast *S. cerevisiae*. In yeast *S. cerevisiae*, the transcriptional activator GCN4 mRNA is derepressed in amino acid deprived cells, and Gcn4p is responsible for activation of all genes responsible for amino acid biosynthesis. GCN4 translation is regulated by eIF2 (an initiation factor for protein synthesis), so when Gcn4p is derepressed the general rate of protein synthesis is also

reduced (15). This regulation is important for cells in order to limit their consumption of amino acids while activating machineries to synthesize amino acids under nitrogen starvation conditions. In case of yeast *S. cerevisiae* Gcn4p regulates more than 500 genes for that purpose via GCRE elements (15). Like *S. cerevisiae*, *CaGCN4* also activates the transcription of amino acid biosynthetic genes. But *C. albicans* also induce the Ras-cAMP pathway by *GCN4* and thereby induce hyphal formation (44). Thus *GCN4* in *C. albicans* acts as a global regulator of morphogenetic and metabolic responses to nitrogen starvation. In this thesis we report that during germ tube formation the kinetics of germ tube formation were faster when *GCN4* was repressed and *vice versa*, confirming its role towards morphogenesis. Moreover we found under special conditions, such as when a *C. albicans* yeast cell is phagocytized by a macrophage, the cell exhibits NCR but not GAAC. In our study we found that the genes *CARI*, and *DURI,2* are regulated by NCR. Arginine converts to urea by the enzyme Car1p and then urea degrades to ammonia and CO₂ by the enzyme Dur1,2p. This conversion from arginine to CO₂ is important for the yeast to hyphae switch inside a macrophage, an important factor for virulence. Similarly, our studies with *gcn4/gcn4* strains, which were able to form hyphae inside macrophage, suggest that the arginine biosynthetic genes are not regulated by Gcn4p inside macrophage. During those conditions *C. albicans* cells specifically up-regulate arginine biosynthetic genes about 3-5 folds, with the exception of *ARG2* (30). In *C. albicans* cells there are only 1 set of arginine biosynthetic genes. Thus, these arginine biosynthetic enzymes are regulated by some other mechanism in addition to the GAAC response, so that the *C. albicans* cells regulate arginine biosynthesis in more than one way.

Eight amino acids; phenylalanine, tryptophan, lysine, leucine, isoleucine, valine, methionine, and valine, are essential for humans because humans cannot synthesize them. Cysteine, tyrosine, histidine, and arginine are conditionally essential because they are additionally required by children but not adults. These amino acid requirements are generally met through diet. Because aromatic amino acids are not synthesized by humans, there are no human or mouse homologues of the aromatic amino acid biosynthetic genes that are present in *S. cerevisiae* (Saccharomyces Genome Database), *C. albicans* (Candida Genome Database) and other fungi (5). Thus it is likely that opportunistic fungal pathogens of humans like *C. albicans* have to synthesize these aromatic amino acids rather than acquire them from their hosts. In case of pathogenic *S. cerevisiae*, it has been reported that some nitrogenous compounds (polyamines, methionine, and lysine) can be acquired from the host, while others (aromatic amino acids, threonine, isoleucine, and valine) must be synthesized by the pathogen (26). This is consistent with the observation that a *aro7* mutant that cannot synthesize aromatic amino acids is less virulent in mouse model (26). Thus it is important for the pathogen to regulate biosynthesis and metabolism of these amino acids. We have focused on the aromatic amino acid metabolism of *C. albicans* to understand how this pathway is being regulated. Our goal is to find a unique mechanism or target specific to this opportunistic fungal pathogen. We have examined the regulation of aromatic amino acids in cells grown under different physiological conditions, focusing on their genetic regulation and the role of NCR in the utilization of aromatic amino acids. The *C. albicans* like yeast *S. cerevisiae* utilizes the amino groups of aromatic amino acids but cannot use the carbon skeleton. Instead, they secrete these carbon skeleton as aromatic alcohols, otherwise known as

fusel alcohols or fusel oils, by a very common mechanism known as Ehrlich's pathway (13). By measuring the amount of aromatic alcohols secreted by the cells we evaluated the levels of aromatic amino acid metabolic genes.

Inoculum size effect is a very well studied phenomenon in that when $\geq 10^6$ cells are inoculated in a medium, *C. albicans* and other fungi such as *Ceratocystis ulmi* grow as yeasts whereas in case of inoculation of $< 10^6$ cells they grow as mycelia (17). The inoculum size effect is not affected by the spore type, age, temperature, pH, oxygen availability, trace metals, sulfur or phosphorous sources, or the concentration of carbon or nitrogen (17). The growing cells excrete quorum sensing factors that cause morphological shifts from mycelia to budding yeast (17). For the *C. albicans* quorum sensing system, farnesol was identified as a quorum sensing molecule (QSM) that blocked the yeast to hyphae switch in *C. albicans* (18). This was the first eukaryotic QSM to be identified. Farnesol is secreted as a byproduct in the ergosterol biosynthetic pathway (19, 34, 36). Farnesyl pyrophosphate is converted to farnesol by two pyrophosphate phosphatases (*DPP2* and *DPP3*) (19, 34, 36) and this scenario was confirmed by constructing a *dpp3/dpp3* (KWN2) mutant and a *dpp3::DPP3/dpp3::DPP3* (KWN4) revertant, that produced six times less and twice as much farnesol as their parent (BWP17) respectively (34). There have been significant efforts in finding the mode of action of farnesol as a quorum sensing molecule. Several groups have reported the effects of farnesol on several components of morphogenetic pathways. In cultures containing farnesol, hyphae-forming ability was restored by cAMP (9) and a strain with dominant active variant of Ras1p grew as hyphae that could not be blocked by farnesol (9). Both of these observations suggest that Ras1-cAMP-Efg1 signaling cascade is inhibited by farnesol (9). Another

report suggests that farnesol causes small but consistent increase in both TUP1 mRNA and Tup1p protein levels, which serve as negative regulators of hyphal morphogenesis when acting in conjunction with *RFG1*, *NRG1*, and *RBF1* (25). Farnesol has also been reported to phosphorylate Hog1p, suggesting a possible interaction between farnesol and osmotic and oxidative stress (41). In the literature farnesol is also reported to act by blocking the MAP kinase cascade (40). CPH1 and HST7 mRNA, components of the MAP kinase cascade, were decreased in cells treated with farnesol (40). Also, farnesol was not able to block germ tube formation of *chk1/chk1* (CHK21), suggesting that farnesol might act via a two component signal transduction pathway (28). Our study showed that farnesol competes with amino acid induced germ tube formation. In our germ tube assay, when Gcn4p is derepressed, thus slowing down the general rate of protein synthesis, we also see slower germ tube kinetics. In contrast, in the presence of added amino acids, Gcn4p is repressed and GATA transcription factor genes will be activated resulting in inhibition of NCR leading to faster germ tube formation. In our N-acetyl glucosamine (GlcNAc) induced germ tube assay at 37 °C, 5 μM farnesol was sufficient to block the yeast to hyphae switch. But, in the presence of added amino acid germ tube assay, 20 μM farnesol was not sufficient to block hyphal morphogenesis. Moreover, if the amino acids were added after 30 minutes then 20 μM farnesol could block hyphae formation, suggesting that farnesol might also play a role in NCR induced hyphal morphogenesis. Based on these data taken together, it is tempting to hypothesize that farnesol acts far upstream and thus affects most of these signal transaction pathways important for morphogenesis.

C. albicans strains that are locked in either the yeast or hyphae morphology are less virulent (30). This fact led us to think that if farnesol can block the mycelia form of growth then it could be used as a therapy for candidiasis, either by itself or in combination with other drugs. However, several mouse studies revealed that farnesol acts as a virulence factor *in vivo*. When *C. albicans* cells were pre-treated with fluconazole, they secreted more farnesol and were more virulent than untreated *C. albicans* cells (33). Similarly the strain lacking *DPP3*, which produced 7-fold less farnesol was 4-5 fold less virulent in a mouse model of disseminated candidiasis (34). Mice treated with farnesol also inhibited Th1 cytokines such as IFN- γ and IL-12, and enhanced Th2 cytokines such as IL-5 (35). Taken together, even though farnesol blocks the yeast to hyphal switch of *C. albicans in vitro*, it acts as a virulence factor in the mouse model of systemic candidiasis. So there is an urgent need of understanding the mode of action of farnesol in *C. albicans* as well as its broader effects in candidiasis.

The main reservoir of the opportunistic pathogen *C. albicans* is the mammalian body where the fungus normally resides as commensal. In compromised immune conditions *C. albicans* can cause superficial to deep rooted infection candidiasis. In some cases *C. albicans* can also reach bloodstream and cause disseminated candidiasis which is fatal to immune-compromised patients. Once *C. albicans* cells reach the bloodstream, they interact with the components of immune system. So it is critical to understand how different components of immunity interact with the pathogen *C. albicans*. Interaction of different innate and adaptive immune components with *C. albicans* is a major topic of current research. When the *C. albicans* reaches blood stream, the cells interact initially with innate components such as macrophages, neutrophils, dendritic cells, and natural

killer cells. These innate immune components try to control the infection while inducing several cytokines and chemokines for induction of proper adaptive immune components. Many groups have studied the interaction of *C. albicans* with immune components. Macrophages are among the first innate immune components that phagocytize *C. albicans*. Within six hours of being engulfed by the macrophages, *C. albicans* shifts its metabolism from glycolysis to gluconeogenesis, down-regulates translation, switches from yeast to hyphae, pierces the phagolysosome, and comes out of macrophage by killing it (30). Prominent among the early responses is up-regulation of all the arginine biosynthetic genes except *ARG2* (30). This type of response probably was not because of nitrogen starvation, because only the arginine biosynthetic genes were dramatically up-regulated. As previously explained, in case of GAAC all the amino acid biosynthetic genes would be up-regulated. In the fourth part of this thesis we describe how *C. albicans* can utilize arginine and induce hyphal morphogenesis that is critical for their escape from macrophages. There are evidences that this arginine utilization pathway is also regulated by GATA factors important for NCR. *C. albicans* also induce arginine and methionine biosynthetic genes, resembling amino acid deprivation response, while interacting with neutrophils (39). Neutrophils use reactive oxygen species (ROS) and reaction nitrogen species (RNS) to kill a pathogen in a much more effective way than macrophages.

After coming into contact with the pathogenic fungus *C. albicans*, macrophages go through a series of coordinated events, induce several signal transduction pathways to contain the infection. Macrophages, like other innate immune components, express Toll-like receptors (TLRs), which can recognize pathogen associated molecular patterns (PAMPs) (21, 22). Beta-glucan (12), phospholipomannan (24), alpha-mannan (43), and

beta 1,2 mannoside (23) are the components of fungal cell wall that serve as PAMPs and are recognized by TLR2 (12), TLR4 (43), dectin-1 (12), and galectin-3 (23), either alone or in association. After being recognized by the receptors at the surface of the macrophage, the fungal pathogen *C. albicans* cells are internalized and phagosome is formed, while many other signal transduction events occur in the macrophage cells. These series of events cause the production of a set of cytokines that are specific for the fungus. In this thesis we report which cytokines are made that are specific for *C. albicans*. Although we found that macrophages die within four to six hours after phagocytosis of *C. albicans*, that time frame is still sufficient for cytokine induction. The cytokines reported in chapter 5 are important for the induction of T cells. In this thesis we report *C. albicans*; specifically beta-glucan and secreted quorum sensing molecule farnesol, together synergistically activate TLR2 thereby induce several cytokines. These cytokines are effective in stimulating the differentiation of CD4⁺ T cells to T helper cells. In this thesis we report that, in addition to Th1 and Th2 cells, the macrophage induced cytokines are also effective in induction of Th17, a critical event in prevention of candidiasis. In immune-compromised patients such as AIDS patients the T cell counts is severely compromised. So it will be extremely valuable to develop therapies to enhance the appropriate T cell response for effective immunity to *C. albicans*.

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Figure legends**Figure 1-1. Model for Nitrogen Catabolite Repression (NCR)**

A. NCR. Closed and open boxes designate the presence and absence of transport gene expression. Compounds surrounding the yeast cells are all poor nitrogen sources. Taken from (6).

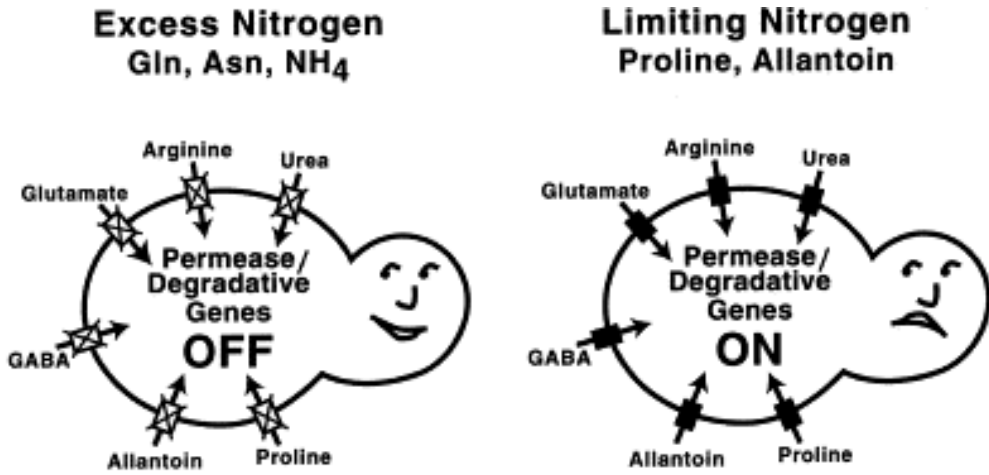
B. Model of reciprocal regulation of GATA factor gene expression and GATA factor regulation of NCR-sensitive gene expression per se. Arrowheads and bars designate positive and negative regulation, respectively. Dashed areas designate weak regulation. Taken from (6).

Figure 1-2. Summary of the major control mechanisms regulating Gcn4p levels in the cell and transcription of target genes subject to GAAC. Signals and factors controlling *GCN4* at the level of translation (*black*), mRNA abundance (*purple*), or protein degradation (*green*) are color-coded as is the induction of GAAC target genes and its consequences (*blue*). Taken from (15).

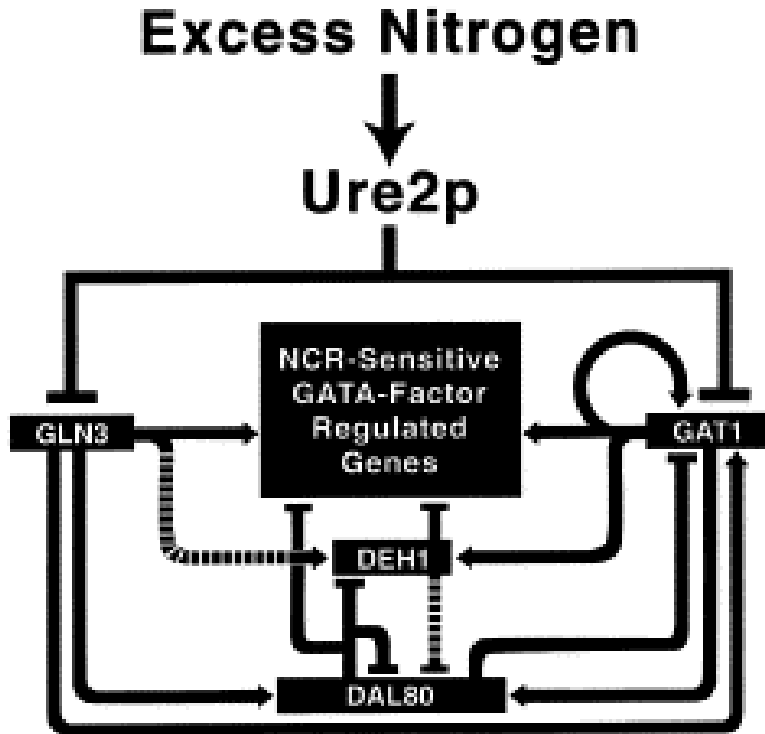
Figures:

Figure 1-1

A

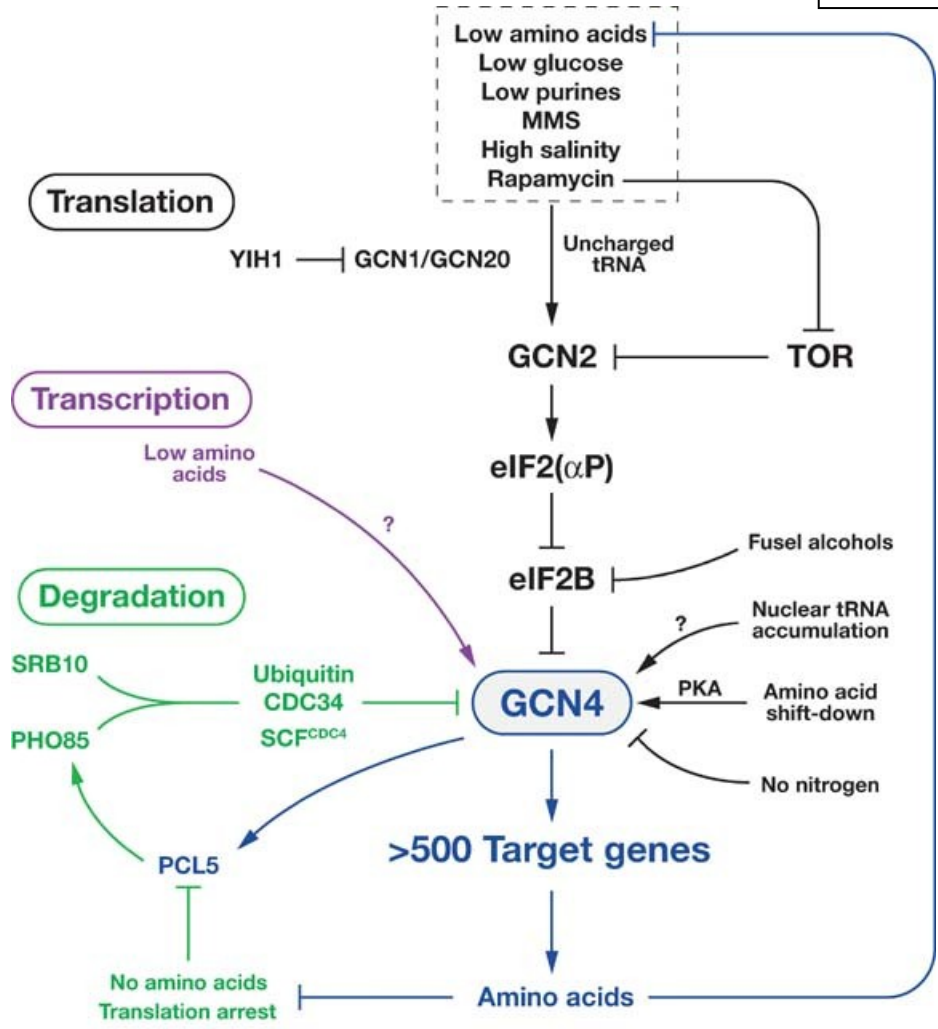


B



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Figure 1-2



Hinnebusch AG. 2005.
 Annu. Rev. Microbiol. 59:407-50

Hinnebusch AG, Annu. Rev. Microbiol. 2005

CHAPTER 2

Regulation of aromatic alcohol production in *Candida albicans*

Reference:

Ghosh, S., B. W. Kebaara, A. L. Atkin, and K. W. Nickerson. 2008. Regulation of aromatic alcohol production in *Candida albicans*. *Appl Environ Microbiol* **74**:7211-8.

Abstract

Colonization by the fungal pathogen *Candida albicans* varies significantly dependent upon the pH and availability of oxygen. Because of our interest in extracellular molecules as potential quorum sensing molecules, we examined the physiological conditions which regulate the production of the aromatic alcohols, i.e. phenethyl alcohol, tyrosol, and tryptophol. The production of these fusel oils has been well studied in *Saccharomyces cerevisiae*. Our data shows that aromatic alcohol yields for *C. albicans* are determined by growth conditions. These conditions include the availability of aromatic amino acids, pH, oxygen levels, and ammonium salts. For example, tyrosol production in wild type *C. albicans* varied 16-fold merely by including tyrosine or ammonium salts in the growth medium. Aromatic alcohol production also depends on the transcription regulator Aro80p. Our results are consistent with aromatic alcohol production via the fusel oil pathway: aromatic transaminases (*ARO8* and *ARO9*), aromatic decarboxylase (*ARO10*), and alcohol dehydrogenase (*ADH*). Expression of *ARO8*, *ARO9* and *ARO10* is also pH dependent. *ARO8* and *ARO9* were alkaline up regulated while *ARO10* was alkaline down regulated. The alkaline dependent change in expression of *ARO8* was Rim101-independent while expression of *ARO9* was Rim101-dependent.

Short Title: Fusel Oils in *Candida albicans*

Key words: tyrosol, tryptophol, phenethyl alcohol, Aro80p, *RIM101* pathway,

Introduction

The dimorphic fungal pathogen *Candida albicans* grows and colonizes different niches of human hosts (41), which differ significantly both physically and chemically. The pH of the oral cavity varies according to diet, the metabolism of other microflora, and salivary flow. Stomach pH is less than 3 while the duodenum is pH 5 and the large intestine is pH 7.7 (4). Blood is around pH 7.4. *C. albicans* can thrive in all these varying pH conditions. Similarly *C. albicans* can adapt to aerobic, anaerobic, or hypoxic microenvironments as is evident from its ability to exist in the anaerobic gastrointestinal tract (14, 15) and still cause infections ranging from superficial skin infections to deep-seated generalized infections where multiple internal tissues and host cells are invaded and colonized. The interiors of biofilms also may be partially anaerobic environment (31). The ability to adapt in all these different conditions is one of the most important attributes of severe fungal pathogens. Our long-standing interest is in extracellular molecules produced by *C. albicans* (31). Does their production differ under these different growth conditions and do they have a role in cellular adaptations from one condition to another?

It has been well established that *Saccharomyces cerevisiae* secretes fusel oils, a name derived from the old German word fousel meaning bad spirit (40). The components of fusel oils and the mechanism of formation of these higher alcohols from amino acids have been well characterized in *S. cerevisiae* (18, 34, 40). Fusel oil formation from amino acids proceeds through the Ehrlich pathway, which was first proposed 100 years ago (16, 30). This pathway consists of three enzymatic steps: transamination to form an α -keto acid which would then be decarboxylated to an

aldehyde and reduced to the fusel alcohol (Fig. 1-1). This pathway has been confirmed by more recent work (34, 39) including elegant studies using ^{13}C -labeled amino acids and ^{13}C NMR spectroscopy (13, 18). Ehrlich also showed that addition of ammonium salts and asparagine inhibited the formation of fusel oils (16, 40) and that yeasts produced tyrosol (p-hydroxy-phenyl ethanol) if tyrosine was added to the fermenting mixture and tryptophol if tryptophan was added (16, 40). *S. cerevisiae* can use tryptophan, phenylalanine, or tyrosine as the only source of cellular nitrogen (8) with the main products of their catabolism being tryptophol, phenethyl alcohol, and tyrosol, respectively (22, 27, 34, 36).

The present paper examines aromatic alcohol production in *C. albicans* and finds that the production characteristics fit those expected of fusel oils. That is, aromatic alcohol production uses homologous genes and enzymes as in *S. cerevisiae*, is dependent on the availability of amino acid precursors, and is regulated by pH, oxygen availability, and nitrogen repression by ammonium. For instance, tyrosol production per g dry wt of cells varied over 16-fold for wild type cells. Studies with null mutants revealed that transaminases and decarboxylase are under dual control of the Aro80p and pH pathways. *ARO8* and *ARO9* (transaminases) were alkaline up regulated while *ARO10* (decarboxylase) was alkaline down regulated. *ARO8* was Rim 101p independent while *ARO9* was Rim101p dependent.

Methods

Strains and growth media

The *C. albicans* strains SC5314, CAI4 (*ura3Δ::imm434/ura3Δ::imm434*), and BWP17 (*ura3Δ::imm434/ura3Δ::imm434, arg4::hisG/arg4::hisG, his1::hisG/his1::hisG*) (42)

were obtained from Alexander Johnson, University of California at San Francisco. The *aro80* (orf19.3012) and *rim13* (orf19.3995) insertion mutants were obtained from Dr. Aaron Mitchell's collection (32); they were derived from strain BWP17. CAR2 (*rim101::hisG/rim101::hisG-URA3-hisG ura3::imm434/ura3::imm434*) (33) was obtained from Dr. Fritz A. Muhlschlegel, Canterbury, UK.

For routine growth of strains, YPD medium (10 g of yeast extract per liter, 20 g of peptone per liter, 20 g of glucose per liter) was used. To quantify aromatic alcohols in culture supernatants, GPP, GPA, or GPP+A media (23) were used. GPP medium contains the following (per 900 ml of distilled water): 4.0 g of KH_2PO_4 , 3.2 g of Na_2HPO_4 , 1.2 g of L-proline, and 0.7 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. After the medium was autoclaved, 100 ml of 20% (wt/vol) glucose, 1 ml of a vitamin mix, and 0.25 ml of a mineral mix were added. The vitamin mix contains the following (per 100 ml of 20% ethanol): 2 mg of biotin, 20 mg of thiamine-HCl, and 20 mg of pyridoxine-HCl. The mineral mix contains the following (per 100 ml of 0.1 N HCl): 0.5 g of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.5 g of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.8 g of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, and 0.5 g of FeSO_4 . The vitamin mix and the mineral mix were filter sterilized through 0.2- μm -pore-size Whatman (Maidstone, United Kingdom) cellulose nitrate filters and stored at 4°C. For GPA ammonium sulphate (10mM) replaced proline and for GPP+A ammonium sulphate was added to GPP. Anaerobic growth employed the Hungate technique for growing stringent anaerobes as adapted for *C. albicans* by Dumitru et al (14). Thus, our regular GPP medium (50ml) was supplemented with 200 μl of 1 mM oleic acid in 100% methanol, 200 μl of 4 mM nicotinic acid, and 1 ml of 500mM NH_4Cl (14). The cells were harvested after 5 days at 30°C.

Quantification of Excreted Aromatic Alcohols

C. albicans strains were cultured for 24 – 28 hours in 50 ml of defined medium (GPP, pH6.8) while shaking at 250 rpm. Cells were grown at 30 or 37°C as specified. When necessary, pH values were adjusted using 1N HCl or 1N NaOH. After growth the fungal cultures were harvested by centrifugation at 6,000 rpm for 10 min. The supernatants were filtered through 0.2 μ Millipore filters, extracted with ethyl acetate, filtered, and concentrated by rotary evaporation to 50 μ l. Then 1 μ l of sample was injected into a HP6890 GC/MS with 50 m Capillary column HP 19091B-005. The flow rate was 1.0 ml/min. GC used an inlet temperature of 280°C and temperature program of 80°C for 2 min, then 60°C/min until 160°C and holding for 2 minutes and then 10°C/min until 300°C, and holding for 5 minutes with total run time 24.33 minutes. MS used a 5-min solvent delay. Ethyl acetate extraction is suitable for the three aromatic alcohols as well as farnesol, whereas hexane or 1:4 ethylacetate/hexane is suitable for farnesol (20) but not for the aromatic alcohols. The phenethyl alcohol was purchased from Aldrich Chemical Co., Milwaukee, WI; tyrosol from Avocado Research Chemicals Ltd., Heysham, UK; and tryptophol from TCI-EP, Tokyo.

Northern Analysis

C. albicans total RNA used for mRNA accumulation was extracted by the hot phenol extraction method using yeast cells harvested at mid-log phase (24). Equal amounts of RNA (15 μ g) were resolved on 1.0% agarose–formaldehyde gels and transferred to GeneScreen Plus membranes (NEN Life Science Products, Boston, MA). The NorthernMax complete Northern blotting kit (Ambion, Austin, TX) was used for transfer and hybridization. The DNA templates for probe synthesis were prepared using PCR

with *C. albicans* SC5314 genomic DNA and the following primers (5' to 3'): *ARO8* – TATTCCAACACCGTCGTTCA and ACAAACTGGTCCAAGGCATC, *ARO9* – CAAAACCTCCGCCTTCCAGTA and AGCCATCCATCAACACCTTT, *ARO10* – GTGCTTATGCTGCTGATGGA and TCTTTTTGGGTTCTGCTGCTG, *RIM101* – AGTCCATGTCCCATTGAAGC and ACACCGCCAAACTCTAATGC, *ACT1* – AGTTATCGATAACGGTTCTG and AGATTTCCAGAATTTCACTC. The DNA templates were used to synthesize DNA probes labeled with ³²P using an oligolabeling kit (Rad Prime DNA labeling system; Invitrogen Life Technologies, Carlsbad, CA), as described by Atkin et al. (3). Northern blots were PhosphorImaged using a Storm Phosphorimager (Amersham Pharmacia Biotech) and measurements of *ARO8*, *ARO9*, *ARO10*, *RIM101* mRNA were normalized with *ACT1* mRNA control.

Results

Environmental control of aromatic alcohol production by *C. albicans*. The aromatic alcohol yields for *S. cerevisiae* vary by growth conditions (18). The rationale for the environmental variables tested here was to see if the control mechanisms operative in *C. albicans* paralleled those known for fusel oil production in *S. cerevisiae* (21).

Temperature, anaerobiosis, precursor availability, ammonium ions, and pH were examined. In each case, supernatants from *C. albicans* SC5314 grown in GPP were analyzed for the three aromatic alcohols, phenethyl alcohol, tyrosol, and tryptophol (Fig. 2-2). Peaks were identified by comparing their retention times and MS spectra versus those of the pure compounds (Fig. 2-2 C-F).

Aromatic alcohol production was not affected by growth temperature. With regard to temperature, there was little difference in aromatic alcohol production between 30°C (Fig. 2-2 A) and 37°C (Fig. 2-2 B). The concentrations of phenethyl alcohol, tyrosol, and tryptophol were 830, 2120, and 440 µg/g at 30°C and 1030, 2530, and 660 µg/g at 37°C, respectively (Fig. 2-3 A). For comparison, farnesol was present at 17 and 20 µg/g at 30°C and 37°C, respectively (Fig. 2-2 A-B). The identities of the other peaks in Fig. 2-2 remain unknown. Their mass values do not correspond with those expected for any of the other fusel oils.

Increased aromatic alcohols are produced anaerobically. Another of the environmental variables examined was anaerobiosis. Anaerobic growth conditions (14) should be relevant for *C. albicans* growing in animal gastrointestinal tracts (15) and biofilms (31). Alem et al (1) recently reported that *C. albicans* biofilms produced 1.5-fold more tyrosol than did the corresponding planktonic cells. We found that cells of *C. albicans* grown anaerobically at 30°C produced roughly twice as much of each of the three aromatic alcohols as did aerobically grown cells (Fig. 2-3 B).

Aromatic amino acid precursors elevate aromatic alcohol production. We examined aromatic alcohol production by cells grown at 37°C in GPP supplemented with the aromatic amino acids phenylalanine, tyrosine, or tryptophan which are precursors for aromatic alcohol production (Fig. 2-3 C). In each case, the expected alcohol increased in abundance. At 37°C tyrosol production was increased 2-fold in the presence of tyrosine, and tryptophol production was increased 2.5-fold in the presence of tryptophan (Fig. 2-3 C). No significant further changes in tyrosol levels were observed as tyrosine was

increased from 50 to 150 $\mu\text{g/ml}$ (Fig. 2-3 C). Throughout, the cell morphologies remained unchanged by the amino acid additions; the cells were 90-95% hyphal.

Ammonia suppresses aromatic alcohol production. Another environmental variable expected to influence fusel oils is the ammonia effect (6, 21), whereby aromatic alcohol production is inhibited by ammonia. *C. albicans* SC5314 produced 5-7-times less aromatic alcohols when grown at 30°C in GPA than when grown in GPP (Fig. 2-3 D). The two media differ only in whether ammonium sulfate or L-proline (both at 10 mM) is the nitrogen source. Aromatic alcohol production was also 5 to 7-fold lower when the cells were grown in L-proline and ammonium sulfate together (Fig. 2-3 D), showing that the ammonia effect is operative even in the presence of proline. In the upstream regions for *C. albicans* *ARO8-10* we found several GAT (A/T) (A/G) sequences, putative binding sites for the GATA transcription factors Gln3p and Gat1p (9, 25). These conserved regulators mediate nitrogen catabolite repression by activating genes whose products are required for nitrogen catabolism.

Decreased production of aromatic alcohols by an *aro80* mutant. By analogy with aromatic alcohol production in *S. cerevisiae* (21), aromatic alcohol production in *C. albicans* is expected to depend on Aro80p. *S. cerevisiae* Aro80p is a member of the Zn_2Cys_6 transcription activator family which increases synthesis of Aro9p (aromatic transaminase) and Aro10p (aromatic decarboxylase). Aro80p is activated by three aromatic amino acids (21). The *C. albicans* Aro80p is 32% identical with its *S. cerevisiae* homolog. Importantly, however, the conservation is much higher (67% identical) at the N-terminus that contains the zinc binuclear DNA binding domain (Fig. 2-4 A). Thus, both the *S. cerevisiae* and *C. albicans* Aro80p are bimetal thiolate cluster

proteins/transcription regulators (28, 38) and they probably recognize the same or similar sequences. Aro80p is a transcription activator of *ARO8*, *9*, and *10* in *S. cerevisiae* (21) and by analogy it may be in *C. albicans* also. We also chose the *ARO8*, *9*, and *10* genes for study because *ARO9* was consistently up regulated when *S. cerevisiae* was grown in a glucose-limited chemostat with phenylalanine as the sole nitrogen source (5) while *ARO10* was the only decarboxylase gene whose transcript profile correlated strongly with α -ketoacid decarboxylase activity in chemostat culture (5, 18, 39).

When grown in GPP medium, *C. albicans aro80* produced ca. 3.5 times less phenethyl alcohol, 4.5 times less tyrosol, and 2.5 times less tryptophol than did the wild type SC5314 and ca. 5 times less phenethyl alcohol, 2.5 times less tyrosol, and 3.5 times less tryptophol than did the parent BWP17 (Fig. 2-4 B). In the presence of ammonia (GPA instead of GPP), the *aro80* production levels for phenethyl alcohol, tyrosol, and tryptophol were reduced a further 1.2, 4, and 20-fold, respectively (Fig. 2-4 B). The fact that the ammonia effect is still observed in *aro80* suggests that the ammonia effect on the *ARO8* and *ARO9* (transaminases) and *ARO10* (decarboxylase) genes and/or proteins (Fig. 2-1) is independent of *aro80*. These findings are consistent with the conclusion that aromatic amino acid metabolism in *C. albicans*, like *S. cerevisiae* (21), is both stimulated by transcription activation by Aro80p and subject to nitrogen catabolite repression by ammonia.

Alkaline pH elevates aromatic alcohol yield. The final environmental variable we explored was pH. *C. albicans* grows over a pH range from ca. 1.5 to 10 and culture pH is strongly influenced by the nitrogen source. For both *C. albicans* and *Ceratocystis ulmi* (23), the pH remains constant at ca. 6.5 throughout growth in both proline and arginine-

containing media. Indeed, GPP and GPR were designed to study fungal dimorphism without concurrent changes in pH (23). In contrast, with ammonium salts as the nitrogen source, the pH drops to 2-3 with $(\text{NH}_4)_2 \text{SO}_4$ or NH_4Cl , but remains at pH 6.5 with ammonium tartrate (23). Many bacteria respond to pH extremes by synthesizing amino acid decarboxylases at low external pH and amino acid deaminases at high external pH (17). *C. albicans* may have similar mechanisms. In unbuffered medium 199 (a glutamine-containing medium), cultures that started at pH values ranging from 4 to 10 returned to pH 7 within 6 hrs (M.C. Lorenz, personal communication). Accordingly, production of the aromatic alcohols by wild type cells was examined in highly buffered cultures grown in GPP at pH 3, 7, or 8, so that pH 3 constitutes acid stress and pH 7 and 8 constitute more alkaline conditions (11). Production of the three aromatic alcohols by SC5314 was 2-3 fold higher for cells grown at pH 7 (Fig. 2-5 A) or pH 8 (data not shown) than by cells grown under acid stress (Fig. 2-5 A). Northern blots for these cells showed that *ARO8* and *ARO9* were alkaline up regulated while *ARO10* was alkaline down regulated (Fig. 2-5 B, lanes 1 and 2).

Rim101p is required for maximal aromatic alcohol production. Alkaline pH responses can be either Rim101p dependent or independent (11). For the Rim101p dependent alkaline response, Rim101p needs to be proteolytically cleaved to its active form by Rim13p (Fig. 2-1), a calpain protease (10). Then Rim101p activates transcription of a variety of genes including *PHR1* and *PRA1*, as well as its own gene *RIM101*. Thus, in the absence of *RIM13*, Rim101p would remain inactive and genes such as *PHR1* and *PRA1* would not be expressed at alkaline pH (11).

The *rim13* and *rim101* mutant cells grew as yeasts in GPP at 37°C at pH values from 3 to 8, thus confirming that the *RIM101* pathway is required for alkaline-induced filamentation (4). However, the absence of filamentation was not due to growth defects. The *rim13* and *rim101* dry weights after 24 hrs were very close to those for wild type cells and in the GlcNAc-induced filamentation assay (20), histidine supplemented *rim13* and uridine supplemented *rim101* produced germ tubes just like wild type cells (data not shown). Thus, *RIM101* is not essential for filamentation, other pathways are available for hypha formation.

With wild type SC5314, the aromatic alcohol yields *in vitro* were elevated at pH 7 compared to pH 3 (Fig. 2-5 A) and thus we wanted to see if this pH effect was Rim101p dependent or not. We tested aromatic alcohol production of *rim13* and *rim101* at pH 3 and pH 7 (Fig. 2-5 A) and then compared these values with their parents BWP17 and CAI4, respectively, as well as with the wild type SC5314 (Fig. 2-5 A). The observation that two independent mutants in the RIM101 pathway, i.e. *rim13* and *rim101*, both curtail fusel alcohol synthesis implicates this pathway in their synthesis.

For the wild type SC5314 and the two parental strains, BWP17 and CAI4, the aromatic alcohol yields *in vitro* were pH dependent, being at least 2.0 fold higher at pH 7 than pH 3. In contrast, pH regulation was lost for *rim13* (Fig. 2-5 A). Two independent *rim13* mutants were tested and they behaved similarly. Although the *rim13* and *rim101* mutants produced less aromatic alcohols, the pH regulation was not completely lost for *rim101* (Fig. 2-5 A). As a control, farnesol production by *rim13* and *rim101* were similar to the wild type SC5314 and their parents BWP17 and CAI4 at both pH values (data not shown).

We further tested *C. albicans* with regard to whether the genes for aromatic alcohol production were pH regulated and, if so, whether the pH effects observed were Rim101p - dependent or - independent. This latter distinction was made using *rim13* and *rim101* mutants; *rim13* is the mutant in which Rim101p cannot be activated. For wild type SC5314, *ARO8* and *ARO9* were alkaline up regulated while *ARO10* was alkaline down regulated (Fig. 2-5 B, lanes 1 and 2). This pattern was also seen for BWP17 (Fig. 2-5 B, lanes 3 and 4). *ARO9* was regulated in a RIM101p dependent manner because its alkaline up regulation was lost in both *rim13* (lanes 5 and 6) and *rim101* (lanes 7 and 8). In contrast, *ARO8* was regulated in a Rim101p independent manner because it was still alkaline regulated in both *rim13* and *rim101* (Fig. 2-5 B, lanes 5-8). The situation with *ARO10* is more complicated in that its alkaline down regulation was lost in *rim101* (lanes 7 and 8) but not in *rim13* (lanes 5 and 6). These findings reinforce the microarray data of Bensen et al (4) who found that *ARO8* was alkaline up regulated and *ARO10* was alkaline down regulated. Alkaline induction of *RIM101* can also be seen in Fig. 2-5 B (lanes 1-2).

Discussion

Fusel alcohols are the natural products of amino acid catabolism. Yeasts cannot use branched chain or aromatic amino acids as their sole carbon source (8). However, they can be used as nitrogen sources under otherwise nitrogen limiting conditions, with the consequent production of fusel alcohols as potentially toxic or regulatory by-products (2, 19). Our studies on aromatic alcohol production showed that *C. albicans* produced three aromatic alcohols, phenethyl alcohol, tyrosol, and tryptophol, using a similar pathway as in *S. cerevisiae*, i.e. transamination (*ARO8*, *ARO9*), decarboxylation

(*ARO10*), and then reduction by alcohol dehydrogenase (*ADH*) (18, 34). This pathway is summarized in Fig. 2-1. We found that *C. albicans* produced the three expected aromatic alcohols in roughly constant proportions under all conditions studied. Previously, Lingappa et al (26) reported production of phenethyl alcohol and tryptophol whereas Chen et al (7) detected tyrosol and Martins et al (29) detected phenethyl alcohol and isoamyl alcohol. Isoamyl alcohol is the fusel alcohol derived from leucine (18).

Aromatic alcohol production was dependent on the transcription factor Aro80p. It was repressed by ammonium ions but elevated under anaerobic conditions or whenever the appropriate amino acids, phenylalanine, tyrosine, or tryptophan, were provided in the growth medium. Aromatic alcohol production was determined by growth conditions. For example, for wild type *C. albicans* tyrosol production varied 16-fold merely by including tyrosine (Fig. 2-3 C) or ammonium ions (Fig. 2-3 D) in the growth medium. Also, we found that cells of *C. albicans* grown anaerobically at 30°C produced roughly twice as much of each of the three aromatic alcohols as did aerobically grown cells (Fig. 2-3 B). This increased production occurred despite the fact that our anaerobic growth medium is a modified GPP containing 10 mM ammonium salts (14). *C. albicans* up regulates three alcohol dehydrogenase genes (*ADH1*, *ADH2*, and *ADH5*) during hypoxic growth (35), a finding which is consistent with the fact that higher amounts of aromatic alcohols are secreted under anaerobic conditions (Fig. 2-3 B). Aromatic alcohol production would be energetically favorable under anaerobic conditions. The aromatic aldehydes would be electron acceptors and substrates for one or more of the alcohol dehydrogenases (Fig. 2-1). Higher aromatic alcohol production under anaerobic conditions would also explain the observation of Alem et al (1) that on a per weight basis

biofilm cells secreted 50% more tyrosol than did planktonic cells. This 50% increase would be expected if 30-40% of the biofilm cells were growing in anaerobic conditions.

Because aromatic alcohols are formed from aromatic amino acids by a pathway which includes decarboxylation, we also considered whether their production was part of a pH response by *C. albicans*. All microbes have an optimal pH for growth and many use pH-regulated genes to bring external pH close to this optimal range (37). These studies were pioneered by Ernest Gale and Helen Epps (17) who showed that in an amino acid or protein rich environment many bacteria made amino acid decarboxylases at low external pHs and amino acid deaminases at high external pHs, in each case acting to neutralize the pH of the growth medium. *C. albicans* is also capable of neutralizing unbuffered growth media. However, compensating for pH extremes is clearly not the dominant reason for aromatic alcohol production by *C. albicans*. Aromatic alcohol production was actually reduced during growth at low pH (Fig. 2-5 A) and the transaminase and decarboxylase genes were regulated in an opposite manner by pH (Fig. 2-5 B). *ARO8* and *ARO9* were alkaline up regulated whereas *ARO10* was alkaline down regulated. This pH regulation in opposite directions is consistent with aromatic alcohol production being maximal at pH 7 (Fig. 2-5 A).

Finally, aromatic alcohol production was insensitive to pH in the *rim13* mutant (Fig. 2-5 A) and the pH dependent up regulation of *ARO9* was lost in both *rim13* and *rim101* (Fig. 2-5 B). Thus we suggest that *ARO9* should be added to the list of Rim101p regulated genes. Dual regulation of *ARO9* by Aro80p and Rim101p suggests that Aro9p is a critical step for the regulation of fusel oils, a reasonable possibility because the following decarboxylation step is effectively irreversible (12, 18, 34). For *ARO10*,

regulation was lost in *rim101* but not in *rim13* (Fig. 2-5 B). This juxtaposition could mean that *ARO10* expression is dependent on Rim101p but not on the activation / processing of that protein by Rim13p.

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Figure legends

Figure 2-1. Regulation of the production of aromatic alcohols from aromatic amino acids.

C. albicans can use the aromatic amino acids tryptophan, phenylalanine and tyrosine as cellular nitrogen sources. This results in the production of tryptophol, phenylethanol and tyrosol, collectively known as fusel oils. Fusel oil production depends on environmental factors including the availability of aromatic amino acids, ammonia, oxygen level, and alkaline pH (indicated by dotted lines). Aromatic amino acids stimulate Aro80p, a transcription activator required for full expression of *ARO8* and *ARO9* (encoding aromatic transaminases) and *ARO10* (aromatic decarboxylase). Genes are in boxes; enzymes/proteins are in ellipses. The scheme is based on our findings, as well as on pathways reported for both *S. cerevisiae* (6, 13, 16, 21) and *C. albicans* (10, 11, 33).

Figure 2-2. GC/MS analysis of ethyl acetate extracts from cell free supernatants of *C. albicans*

Cells were grown overnight at (a) 30°C or (b) 37°C prior to GC/MS analysis. The GC peaks labeled I, II, III, and IV were identified by MS as (c) phenethyl alcohol, (d) tyrosol, (e) tryptophol, and (f) farnesol, respectively.

Figure 2-3. Effects of environmental conditions on production of aromatic alcohols.

GC/MS analysis of cell free supernatants of *C. albicans*: (a) 30°C and 37°C (from Fig. 2a and b, respectively); (b) grown at 30°C aerobically or anaerobically; (c) defined GPP medium (37°C) supplemented with the indicated amino acid(s), at 50µg/ml unless

otherwise indicated; (d) with proline (GPP) or ammonia (GPA) or both (GPP+A) as the nitrogen source. Throughout, phenethyl alcohol (PEA, white bars), tyrosol (TOH, black bars), and tryptophol (TrpOH, patterned bars) are expressed as μg per g dry weight of fungal cells. Data for b and d (all at 30°C and pH7) are the average of triplicate experiments, with bars representing standard error, whereas a and c are the average of two experiments which agreed within $\pm 10\%$.

Figure 2-4. Effects of Aro80p on production of aromatic alcohols.

(a) Comparison of the N-terminal portions of Aro80p from *C. albicans* and *S. cerevisiae*, showing the *bimetal thiolate* cluster expected in $\text{Zn}_2 \text{Cys}_6$ type transcription activators. The six cysteine residues are shown in ash color and the other conserved amino acids in the DNA binding domain are shown in black. (b) GC/MS analyses of *C. albicans* (30°C) supernatants from SC5314, CAI4, BWP17, and *aro80* grown in proline (GPP) or ammonia (GPA)-containing media. Tryptophol was below the detection limits for *aro80* GPA. Bars represent standard error.

Figure 2-5. Effects of pH on production of aromatic alcohols. (a) GC/MS analysis of *C. albicans* (30°C). SC5314, BWP17, CAI4, *rim13*, and *rim101* grown in GPP at pH3 or pH7. Values are the average of triplicate experiments. Bars represent standard error. (b) Northern blot analysis of RNA prepared from mid-log phase cells grown at 30°C. Blots were probed for the transcripts indicated along the left side. The numbers below represent the signal quantified with a Phosphor Imager™, normalized for *ACT1* loading

control (average of three replicates). Lane numbers at bottom. Note that lanes 7 and 8 are from the same blot and exposed for the same time.

Figure 2-1

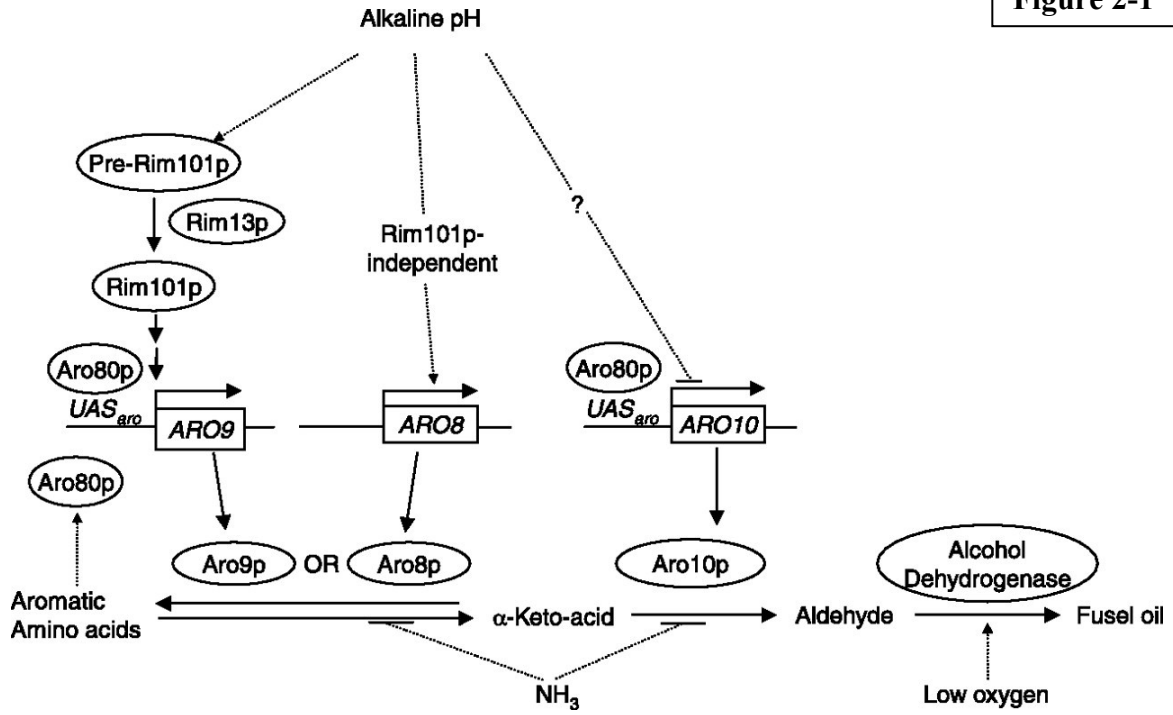


Figure 2-2

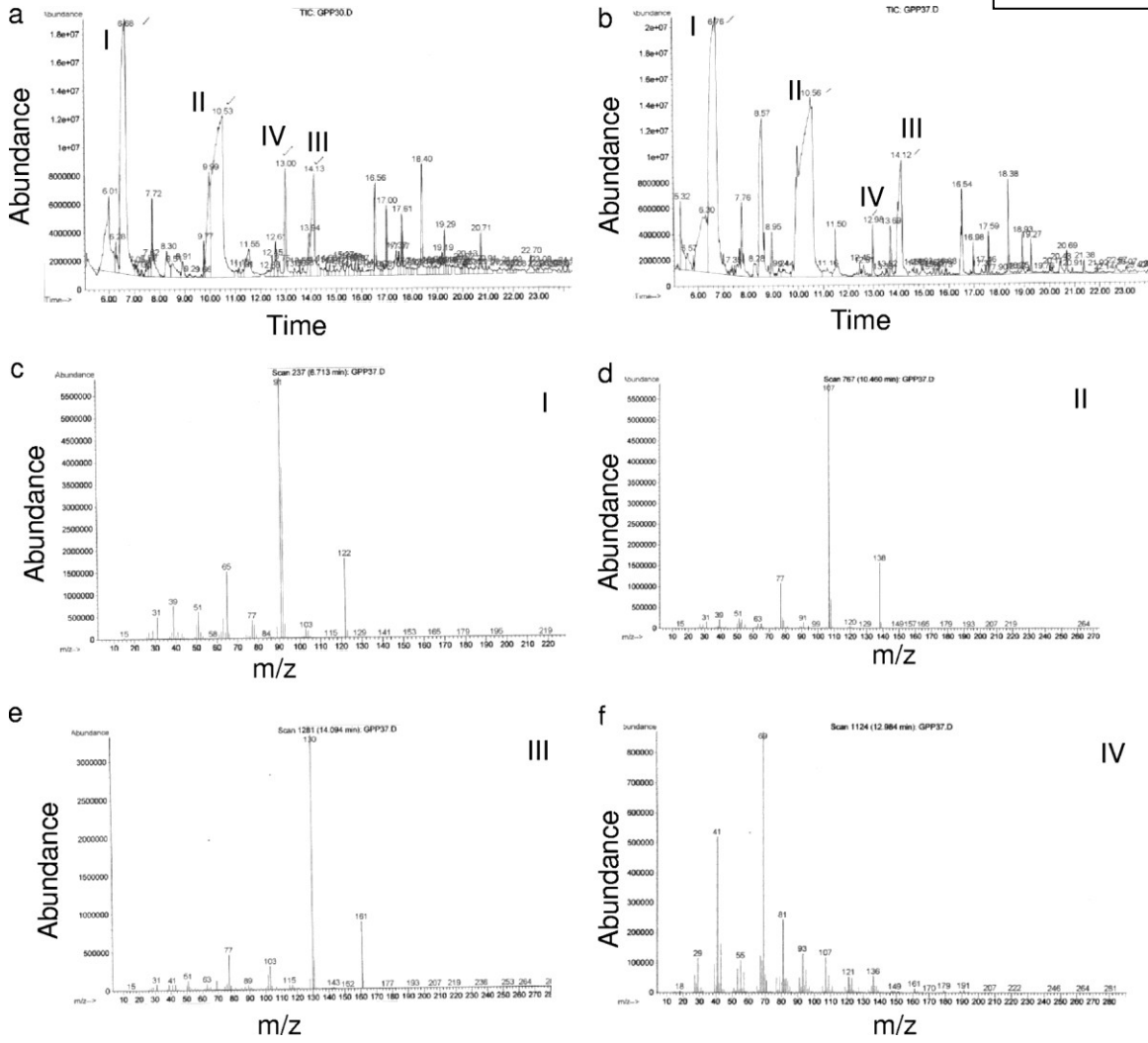


Figure 2-3

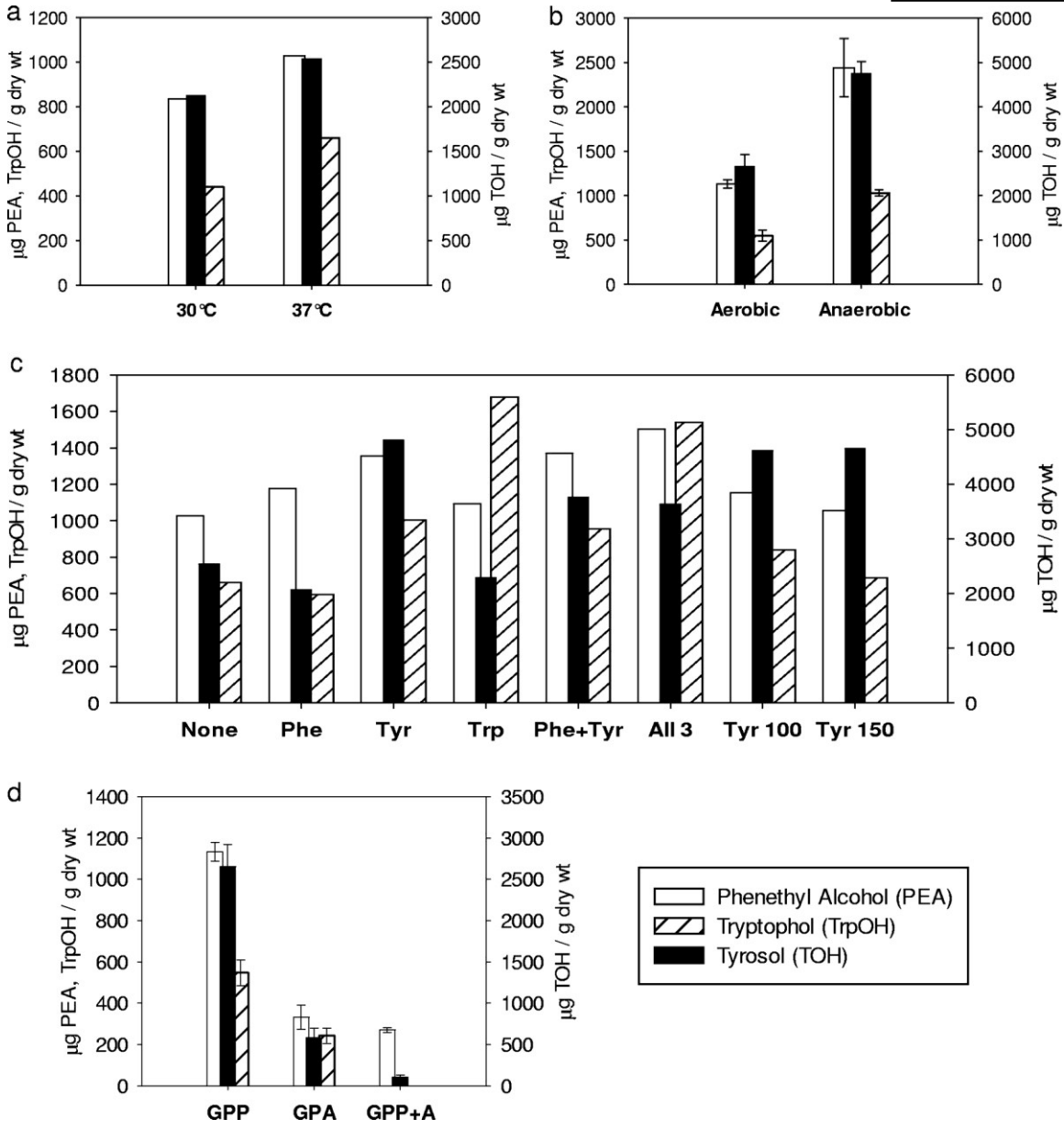


Figure 2-4

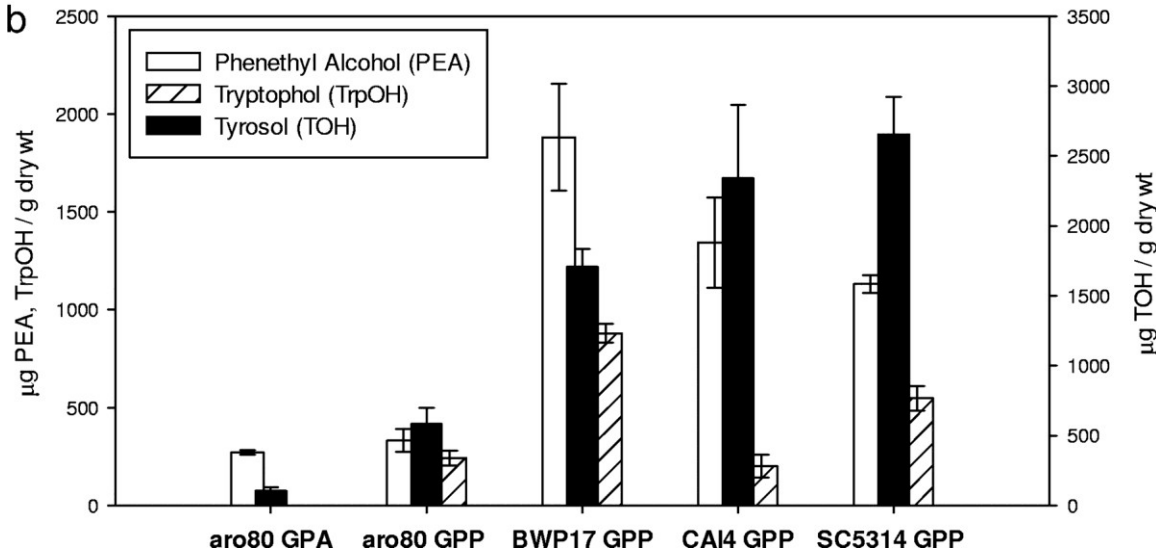
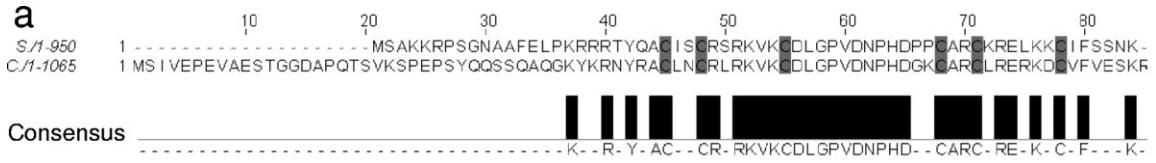
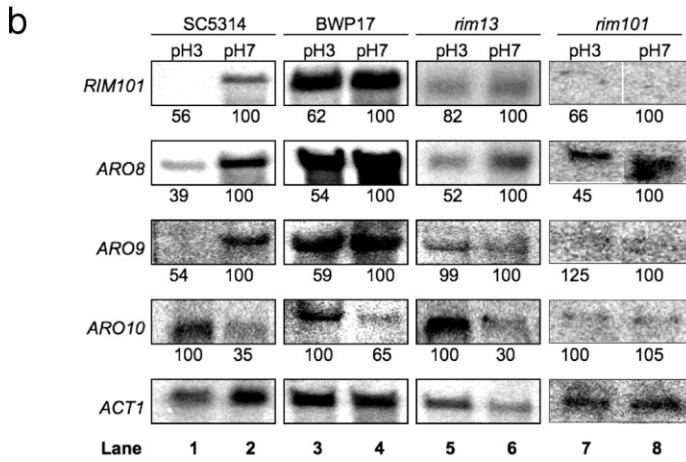
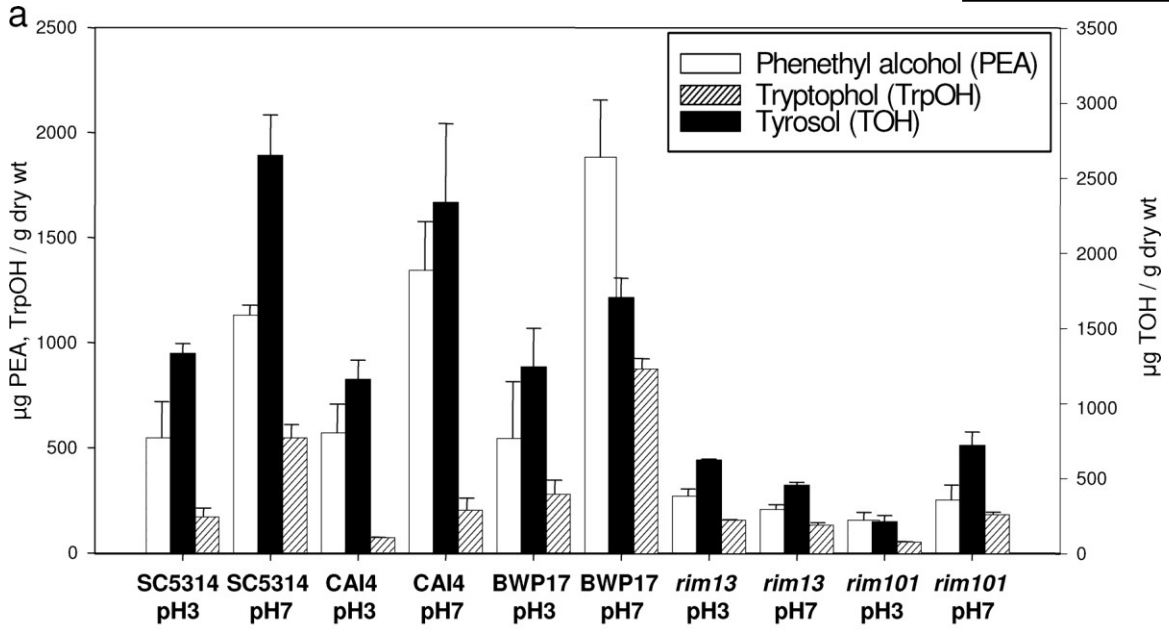


Figure 2-5



CHAPTER 3

The Regulation of Aro80p in *Candida albicans*

Reference:

Ghosh, S., B. W. Kebaara, K. W. Nickerson, and A. L. Atkin. The Regulation of Aro80p in *Candida albicans*. To be submitted.

Abstract:

The opportunistic pathogen *Candida albicans* can metabolize aromatic amino acids in poor nitrogen conditions and excrete the non-metabolized carbon skeletons as aromatic alcohols or fusel alcohols via a well known Ehrlich's pathway conserved in fungi. The aromatic amino acids are metabolized first by aromatic transaminases (*ARO8* and *ARO9*) to produce α -keto acids; which are modified by aromatic decarboxylase (*ARO10*) to produce aromatic aldehydes; which are then reduced by alcohol dehydrogenases (*ADH*) to produce aromatic alcohols otherwise known as fusel alcohols. Aro80p is a Zn₂Cys₆ transcription activator for *ARO9* and *ARO10* in the yeast *Saccharomyces cerevisiae* and is required for the full activation of aromatic amino acid metabolism genes. In this report we studied the *ARO80* homolog in *C. albicans* and its regulation in different physiological conditions. In contrast to the situation in the yeast *S. cerevisiae*, we found the Aro80p in *C. albicans*, is regulated by nitrogen catabolite repression genes as well as by pH pathways. Aro80p was activated by poor nitrogen sources such as proline and arginine and was repressed by ammonia. The *aro80/aro80* strain was also able to grow in the presence of phenylalanine, tyrosine, or tryptophan as sole sources of nitrogen. The production of aromatic alcohol is pH dependent and alkaline up-regulated. The pH regulation of the aromatic alcohol production was also lost *aro80/aro80* strains. Northern analysis suggests that the alkaline up-regulation of *ARO8*, *ARO9* and alkaline down-regulation of *ARO10* was lost in *aro80/aro80* strains.

Key words: phenethyl alcohol, tyrosol, tryptophol, Aro80p, nitrogen catabolite repression (NCR), pH pathway

Introduction:

In yeast *Saccharomyces cerevisiae*, aromatic amino acid metabolism is mainly regulated by a Zn₂Cys₆ transcriptional factor *ARO80*. Aro80p is activated in the presence of aromatic amino acids. Once activated it induces expression of aromatic transaminase (*ARO9*) and aromatic decarboxylase (*ARO10*) (7). The *S. cerevisiae aro9* and *aro80* mutants were impaired in growth when tyrosine or tryptophan was used as the sole source of nitrogen (7). Again, *ARO9* and *ARO10* were induced only in the presence of the aromatic amino acid tryptophan; they were inactivated in the presence of other nitrogen sources such as ammonia or urea. In the presence of ammonia, a preferred nitrogen source, the transcription of *ARO9* and *ARO10* was repressed. Similarly, the transcription of both *ARO9* and *ARO10* was repressed in the presence of urea, another poor nitrogen source (7). This suggests that the activation of aromatic transaminase (*ARO9*) and decarboxylase (*ARO10*) depends on the presence of aromatic amino acids, not just the lifting of nitrogen catabolite repression (NCR). In the case of *aro80* mutant *ARO9* and *ARO10* were not activated (7). This suggests that the activation of both *ARO9* and *ARO10* are dependent on *ARO80*, the Zn₂Cys₆ transcription factor.

We are interested to study Aro80p in *C. albicans* to see if its regulation of aromatic amino acid metabolism is different from *S. cerevisiae*. In this study we have specifically looked at how Aro80p is regulated in *C. albicans* cells and their effects on aromatic alcohol production levels. We found that in *C. albicans*, unlike in *S. cerevisiae*, Aro80p is activated by poor nitrogen sources whenever NCR genes are induced, thereby influencing the secretion of fusel alcohols. We also report that the pH regulation of the aromatic alcohol production is lost in *aro80/aro80* strains.

Materials and Methods:

Strains and growth conditions

The *C. albicans* strains SC5314, CAI4 (*ura3Δ::imm434/ura3Δ::imm434*), and BWP17 (*ura3Δ::imm434/ura3Δ::imm434, arg4::hisG/arg4::hisG, his1::hisG/his1::hisG*) (11) were obtained from Alexander Johnson, University of California at San Francisco. The *aro80/aro80* (orf19.3012) insertion mutants were obtained from Dr. Aaron Mitchell's collection (9); they were derived from strain BWP17.

For routine growth, YPD medium (10 g of yeast extract, 20 g of peptone, and 20 g of glucose per liter) was used. To quantify aromatic alcohols in culture supernatants, GPP, GPR, or GPA media (4) were used. GPP medium contains the following (per 900 ml of distilled water): 4.0 g of KH_2PO_4 , 3.2 g of Na_2HPO_4 , 1.2 g of L-proline, and 0.7 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. After the medium was autoclaved, 100 ml of 20% (wt/vol) glucose, 1 ml of a vitamin mix, and 0.25 ml of a mineral mix were added. The vitamin mix contains the following (per 100 ml of 20% ethanol): 2 mg of biotin, 20 mg of thiamine-HCl, and 20 mg of pyridoxine-HCl. The mineral mix contains the following (per 100 ml of 0.1 N HCl): 0.5 g of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.5 g of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.8 g of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, and 0.5 g of FeSO_4 . The vitamin mix and the mineral mix were filter sterilized through 0.2 μm Whatman (Maidstone, United Kingdom) cellulose nitrate filters and stored at 4°C. For GPR, arginine (10 mM) replaced proline and for GPA, ammonium sulphate (10 mM) replaced proline.

Quantification of Excreted Aromatic Alcohols

C. albicans strains were cultured for 24 – 28 hours in 50 ml of defined medium (GPP, pH6.8) while shaking at 250 rpm at 30 °C. The pH values were adjusted to 3.0 or

7.0 using 1N HCl or 1N NaOH to study the pH effects. After growth the fungal cultures were harvested by centrifugation at 6,000 rpm for 10 min. The supernatants were filtered through 0.2 μ Millipore filters, extracted with ethyl acetate, filtered, and concentrated by rotary evaporation to 50 μ l. Then 1 μ l of sample was injected into a HP6890 GC/MS with 50 m Capillary column HP 19091B-005. The flow rate was 1.0 ml/min. The GC used an inlet temperature of 280°C and temperature program of 80°C for 2 min, then 60°C/min until 160°C and holding for 2 minutes and then 10°C/min until 300°C, and holding for 5 minutes with total run time 24.33 minutes. MS used a 5-min solvent delay. Ethyl acetate extraction is suitable for the three aromatic alcohols as well as farnesol, whereas hexane or 1:4 ethylacetate/hexane is suitable for farnesol (6) but not for the aromatic alcohols. The phenethyl alcohol was purchased from Aldrich Chemical Co., Milwaukee, WI; tyrosol from Avocado Research Chemicals Ltd., Heysham, UK; and tryptophol from TCI-EP, Tokyo.

Northern Analysis

C. albicans total RNA used for mRNA accumulation was extracted by the hot phenol extraction method using yeast cells harvested at mid-log phase (8). Equal amounts of RNA (15 μ g) were resolved on 1.0% agarose–formaldehyde gels and transferred to GeneScreen Plus membranes (NEN Life Science Products, Boston, MA). The NorthernMax complete Northern blotting kit (Ambion, Austin, TX) was used for transfer and hybridization. The DNA templates for probe synthesis were prepared using PCR with *C. albicans* SC5314 genomic DNA and the following primer pairs (5' to 3'):
ARO80 – ATGTCAATTGTCGAACCAG and TCAATTCAAAAACTCCACAAG,
ARO8 – TATTCCAACACCGTCGTTCA and ACAAACTGGTCCAAGGCATC, *ARO9*

– CAAAACCTCCGCCTTCCAGTA and AGCCATCCATCAACACCTTT, *ARO10* – GTGCTTATGCTGCTGATGGA and TCTTTTTGGGTTCTGCTGCTG, *RIM101* – AGTCCATGTCCCATTGAAGC and ACACCGCCAAACTCTAATGC, *ACT1* – AGTTATCGATAACGGTTCTG and AGATTTCCAGAATTTCACTC. The DNA templates were used to synthesize DNA probes labeled with ³²P using an oligolabeling kit (Rad Prime DNA labeling system; Invitrogen Life Technologies, Carlsbad, CA), as described by Atkin et al. (2). Northern blots were PhosphorImaged using a Storm Phosphorimager (Amersham Pharmacia Biotech) and measurements of *ARO80*, *ARO8*, *ARO9*, *ARO10*, *RIM101* mRNA were normalized with *ACT1* mRNA control.

Results:

Aro80p is regulated by NCR transcription factors:

We have earlier reported that the Aro80p in *C. albicans* is only 32% identical to *S. cerevisiae*, but the DNA binding region, including six cysteine residues, is conserved in both species (4). First we wanted to confirm if Aro80p is activated solely in the presence of aromatic amino acids, as is the case in the yeast *S. cerevisiae*. Instead, we found that, the *C. albicans* Aro80p is also regulated by nitrogen catabolite repression genes. When the *C. albicans* cells were inoculated in a defined media with aromatic amino acids as the sole nitrogen source, all the wild type SC5314, control DAY286, and the *aro80/aro80* strains of *C. albicans* were able to grow using tyrosine, tryptophan, or phenylalanine as the sole nitrogen source (Fig. 3-1 A). Wild type SC5314 (parent), DAY286 (control), and *aro80/aro80* were able to utilize phenylalanine, tyrosine, or tryptophan as the sole source of nitrogen. They also had no defects in growth when all

three aromatic amino acids and ammonia were present as nitrogen sources (Fig. 3-1 A). The *aro80/aro80* strain and the control DAY286 are histidine auxotrophs. So, in these experiments 40 µg/ml of histidine was added in the media to fulfill the auxotrophic requirement. To check if this minimal amount of histidine was contributing to the growth, all the strains were also grown without the aromatic amino acids but with 40 µg/ml histidine (Fig. 3-1 A, plate labeled nothing). There was only residual growth for all strains, suggests that the histidine used to fulfill the auxotrophy did not contribute to the growth of the *C. albicans* strains. In contrast, *S. cerevisiae* was not able to use these aromatic nitrogen sources when the *ARO80* gene was disrupted (7).

Similarly, *C. albicans* Aro80p is activated in the presence of poor nitrogen sources like proline or arginine (Fig. 1 B) but it is also repressed by ammonia (Fig. 3-1 B). These results suggest that *C. albicans* *ARO80* is NCR regulated, unlike *S. cerevisiae*. We also found that the *ARO9* transcript was induced 68% in the presence of ammonia as a sole nitrogen source (GPA) compared to proline (GPP) (100%) as a sole source of nitrogen (Fig. 3-1 B). This comparatively high level of *ARO9* transcript suggests that, although down-regulated, *ARO9* was not completely inhibited by the presence of ammonia. This could be the reason for continued production of some aromatic alcohols in GPA (4). However, *ARO80* was completely repressed by ammonia. Thus, the regulation of aromatic alcohol biosynthesis was completely different in *C. albicans* than in the yeast *S. cerevisiae* where Iraqui et al, 1999 reported that the *ARO9* and *ARO10* transcripts were only expressed in the presence of tryptophan. *ARO9* and *ARO10* were not expressed in the presence of either poor nitrogen sources like urea or good nitrogen sources such as

ammonia. Unlike the pathogenic fungus *C. albicans* (Fig. 3-2 B) also, *ARO9* and *ARO10* were completely repressed in *aro80* strains of *S. cerevisiae* (7).

The pH regulation is lost in *aro80* Δ /*aro80* Δ strain:

Previously we showed that aromatic transaminases (*ARO8*, *ARO9*) are alkaline up regulated while the aromatic decarboxylase (*ARO10*) is alkaline down regulated (4). Thus we saw higher aromatic alcohol levels at pH 7 than at pH 3 (4). We also reported that this alkaline up regulation was lost in *rim13/rim13* and *rim101/rim101* mutants (4), suggesting a role for pH and the Rim101 pathway in aromatic amino acid metabolism. Here we report that in *aro80/aro80* mutants of *C. albicans* the aromatic alcohol levels were low and their pH regulation had also been lost (Fig. 3-2 A). The production of phenethyl alcohol and tyrosol were very similar at pH 3 and pH 7 (Fig. 3-2 A) whereas the production of tryptophol was still alkaline up regulated, although less so than for the wild type SC5314, CAI4, and parent BWP17 (Fig. 3-2 A).

Northern analysis revealed that *ARO8* and *ARO9* mRNAs were alkaline up regulated (~ 2 fold higher in alkaline conditions) in SC5314 and BWP17 (Fig. 3-2 B, Lanes 1-4) but not in *aro80/aro80* (Fig. 3-2 B, Lanes 5, 6). Similarly, *ARO10* was alkaline down regulated (~ 2 fold lower in alkaline conditions) in SC5314 and BWP17 (Fig. 3-2 B, Lanes 1-4) but not in *aro80/aro80* (Fig. 3-2 B, Lanes 5, 6). The aromatic alcohol production levels (Fig. 3-2 A) and the northern data (Fig. 3-2 B) are the average of three replicates. This data suggests that indeed the pH regulation of aromatic alcohol production was lost in *aro80/aro80* mutants.

Discussion:

Earlier we reported that aromatic transaminases (*ARO8*, *ARO9*) and aromatic decarboxylase (*ARO10*) are alkaline up regulated and alkaline down regulated respectively (4). At that time we proposed a model which suggested that aromatic amino acid metabolism genes were regulated by several transcription factors, including Gcn4p and the GATA transcription factors, along with Rim101p (4). In this report we have studied the regulation of the Zn₂Cys₆ transcription factor Aro80p and its role in aromatic amino acid metabolism. This study suggests that Aro80p is also under the control of at least two types of transcriptional activators, viz. the GATA transcription factors Gln3p and Gat1p, required for NCR, and Rim101p, required for alkaline activation. In the first part we observed that *C. albicans* can use phenylalanine, tyrosine, or tryptophan as the sole nitrogen source even when the Zn₂Cys₆ transcription activator Aro80p is absent (Fig. 3-1 A). So, Aro80p is not required to activate the aromatic amino acid metabolism genes. We hypothesized that when grown in the presence of aromatic amino acids the conditions will be similar to growth under poor nitrogen sources. In that case the aromatic amino acid metabolism genes are also under the control of NCR. Our northern analysis data (Fig. 3-1 B) revealed that the *ARO80* mRNA is also regulated by NCR as well as the *ARO9* mRNA.

In our previous study we reported that *ARO8*, *ARO9*, and *ARO10* probably were regulated by NCR as well. The production of aromatic alcohols by *aro80/aro80* was greatly reduced when grown in the presence of ammonia compared with proline as the sole nitrogen source (4). We also found GATAA binding sites in the upstream promoter regions of the aromatic transaminases and decarboxylase, suggesting that these enzymes

are also among the NCR sensitive genes (4). Here we report that the *ARO9* transcript was slightly down-regulated in the presence of ammonia (68%), as opposed to proline (100%). This explains the high levels of the aromatic alcohols observed in the presence of poor nitrogen sources like proline. The aromatic alcohol yield is down-regulated, but not completely inhibited by a preferred nitrogen source like ammonia (4). The transcription factor Aro80p was inhibited by ammonia. The detection of *ARO8*, *ARO9*, and *ARO10* in *aro80/aro80* (Fig. 3-2 B) also testifies that in *C. albicans* cells aromatic transaminases and decarboxylase are regulated by NCR transcription factors like Gln3p and Gat1p. We hypothesize that in the presence of poor nitrogen sources GATA transcription factors such as Gln3p and Gat1p will bind to the upstream of aromatic transaminases (*ARO8*, *ARO9*) and aromatic decarboxylase (*ARO10*) as well as their transcription activator Aro80p. Aro80p is also under the control of NCR. Therefore, when the preferred nitrogen is used up the cells shift their machinery to use poor nitrogen sources. Aro80p will be activated, which will increase the levels of aromatic transaminases and decarboxylase. This will increase the production of aromatic alcohols.

The availability of good nitrogen sources inside host varies with the site of infection. Moreover the physiological niches of the pathogenic fungus *C. albicans* and the yeast *S. cerevisiae* are different. Although both species uses same machinery to metabolize aromatic amino acids, the regulation of the aromatic amino acid metabolism genes is different in each case. The opportunistic pathogen *C. albicans* has evolved in such a way so that it can use aromatic amino acids in more than one way when good nitrogen sources are unavailable. Interestingly, fusel alcohols inhibit initiation factor eIF2B activity in the yeast *S. cerevisiae* (5). The initiation factor eIF2B is required for

general protein synthesis in a cell. It also serves to derepress the transcription factor Gcn4p required for general amino acid control response (5). Thus, when the fusel alcohol is accumulated in large amount outside the cell, it can serve as a nitrogen starvation signal. It would be very interesting to find out if a similar response occurs in the pathogenic fungus *C. albicans*. If the fusel alcohols can derepress Gcn4p in *C. albicans*, then the amino acid biosynthetic genes can be activated by this feedback control mechanism. In *C. albicans* cells, Gcn4p is also reported to induce hyphal morphogenesis by interacting with Ras-cAMP pathway (10). This can be an explanation of how tyrosol might induce hyphal morphogenesis in favorable conditions as has been reported by some other groups (1, 3).

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Figure Legends:

Figure 3-1. Effects of nitrogen catabolite repression (NCR) on *ARO80*. (a) Growth of *aro80/aro80* mutant on aromatic amino acids. Parent strain SC5314 (*ARO80/ARO80*), the control DAY286 and *aro80/aro80* strain streaked on defined media with phenyl alanine, tyrosine, Tryptophan, all 3 aromatic amino acids and ammonia, and nothing as sole nitrogen sources and incubated at 30°C. All the media were supplemented with 40 µg/ml of histidine to meet auxotrophic requirements. The positions of each strain in the plate are shown in the bottom right panel.

(b) Northern blot analysis of RNA prepared from SC5314 mid-log phase cells grown in GPP (P), GPR (R), or GPA (A) at 30°C. Blots were probed for the transcripts indicated along the right side. The numbers below represent the signal quantified with a Phosphor Imager™, normalized for *ACT1* loading control (average of three replicates).

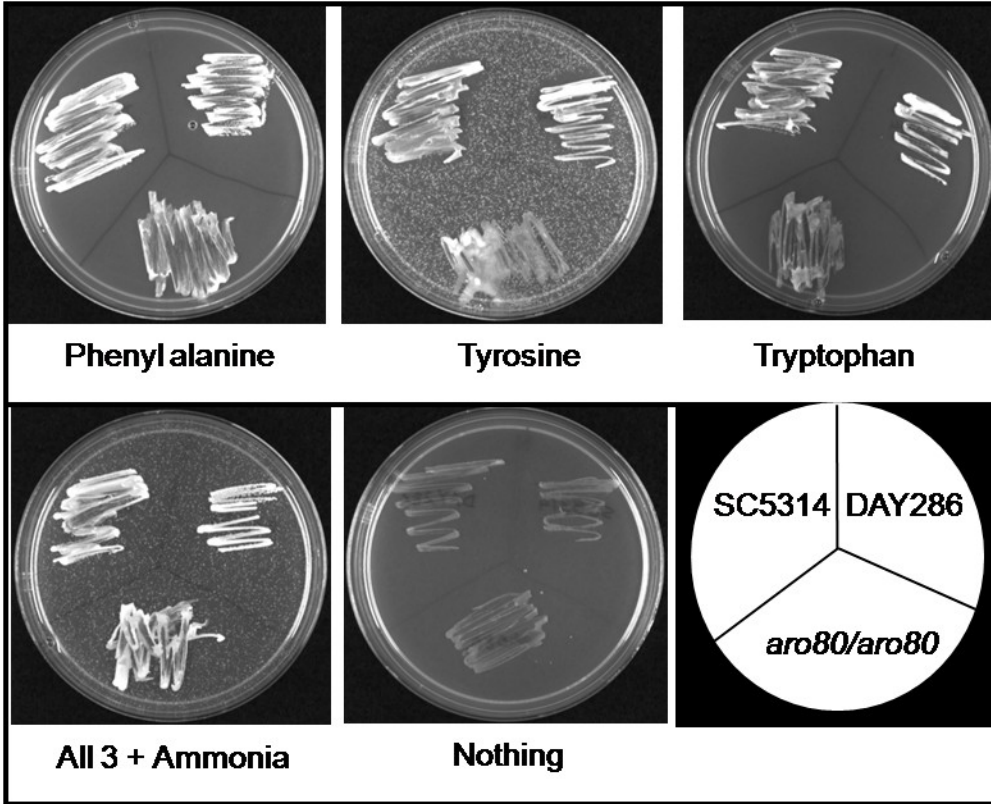
Figure 3-2. Effects of pH on production of aromatic alcohols in *aro80/aro80* strains.

(a) GC/MS analysis of *C. albicans* (30°C). SC5314, CAI4, BWP17, and *aro80/aro80* strains were grown in GPP at pH3 or pH7. Values are the average of triplicate experiments. Bars represent standard error. (b) Northern blot analysis of RNA prepared from mid-log phase cells grown at 30°C. Blots were probed for the transcripts indicated along the left side. The numbers below represent the signal quantified with a Phosphor Imager™, normalized for *ACT1* loading control (average of three replicates). Lane numbers at bottom.

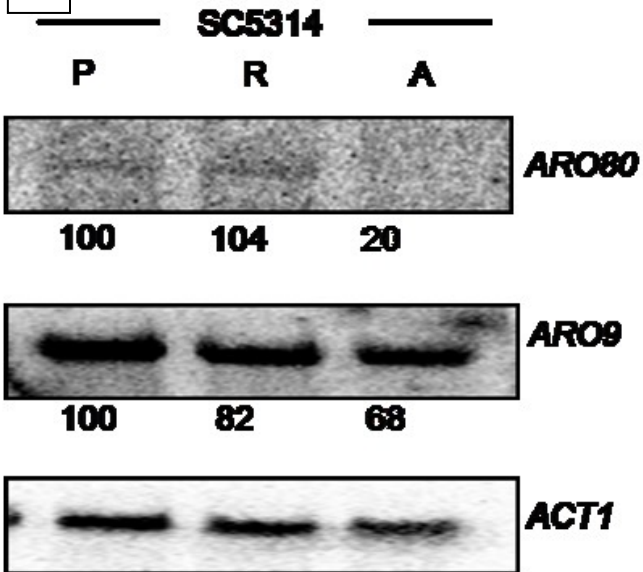
Figure 3-1

Figures:

A

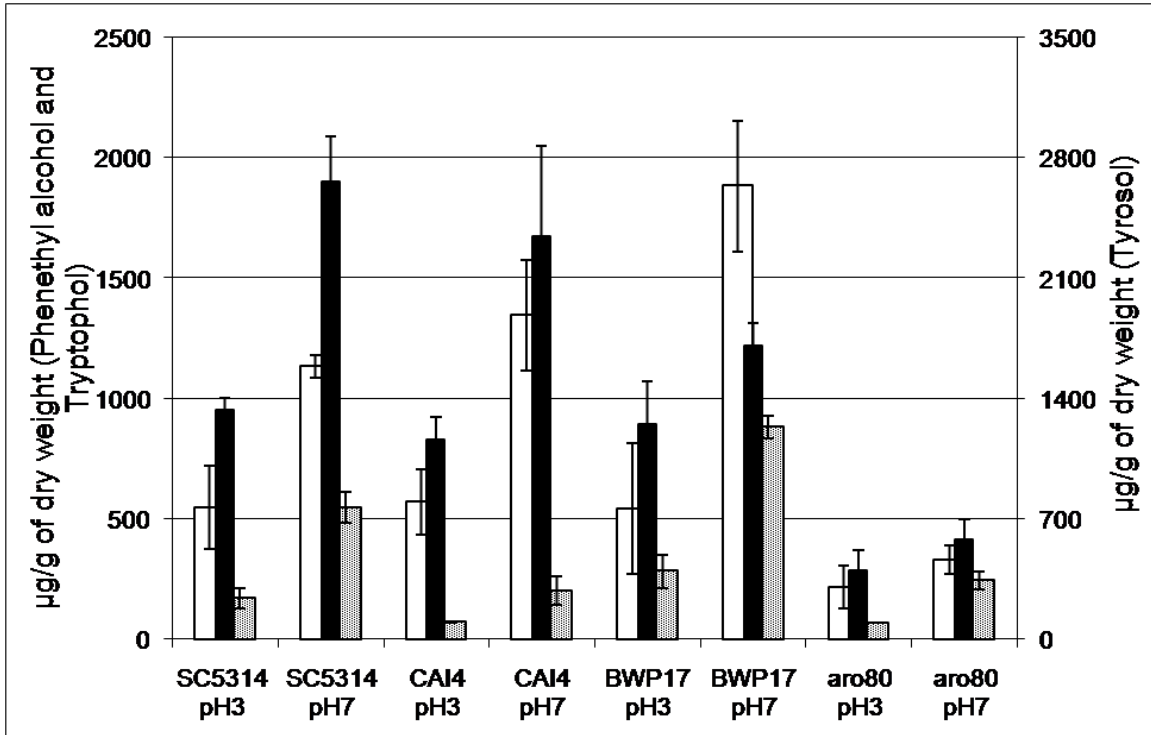


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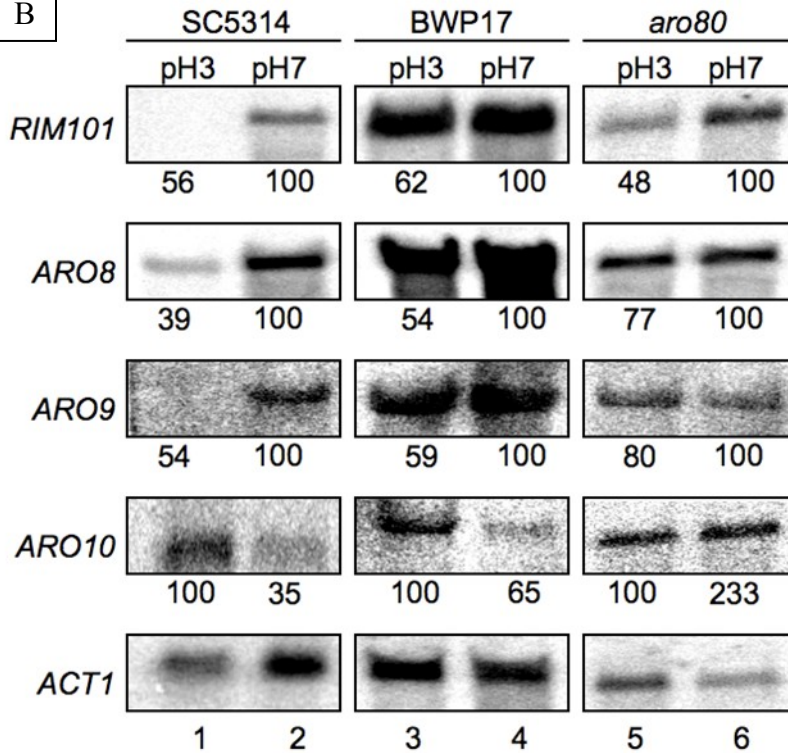


A

Figure 3-2



B



CHAPTER 4

Arginine induced germ tube formation in *Candida albicans* is essential for escape from murine macrophage RAW264.7 line

Reference:

Ghosh, S., D. H. Navarathna, D. D. Roberts, J. T. Cooper, A. L. Atkin, T. M. Petro, and K. W. Nickerson. 2009. Arginine-induced germ tube formation in *Candida albicans* is essential for escape from murine macrophage line RAW 264.7. *Infect Immun* 77:1596-605.

Abstract

The opportunistic fungal pathogen *Candida albicans* is a part of the normal flora but it also causes systemic candidiasis if it reaches the blood stream. Upon being phagocytized by macrophages, an important component of innate immunity, *C. albicans* rapidly up-regulates a set of arginine biosynthetic genes. Arginine, urea, and CO₂ induced hyphae in a density-dependent manner in wild type, *cph1/cph1*, and *rim101/rim101* strains but not in *efg1/efg1* or *cph1/cph1 efg1/efg1* strains. Arginase (Car1p) converts arginine to urea, which in turn is degraded by urea amidolyase (Dur1,2p) to produce CO₂, a signal for hyphal switching. We used a *dur1,2/dur1,2* mutant (KWN6) and the complemented strain KWN8 (*dur1,2/dur1,2 :: DUR1,2/DUR1,2*) to study germ tube formation. KWN6 could not make germ tubes in the presence of arginine or urea but did make germ tubes in the presence of 5% CO₂, which bypasses Dur1,2p. We also tested the effect of arginine on the interaction between the macrophage cell line RAW264.7 and several strains of *C. albicans*. Arginine activated an Efg1p-dependent yeast to hyphae switch, enabling wild type *C. albicans* and KWN8 to escape from macrophages within 6 h, whereas KWN6 was defective in this regard. Additionally, two mutants that cannot synthesize arginine, BWP17 and SN152, were defective in making hyphae inside the macrophages, whereas the corresponding arginine prototrophs, DAY286 and SN87, formed germ tubes and escaped from macrophages. Therefore, metabolism of arginine by *C. albicans* controls hyphal switching and provides an important mechanism for escaping host defense.

Key words: arginine, *DUR1,2*, phagocytosis, Efg1p-dependent pathway, urea amidolyase

Introduction

In immunocompromised patients such as those with AIDS, the innate immune system has an increased role in resisting infectious diseases. However, the opportunistic fungal pathogen *Candida albicans* has evolved mechanisms to evade innate immunity, which is an important reason that candidiasis is a major complication in AIDS patients. *C. albicans* resists macrophage phagocytosis via a mechanism that does not stimulate apoptosis in macrophages (22). *C. albicans* induces hyphae inside macrophages, thereby penetrating the cell membrane and escaping macrophages (21). *C. albicans* cells that are defective in making germ tubes, such as *cph1/cph1 efg1/efg1* (21) and *cdc35/cdc35* (22), cannot escape the macrophages following phagocytosis and are killed. Thus, the interaction between *C. albicans* and macrophages is critical in determining its pathogenicity in immunocompromised patients.

Lorenz et al (21) used DNA arrays to follow the transcriptional response by *C. albicans* to internalization in macrophages. Their transcriptional analysis suggested that once inside the macrophage *C. albicans* shifts from glycolysis to gluconeogenesis, activates fatty acid degradation, down-regulates transcription, and up-regulates arginine biosynthesis. In the later stages following internalization, hyphal growth is important to piercing the macrophage cell membrane, which at that time the cells resume glycolytic growth (21). Clearly switching from yeast to hyphae is a critical factor in escaping from macrophages after phagocytosis. Thus, one important question is: what triggers the morphological switch in *C. albicans* inside the macrophage?

In *C. albicans* the yeast to hyphae switch has been very well studied (3, 4, 18, 38). It is a carefully coordinated event which is regulated by multiple factors and several

signal transduction pathways. The environmental triggers for hyphal development include growth at 37°C, the presence of serum or *N*-acetylglucosamine (GlcNAc), neutral pH, CO₂, and nitrogen starvation (3, 4, 18, 38). These environmental stimuli act by turning on one or more signal transduction pathways that either stimulate or repress hyphal specific genes. These pathways include the Cph1p-mediated MAPK pathway and the Efg1p-mediated cAMP dependent protein kinase A (PKA) pathway, which has two isoforms of PKA, Tpk1p and Tpk2p, with differential effects on hyphal morphogenesis. Two other hyphal regulators, Rim101p and Czf1p, may function through Efg1p or act in parallel with Efg1p, while another transcription factor Tec1p is regulated by Efg1p and Cph1p. The MAPK cascade includes Cst20p (MAPKKK), Hst7p (MAPKK), Cek1p (MAPK) and the downstream transcription factor Cph1p, which is a homolog of the *S. cerevisiae* transcription factor Ste12p. *C. albicans* also has negative regulators of the hyphal transition. Chief among these is Tup1p, which acts in concert with Rfg1p, Nrg1p, or Rbf1p (3, 4, 17, 18, 38). The downstream targets of these environmental sensing pathways include the hyphal wall protein Hwp1p, adhesins of the ALS family, and extracellular hydrolytic enzymes (secreted aspartyl proteases, phospholipases) (3, 4, 18, 38).

Another unusual feature of *C. albicans* is that it uses the cytoplasmic enzyme urea amidolyase, encoded by *DURI,2*, to hydrolyze urea. Dur1,2p (degradation of urea) is a multifunctional, biotin – dependent enzyme (31) that was first characterized in the yeast *Candida utilis* (32). It is also present in *Saccharomyces cerevisiae* (7, 39). Catabolism of urea involves a single protein with two enzymatic activities. The first is an avidin-sensitive urea carboxylase (EC 6.3.4.6); urea is carboxylated in an ATP dependent

reaction forming allophanate, also known as urea carboxylate. The second is allophanate hydrolase or allophanate amidohydrolyase (EC 3.5.1.54), which releases two molecules each of NH_3 and CO_2 (39).

This paper addresses how the macrophage signal for hyphal switching relates to previously known signaling pathways. The transcriptional response analysis by Lorenz et al (21) showed that at an early stage arginine biosynthesis was strongly up-regulated. In this report we link arginine biosynthesis to the hyphal switch necessary for escape from the macrophage. The link is mediated by the enzyme urea amidolyase encoded by *DUR1,2*. Biosynthesis of arginine, which is metabolized by *C. albicans* cells producing CO_2 , is essential and acts as a signal to activate the cAMP-dependent PKA pathway, thereby regulating the yeast to hyphae switch inside the macrophage. This series of events is critical for hyphal development inside macrophage at the initial phase after phagocytosis, thereby piercing the macrophage and escaping.

Methods

Strains, media and growth condition

The *C. albicans* strains A-72 were obtained from Dr. Patrick Sullivan, University of Otago, Dunedin. Wild type clinical isolate SC5314, CAF2-1 (*ura3::imm434/URA3*) (11), CAI4 (*ura3::imm434/ura3::imm434*) (12), SN152 (*URA3/ura3::imm434 his1/his1 arg4/arg4 leu2/leu2 IRO1/iro1::imm436*) (28), SN87 (*URA3/ura3::imm434 his1/his1 leu2/leu2 IRO1/iro1::imm436*) (28) were obtained from Dr. Alexander Johnson, University of California at San Francisco. BWP17 (*ura3::imm434/ura3::imm434, arg4::hisG/arg4::hisG, his1::hisG/his1::hisG*) (27) and DAY286

(ura3::imm434/ura3::imm434, pARG4::URA3:: arg4::hisG/arg4::hisG,
his1::hisG/his1::hisG) (10) were obtained from Dr. Aaron Mitchell's collection. JKC19
(ura3::imm434/ura3::imm434, cph1::hisG/cph1::hisG, URA3::hisG) (20), HLC52
(ura3::imm434/ura3::imm434, efg1::hisG/efg1::hisG, URA3::hisG) (20) and HLC54
(ura3::imm434/ura3::imm434, cph1::hisG/cph1::hisG, efg1::hisG/efg1::hisG,
URA3::hisG) (20) were obtained from Dr. Gerald R. Fink, Massachusetts, USA and
 CAR2 (*rim101::hisG/rim101::hisG-URA3-hisG ura3::imm434/ura3::imm434*) (30) was
 obtained from Dr. Fritz A. Muhlschlegel, Canterbury, UK. GTC41
(ura3::imm434/ura3::imm434, GCN4/gcn4::hisG-URA3-hisG) (37), GTC43
(ura3::imm434/ura3::imm434, gcn4::hisG-URA3-hisG/gcn4::hisG) (37) and GTC45
(ura3::imm434/ura3::imm434, gcn4::hisG/gcn4::hisG, Clp10-GCN4) (37) were obtained
 from Dr. Alistair J. P. Brown, Aberdeen UK. The construction of KWN2
(dpp3::C.d.HIS1/ dpp3::C.m.LEU2, his1/his1, leu2/leu2, arg4/arg4) and KWN4
(dpp3::DPP3/ dpp3::DPP3, his1/his1, leu2/leu2, arg4/arg4) was described previously
 (25). KWN6 (*dur1,2/dur1,2*), KWN7 (*dur1,2/dur1,2::DUR1,2*) and KWN8
(dur1,2/dur1,2::DUR1,2/DUR1,2) were made by adapting the strategy reported by Reuß
 et al (34) using wild type strain A72 (16). All the KWN strains were made by Dhammika
 Navarathna and they are described in details in his thesis.

For routine growth and maintenance of the *C. albicans* strains, YPD medium (10
 g of yeast extract, 5 g of peptone and 20 g of glucose per liter) at 30°C was used.
 Auxotrophic mutants were grown in YPD supplemented with 40 µg/ml of required amino
 acid. RAW264.7 cells were grown in complete culture medium (500 ml of Dulbecco's

Modified Eagle's Medium + 50 ml of Fetal Bovine Serum + 0.55 ml of 50 mg/ml gentamycin) at 37°C in the presence of 5% CO₂.

Germ Tube Formation (GTF) Assay

C. albicans cells from stationary phase were transferred to GlcNAc – Imidazole – Mg buffer, pH 6.8. (11 mM imidazole, 3 mM MgSO₄, and 2.6 mM N-acetyl-D-glucosamine) (16) at 37°C for 4 hours. Germ tube induction by arginine and urea was performed by using 0.004% glucose and 20 mM arginine or 20 mM urea in distilled water at 37°C. There was no GTF in the glucose – only controls, i.e. with no added arginine or urea. GTF assays in the presence of 5% CO₂ were performed in two ways. The first paralleled the arginine and urea experiments in that it used screw-cap flasks containing 0.004% glucose at 37°C whereas the second transferred *C. albicans* cells growing in YPD in 6 well plates at 37°C exposed to atmosphere of air with 5% CO₂. All the assays except GTF assay in the presence of CO₂ were conducted in 25-ml Erlenmeyer flasks using *C. albicans* inoculums, which had been stored at 4°C in 50 mM potassium phosphate buffer (pH 6.5). The cells were added in aliquots to pre-warmed (37°C) assay medium to give a final cell density of 10⁵-10⁷ cells/ml. The flasks were shaken on a New Brunswick Scientific G2 shaker at 37°C and 225 rpm for 4-6 h and examined for GTF by confocal microscopy. At time zero, the inoculated cells are >98% undifferentiated with 0% germ tubes and 0 to 2% budding yeasts.

Co-culture conditions and macrophage ingestion assay

The murine RAW264.7 macrophage-like cell line was grown in DMEM culture media that contained 10% Fetal Bovine Serum and 50 µg/ml gentamycin. One day prior to the experiment, RAW264.7 cells that reached confluence in culture media were

collected, washed and counted with a hemacytometer. 10^6 cells were plated in culture media in 6 well plates and grown overnight in 5% CO₂ at 37°C to allow adherence to the surface. On day zero the non-adherent cells were removed from the plates by aspiration and fresh pre-warmed complete culture medium was added. Two forms of *C. albicans* yeast cells were used; either up to one week-old resting cells, or actively growing, mid-log phase cells. The resting phase cells were prepared by growing *C. albicans* strains overnight in YPD at 30°C, washing the cells 3 times with 50 mM potassium phosphate buffer (16) and storing the cells in the same buffer. In the second case, these yeast cells were diluted 1:100 and grown for 6-8 h in YPD at 30°C whereupon the log phase cells were harvested by centrifugation. Cultures were washed with phosphate-buffered saline and concentrations were measured using a Spectronic 20 spectrophotometer. 10^6 or 2×10^6 cells were added to each well (1:1 or 2:1 *C. albicans* : macrophage ratio), and the plates were incubated for 6 h at 37°C. At 1 h time point the plates were washed with pre-warmed phosphate-buffered saline and fresh pre-warmed complete culture media was added to minimize *C. albicans* cells that were not phagocytized. The co culture conditions, germ tube formation and escape from macrophage were examined by phase contrast microscopy at different time points. Microscopic examination revealed that a small number of *C. albicans* cells remained that were not phagocytized but adhered to the surface.

Results

Arginine, urea and CO₂ stimulate hyphae by a cell density dependent pathway.

Wild type *C. albicans* A72 formed hyphae in the presence of 2.6 mM GlcNAc, 20 mM arginine, 20 mM urea, or 5% CO₂ within 4 – 6 h (Fig. 4-1 B). High (20 mM) levels of arginine or urea consistently induced germ tube formation in 80 – 90% of the cells (Fig. 4-1 B), whereas lower (5 mM) levels stimulated only ca. 30% of the *C. albicans* cells. Germ tube induction by arginine, urea or CO₂ was cell density dependent in that the efficiency of GTF was 80-90% at $\leq 10^6$ cells/ml, ca. 40% at 10^7 cells/ml, and even less at higher cell densities (data not shown). Interestingly, the presence of 5% CO₂ stimulated GTF under both nutrient rich (YPD) (Fig. S4-1) and poor (0.004% glucose) conditions (data not shown), and in both cases GTF was cell density-dependent. Also, for both arginine and urea, GTF was blocked by ammonium sulfate; 5 mM ammonium sulfate reduced GTF to 10 – 35% and 10 mM blocked GTF completely. In contrast, CO₂ stimulated GTF was not blocked by 10 mM ammonium sulfate. These results are consistent with one or more steps in arginine and urea stimulated GTF being subject to nitrogen catabolite repression (NCR). In *S. cerevisiae*, both arginase (36) and urea amidolyase (8) are subject to NCR. Finally, arginine stimulated biofilm formation in *C. albicans* A72 (data not shown).

Germ tube formation is induced by arginine, urea, or CO₂ in an *efg1* dependent way.

We also examined GTF using four strains of *C. albicans* that lack transcription factors responsive to Rim101p dependent signaling (CAR2) (30), MAP kinase signaling (JKC19) (20), cAMP signaling (HLC52) (20), or both (HLC54) (20). These strains are all derived from CAI4, and they are particularly useful in determining the pathway(s) responsible for germ tube induction by any stimulant. The CAI4 parent exhibited GTF

with 2.6 mM GlcNAc, 20 mM arginine, 20 mM urea, or 5% CO₂ (Fig. 4-2). Significantly, the JKC19 (*cph1/cph1*) and CAR2 (*rim101/rim101*) mutants could respond to arginine, urea or 5% CO₂ (Table 4-1), whereas the HLC52 (*efg1/efg1*) and HLC54 (*cph1/cph1::efg1/efg1*) mutants could not (Fig. 4-2 and Table 4-1). These results suggest that arginine, urea, and 5% CO₂ induce GTF by an Efg1p-dependent mechanism (Fig. 4-2). In *C. albicans* external CO₂ is transported inside, either by diffusion or by transporters, and converted to HCO₃⁻ by carbonic anhydrase, thus activating adenylyl cyclase to synthesize cAMP, which in turn triggers the morphogenetic switch from yeast to hyphae (2).

Urea amidolyase mutants (*dur1,2/dur1,2*) cannot utilize urea as a sole nitrogen source

Arginine can be converted to urea and L-ornithine by the enzyme arginase (Car1p) (24, 36), and urea is converted to CO₂ and ammonia by urea amidolyase (Dur1,2p) (7). To explore whether arginine, urea, and CO₂ are parts of a pathway stimulating GTF or if they act separately, we created *C. albicans dur1,2* knockout mutant (KWN6) and the homozygous reconstituted strain (KWN8).

The effects of *DURI,2* knockout and reconstitution on the ability to use urea as a nitrogen source are shown in Fig. 4-1A. The parent strain A72 and the reconstituted strain (KWN8) were able to grow on defined minimal media with L-proline, urea, or L-arginine as the sole nitrogen source, whereas the *dur1,2, dur1,2* knockout strain (KWN6) was unable to grow on urea at either 30°C (Fig. 4-1A) or 37°C (not shown). However, KWN6 grew as well as its A72 parent on four media: YPD (not shown) and the three defined media GPP (L-proline), GPR (L-arginine), and GPPU (L-proline and urea). It is

not surprising that KWN6 grew on GPR; Car1p breaks arginine down to urea and L-ornithine, and even though KWN6 cannot use the nitrogens in urea, they can still use the nitrogens in L-ornithine. Also, all three strains grew on proline and urea together, showing that the inability of KWN6 to grow on urea only (Fig 4-1 A) was not due to the accumulation of toxic components derived from urea. None of the strains grew on thiourea, and thiourea did not inhibit the growth of A72 on either L-proline or urea (data not shown).

Arginine, urea and CO₂ induce germ tube in a single sequential pathway.

A72 and the reconstituted KWN8 behaved identically under all GTF inducing conditions, i.e. GlcNAc, arginine, urea, and 5% CO₂. However, KWN6 was defective in GTF in the presence of arginine or urea (Fig. 4-1 B), even though it exhibited unimpaired GTF in the presence of 5% CO₂ or GlcNAc (Fig. 4-1 B) or 10% serum (data not shown). The 6 hr GTF assay for KWN6 in 5% CO₂ (Fig. 4-1 B) is somewhat misleading in that it shows many budding yeasts along with the hyphae. The 1 and 2 hr samples showed that $\geq 98\%$ of the cells underwent GTF (Fig. S 4-1); the budding yeasts only appeared 2-6 hrs after inoculation. This shift to the yeast morphology is likely a cell density dependent phenomenon (26). These results suggest a pathway whereby arginine is converted to urea and then to CO₂, with CO₂ acting as a common signal for GTF in *C. albicans*. These results are summarized in Table 4-1. They are consistent with a single sequential pathway for stimulating germ tube formation (Fig. 4-3). This pathway merges our data for arginine and urea with the CO₂, cAMP, and Efg1p-dependent pathway developed by the Muhlschlegel laboratory (2).

Arginine biosynthesis is essential for the escape of *C. albicans* from RAW264.7 macrophage cell line.

We used two types of mutants to test whether the arginine to urea to CO₂ signal operates inside macrophages. The first type (this section) cannot convert arginine or urea to CO₂ (*dur1,2/dur1,2*) while the second type (next section) cannot synthesize arginine.

C. albicans A72 (*DUR1,2/DUR1,2*), KWN6 (*dur1,2/dur1,2*), and KWN8 (*dur1,2/dur1,2::DUR1,2/DUR1,2*) all formed hyphae within 1 hr at 37°C in the complete macrophage growth medium with a 5% CO₂ atmosphere. This observation shows that KWN6 is not defective in its hypha forming ability (Table 4-1). Cells from both resting phase and log phase cultures behaved similarly in terms of GTF in the complete culture medium. These observations are not surprising since this culture medium contains a powerful trigger of GTF, 10% serum and the cells are incubated in an atmosphere that contains 5% CO₂, another trigger for GTF. Thus, hyphal growth was also observed in co-culture experiments for any *C. albicans* that had not been ingested by the RAW264.7 macrophage cells.

Wild type *C. albicans* A72 was fully engulfed by the macrophages by 1 hr, but within 4 hrs the fungus had made hyphae inside the macrophage cells, and by 6 hrs it had penetrated the membranes and emerged or escaped from the RAW264.7 cells. In contrast, KWN6 (*dur1,2/dur1,2*), the urea amidolyase knock out mutant, exhibited delayed hyphae formation; by 4 hrs there were mostly yeast cells and very few hyphae inside the RAW264.7 cells. Similarly, by 6 hrs the percentage and length of germ tubes were much less for KWN6 than for the wild type A72. Finding a few hyphae on KWN6 cells likely means that those cells had been triggered for GTF by the serum and CO₂ and

remained committed (26) for GTF even after ingestion. The ability for GTF inside RAW264.7 macrophage cells was fully restored in both the *DURI,2* complemented strains, the singly *DURI,2* reconstituted KWN7 (Fig. S 4-2) and the doubly *DURI,2* reconstituted KWN8 (Fig. 4-4 A).

The wild type clinical isolate SC5314 was also tested and found positive for the yeast to hyphae switch and escape from RAW264.7 cells (Fig. 4-4 B) as was CAF2-1 (*ura3/URA3*), but CAI4 (*ura3/ura3*) was unable to stimulate hyphae and penetrate the RAW264.7 cells (Fig. 4-4 B). This defect might be because of the lack of *iro1* (13), which is required to acquire iron.

Arginine auxotrophic mutants are defective in escaping from RAW264.7 macrophage cell line.

To test our hypothesis further, we selected two genetically related pairs of amino acid auxotrophic mutants. BWP17 requires his, arg, and ura (27) whereas DAY286 requires only his (10). Similarly, SN152 requires his, leu, and arg whereas SN87 requires only his and leu (28). We found that BWP17 and SN152 could not stimulate hyphae inside the RAW264.7 cells; they remained inside the macrophages even after 6 hrs (Fig. 4-4 C). In contrast, DAY286 and SN87 penetrated the membranes and emerged from the macrophages by 6 hrs (Fig. 4-4 C). These data strongly suggest that arginine biosynthesis is a key regulator for the yeast to hyphae switch inside macrophages. This view was confirmed by the inability of two *arg4* mutants, KWN2 (*dpp3/ dpp3, arg4/arg4*) and KWN4 (*dpp3/ dpp3::DPP3/DPP3, his1/his1, leu2/leu2, arg4/arg4*), to escape from RAW264.7 cells (Fig. S 4-3).

Arginine biosynthesis and escape from macrophages are not regulated by Gcn4 and the General Amino Acid Control pathway.

When eukaryotic cells are starved for nitrogen, the cells respond by activating Gcn4p, a transcription factor that targets roughly 500 genes including most of the amino acid biosynthetic genes (15). The macrophage phagosome environment is likely to be nutritionally poor (5). If the phagosome is nitrogen starved, then it should activate Gcn4p, thereby inducing many amino acid biosynthetic genes as well as morphogenesis (37). Thus, we tested a series of *gcn4* related mutants of *C. albicans* (Fig. 4-4 D). Significantly, all four strains, CAF2-1 (*GCN4/GCN4*), GTC41 (*gcn4/GCN4*), GTC43 (*gcn4/gcn4*), and GTC45 (*ura3/ura3, gcn4/gcn4::CIP10-GCN4*) (37), switched from yeasts to hyphae and were able to escape from the RAW264.7 cells (Fig. 4-4 D). Thus, our results are consistent with the DNA array results of Lorenz et al (21). They found that apart from arginine no other amino acid biosynthetic genes were up regulated (21). Taken together, these data suggest that arginine biosynthesis inside the macrophage is not regulated by Gcn4p but by some other pathway, possibly Arg82p and the Arg80p-Mcm1p-Arg81p complex, which are known to regulate arginine biosynthesis in *S. cerevisiae* (23). This pathway, which specifically induces the *ARG* genes just to breach the macrophage membranes, is of enormous importance as this breach might lead to systemic candidiasis.

Discussion

We have elucidated the signaling pathway whereby *C. albicans* initiates hyphal growth after being ingested by macrophages. Lorenz et al (21) showed that the genes for

L-arginine biosynthesis were induced following internalization by macrophages, and Sims (35) and Bahn and Muhlschlegel (2) showed that elevated CO₂ triggered hyphal growth. We have connected these two observations via the enzyme urea amidolyase (Dur1,2p). The key role of urea amidolyase is shown by the inability of a *dur1,2/dur1,2* mutant (KWN6) to escape from mouse macrophages, while this ability is restored in the reconstituted strains KWN7 and KWN8. The suggested signaling pathway is shown in Fig. 4-3.

A critical role for arginine following macrophage internalization was implied by DNA microarray studies (21). We confirmed that hypothesis using two sets of paired mutants (Fig. 4-4 C). Two strains with an arginine auxotrophy could not escape from the macrophages, whereas the corresponding strains without an arginine auxotrophy could. Interestingly, the strains that were able to escape were auxotrophic for amino acids other than arginine. These findings strongly suggest that the induction of germ tube formation, which is essential for escape from macrophages, requires biosynthesis of arginine but not other amino acids inside macrophages. Also, there is an apparent paradox between the inability of SN152 to escape from macrophages within 6 hrs (Fig. 4-4 C) and its pathogenicity in a mouse tail vein model (28). This continued pathogenicity may just demonstrate the artificial nature of the tail vein model or it could reflect the eventual escape of some SN152 from macrophages after a longer period of time.

The last half of the proposed signaling pathway (Fig. 4-3) is similar to that described by Bahn and Muhlschlegel (2). They showed that *C. albicans* can induce germ tubes in the presence of CO₂ by activating adenylate cyclase (2). The presence of CO₂ is important because *C. albicans* can convert CO₂ to bicarbonate inside the cell by the

enzyme carbonic anhydrase (Nce103p). Bicarbonate then activates adenylate cyclase (Cdc35p), which in turn activates cAMP dependent protein kinase A, thereby activating hyphal specific genes in an Efg1p-dependent manner (2). We confirmed that arginine, urea, and CO₂ induce hyphae in an Efg1p-dependent manner (Fig. 4-2). It has been already established that yeast to hyphae switch is a critical virulence factor in *C. albicans* (26).

Once inside a macrophage, arginine is converted to L-ornithine and urea by the enzyme arginase (Car1p) (24) and urea is converted to CO₂ and NH₃ by the enzyme urea amidolyase (Dur1,2p) (7). From microarray data, Lorenz et al (21) observed that 1 hr after ingestion *CARI* (19.3934) and two other related arginase genes (19.10922 and 19.5862) were up-regulated 3.2-, 4.7-, and 5.1- fold respectively. *DURI,2* (19.780) was also up-regulated after 1 hr but only by 1.4- fold (supplementary data for ref 21). These results suggest that inside the macrophage *C. albicans* not only synthesizes arginine but also utilizes arginine. The *dur1,2/dur1,2* mutant could not use arginine or urea for GTF but was able to respond to its downstream product CO₂ (Fig. 4-1). The inability of *arg4* and *dur1,2* mutants to escape from macrophages suggests that, even though the RAW264.7 cells were grown in 5% CO₂, the phagosomes contained significantly less CO₂. Alternatively, the phagosome environment might be altered in an unknown manner that prevents *C. albicans* from responding to high CO₂.

Furthermore, we observed that 5-10 mM ammonium salts prevented GTF as induced by GlcNAc, arginine, or urea but not by 5% CO₂. The explanation for these differences probably resides in the realm of nitrogen catabolite repression (NCR). Comparative study of *C. albicans* and *S. cerevisiae* often sheds light on the genetic

mechanisms by which regulatory mechanisms work. In the case of *S. cerevisiae*, both *CARI* (34, 36) and *DURI,2* (8) are under the control of NCR. When rich nitrogen sources such as ammonia or asparagine are available the cells are designed to utilize them first and repress other genes that are responsible for breaking down poorer nitrogen sources like proline, arginine or urea (40). When cells are starved for nitrogen, these NCR-regulated genes are induced. In *C. albicans* we found several GAT(A/T)(A/G) sites in the 1000 bp upstream of the open reading frames for both *CARI* and *DURI,2*. These are the putative binding sites for the GATA transcription factors Gln3p and Gat1p, which can mediate NCR in *C. albicans* (9). This regulation makes sense because in the presence of arginine and urea *C. albicans* will induce the NCR-regulated genes *CARI* and *DURI,2* which in turn will make enough CO₂ to induce hyphae by the cAMP/PKA pathway (Fig. 4-3). The use of 5% CO₂ bypasses the steps subject to NCR, shown in the box in Fig. 4-3. As a final thought on the significance of *CARI* and *DURI,2* being NCR-regulated, macrophage phagosomes are acidic (21) whereas neutrophil phagosomes are more basic (33). If the greater alkalinity in neutrophils is ammonia-mediated, the resulting repression of *CARI* and *DURI,2* could partially explain why macrophages kill *C. albicans* less effectively than do neutrophils (1, 33).

DNA array analysis after phagocytosis by human neutrophils revealed that both *C. albicans* and *S. cerevisiae* induced genes for methionine and arginine biosynthesis but still could not escape from neutrophils (33). In addition to the NCR-based explanation provided in the previous paragraph, this situation may arise because neutrophils kill, or at least influence, the *C. albicans* cells quickly through a more potent oxidative and nitrosative burst, thus preventing hyphal formation. This explanation is consistent with

the role of macrophages in innate and adaptive immune responses, which in addition to directly killing the invading microbes is to present antigens to T cells and to produce many different cytokines and chemokines that in turn attract other innate and adaptive immune components. For *C. albicans* cells ingested by neutrophils, only about 70% of the cells were still alive by 60 min (33), whereas for macrophages all of the cells had formed hyphae and escaped by 6 hrs (21). In a separate study, Arana et al (1) found that only 24% of *C. albicans* cells survived after 2 hrs inside neutrophils, whereas 234% (cell replication) had survived after 2 hrs inside macrophages (1). These results along with our own suggest that macrophages kill *C. albicans* less effectively than do neutrophils.

C. albicans has at least three putative arginases (encoded by *CARI*, orf 19.3418, and orf 19.5862), all of which are strongly induced in macrophages (21). Why are three arginases needed and do they serve the same function? Murine macrophages, including RAW264.7 cells, primarily kill microbes via nitrosative stress (6, 19, 29). This NO production is mediated by the enzyme iNOS, which requires arginine as a substrate. Some bacteria are known to avoid macrophage killing by inducing arginase in the host macrophages (19), and a similar protection against macrophage killing was attributed to arginase (rocF) production by *Helicobacter pylori* (14). Therefore, induction of arginase upon ingestion by macrophages may provide a second survival benefit to *C. albicans* by depriving macrophages of the substrate required for synthesis of NO. In this regard, it is significant that Car1p and Dur1,2p are both cytoplasmic; they do not have predicted N-terminal signal peptides (<http://www.cbs.dtu.dk/services/SignalP/>). In contrast, the proteins encoded by orf 19.3418 (361 amino acids) and orf 19.5862 (418 amino acids) have predicted signal peptides, with probabilities of 1.00 and 0.97, respectively. Thus, it

seems likely that the three arginases serve at least two functions. Car1p is cytoplasmic, working with Dur1,2p in a pathway for GTF (Fig. 4-3), whereas the other two arginases are excreted. The excreted arginases may curb nitrosative stress in some fashion. This suggestion predicts that mutants defective in the arginases would have reduced survival in macrophages. Arginase induction would not affect killing by neutrophils, which rely instead on myeloperoxidase.

Gcn4p is a transcription factor that activates most of the amino acid biosynthetic genes under nitrogen starving conditions (15). Strains lacking Gcn4p were examined to see if it regulated arginine biosynthesis during the initial phase after phagocytosis. It was clear from our data (Fig. 4-4 D) that Gcn4p is not essential for hyphae formation because *gcn4/gcn4* mutants were fully capable of forming hyphae inside macrophages (Fig. 4-4 D). However, the fact that Gcn4p does not appear to be needed increases the interest in finding the activator/pathway which does induce the arginine biosynthetic genes after phagocytosis. This regulation may be via Arg82p and the Arg80p-Mcm1p-Arg81p complex, which is known to regulate arginine biosynthesis in *S. cerevisiae* (23), or it might be unique to *C. albicans*, in which case it would be a candidate target for future drugs in case of candidiasis.

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We thank all the colleagues for providing us with *C. albicans* strains, specially Dhammika Navarathna for constructing the *dpp3/dpp3* (KWN2), *dur1,2/dur1,2* (KWN4) mutants and their revertants KWN6 and KWN8 respectively. We also thank Dr. Fahd Al-Salleeh for the help with macrophage co-culture system. This work was supported by the

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Table 4-1. Germ tube formation in wild type and mutant *C. albicans*

| Strain names (Relevant Genotypes in brackets) | GlcNAc | Arginine | Urea | CO ₂ |
|---|--------|----------|------|-----------------|
| A72 (Wild Type) | + | + | + | + |
| KWN6 (<i>dur1,2/dur1,2</i>) | + | - | - | + |
| KWN8 (<i>dur1,2/dur1,2::DUR1,2/DUR1,2</i>) | + | + | + | + |
| CAI4 (<i>ura3/ura3</i>) | + | + | + | + |
| JKC19 (<i>cph1/cph1</i>) | - | + | + | + |
| HLC52 (<i>efg1/efg1</i>) | - | - | - | - |
| HLC54 (<i>cph1/cph1, efg1/efg1</i>) | - | - | - | - |
| CAR2 (<i>rim101/rim101</i>) | + | + | + | + |

Figure legends:**Figure 4-1. Urea amidolyase mutants.**

A. Growth of *dur1,2/dur1,2* mutant on urea. Parent strain A72 (*DUR1,2/DUR1,2*), the urea amidolyase mutant KWN6 (*dur1,2/dur1,2*) and *DUR1,2* reconstructed strain KWN8 (*dur1,2/dur1,2::DUR1,2/DUR1,2*) streaked on defined media with proline (GPP), urea (GPU), arginine (GPR), and proline + urea (GPP+U) as sole nitrogen sources and incubated at 30°C.

B. Germ tube formation by GlcNAc, arginine, urea and CO₂ in *dur1,2/dur1,2* mutants. Photomicrographs showing germ tube assay of A72 (*DUR1,2/DUR1,2*), KWN6 (*dur1,2/dur1,2*) and KWN8 (*dur1,2/dur1,2::DUR1,2/DUR1,2*) strains in presence of 2.6 mM N-acetyl glucosamine (GlcNAc) (first row), 20mM arginine (second row), and 20 mM urea (third row), all at 37°C after 4 hrs, and 5% CO₂ (fourth row) at 37°C after 6 hours. Photomicrographs in the first three rows are taken in a confocal microscope and the fourth row is DIC in a bright field microscope.

Figure 4-2. Germ tube formation by GlcNAc, arginine, urea, and CO₂ in non-filamentous mutants. Photomicrographs showing germ tube assays for CAI4, JKC19 (*cph1/cph1*), HLC52 (*efg1/efg1*), HLC54 (*cph1/cph1, efg1/efg1*), and CAR2 (*rim101/rim101*) in the presence of 2.6 mM N-acetyl glucosamine (GlcNAc) (first column), 20mM arginine (second column), 20 mM urea (third column) and 5% CO₂ (fourth column) at 37°C after 4 hours. Representative photomicrographs in the first three

columns are taken in a confocal microscope and the fourth column is DIC in a bright field microscope.

Figure 4-3. Suggested pathway for arginine induced germ tube formation.

Arginine is metabolized to ornithine and urea by arginase (Car1p); urea is degraded to CO₂ and NH₃ by the enzyme urea amidolyase (Dur1,2p); CO₂ activates adenyl cyclase and the cAMP dependent protein kinase A pathway, thereby activating Efg1p which triggers the yeast to hyphal switch inside macrophage. The two steps catalyzed by Car1p and Dur1,2p, are under nitrogen catabolite repression (NCR). L-ornithine can be used as an alternative nitrogen source by *C. albicans*.

Figure 4-4. Interaction of *C. albicans* with macrophages. Yeast cells were incubated *ex vivo* with RAW264.7 cells in complete culture medium (with 10% serum) at 37°C in 5% CO₂ and the DIC photomicrographs were taken at 1 hour (first column), 4 hour (second column) and 6 hour (third column) time points.

A. *C. albicans* A72 (*DUR1,2/DUR1,2*, parental strain) (first row), KWN6 (*dur1,2/dur1,2*) (second row) and KWN8 (*dur1,2/dur1,2::DUR1,2/DUR1,2*) (third row). Arrows at 1 hr show three *C. albicans* which had been phagocytized by macrophages and two non-ingested *C. albicans* which already have visible germ tubes. The arrows at 4 hrs for A72 and KWN8 show *C. albicans* with visible germ tubes in the process of escaping whereas the 4 and 6 hrs arrows for KWN6 show *C. albicans* yeast cells within the macrophages.

B. SC5314 (*URA3/URA3*, wild type) (first row), CAF2-1 (*ura3/URA3*) (second row) and CAI4 (*ura3/ura3, iro1/iro1*) (third row)

C. Auxotrophic mutants, BWP17 (*his1/his1, arg4/arg4, ura3/ura3*) (first row), DAY286 (*his1/his1*) (second row), SN152 (*his1/his1, arg4/arg4, leu2/leu2*) (third row) and SN87 (*his1/his1, leu2/leu2*) (fourth row).

D. *gcn4* mutants, CAF2-1 (*GCN4/GCN4*, parent) (first row), GTC41 (*GCN4/gcn4*) (second row), GTC43 (*gcn4/gcn4*) (third row) and GTC45 (*ura3/ura3, gcn4/gcn4::Cip10-GCN4*) (fourth row).

Supplementary Figure Legend

Figure 4-S1. Germ tube formation in KWN6 (*dur1,2/dur1,2*) as induced by 5% CO₂.

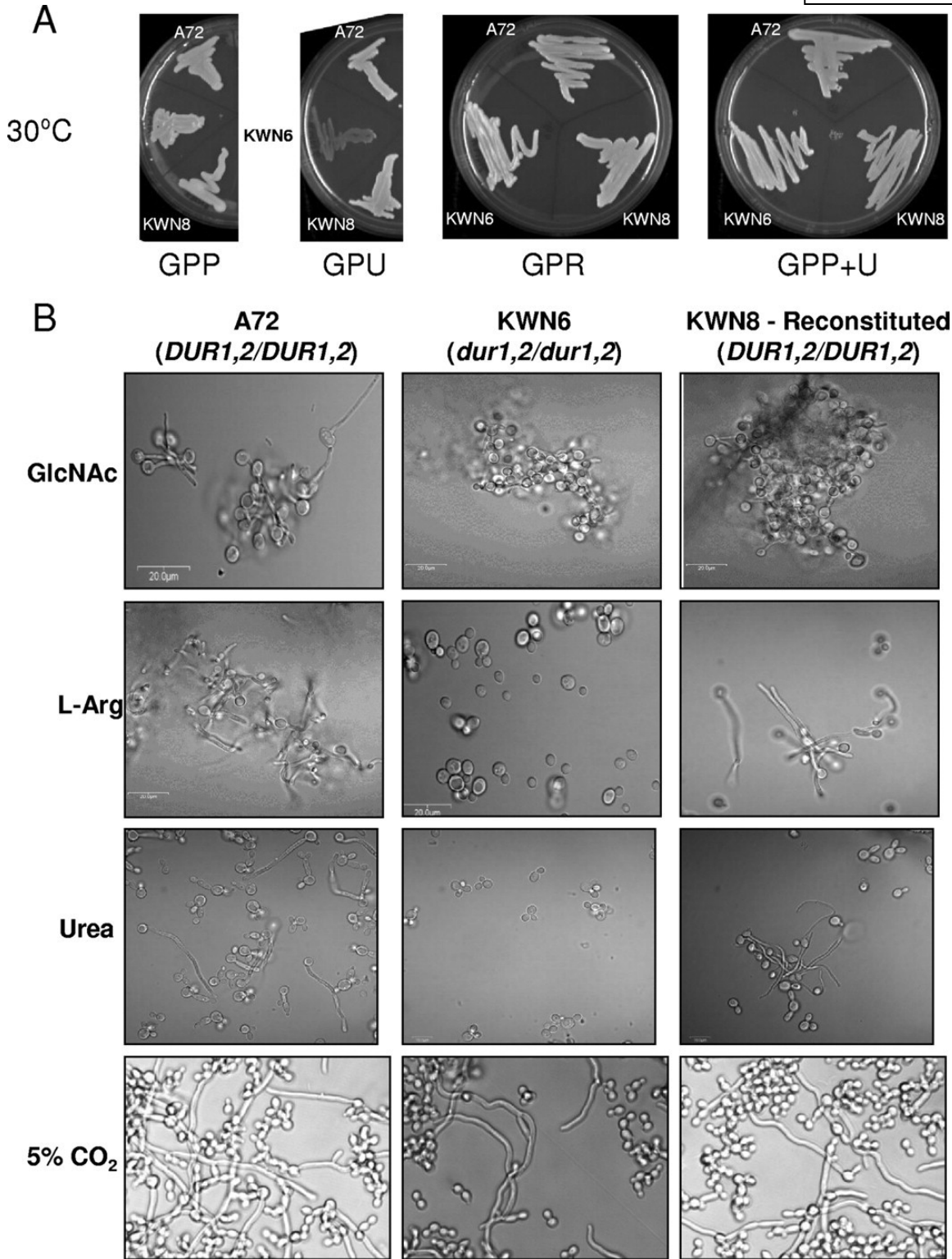
KWN6 cells were inoculated into YPD in the presence and absence (not shown) of 5% CO₂ and shaken at 37°C and 200 rpm for the indicated time. After 1 hr the cells grown with 5% CO₂ had already induced hyphae whereas those without CO₂ had not. By 4 and 6 hrs budding yeasts had developed from the hyphae, probably as the result of cell growth during that time and a cell density dependent conversion to yeast growth.

Figure 4-S2. Interaction of *C. albicans* with macrophages. KWN7

(*dur1,2/dur1,2::DUR1,2*) cells were incubated *ex vivo* with RAW264.7 cells in complete culture medium (with 10% serum) at 37°C in 5% CO₂ and the DIC photomicrographs were taken at 1 hour, 4 hour and 6 hour time points.

Figure 4-S3. Interaction of *C. albicans* with macrophages. Yeast cells were incubated *ex vivo* with RAW264.7 cells in complete culture medium (with 10% serum) at 37°C in 5% CO₂ and the DIC photomicrographs were taken at 1 hour (first column), 4 hour (second column) and 6 hour (third column) time points. SN152 (*his1/his1, leu2/leu2, arg4/arg4*) (first row), KWN2 (*dpp3/dpp3, arg4/arg4*) (second row), and KWN4 (*dpp3/dpp3::DPP3/DPP3, his1/his1, leu2/leu2, arg4/arg4*) (third row).

Figure 4-1



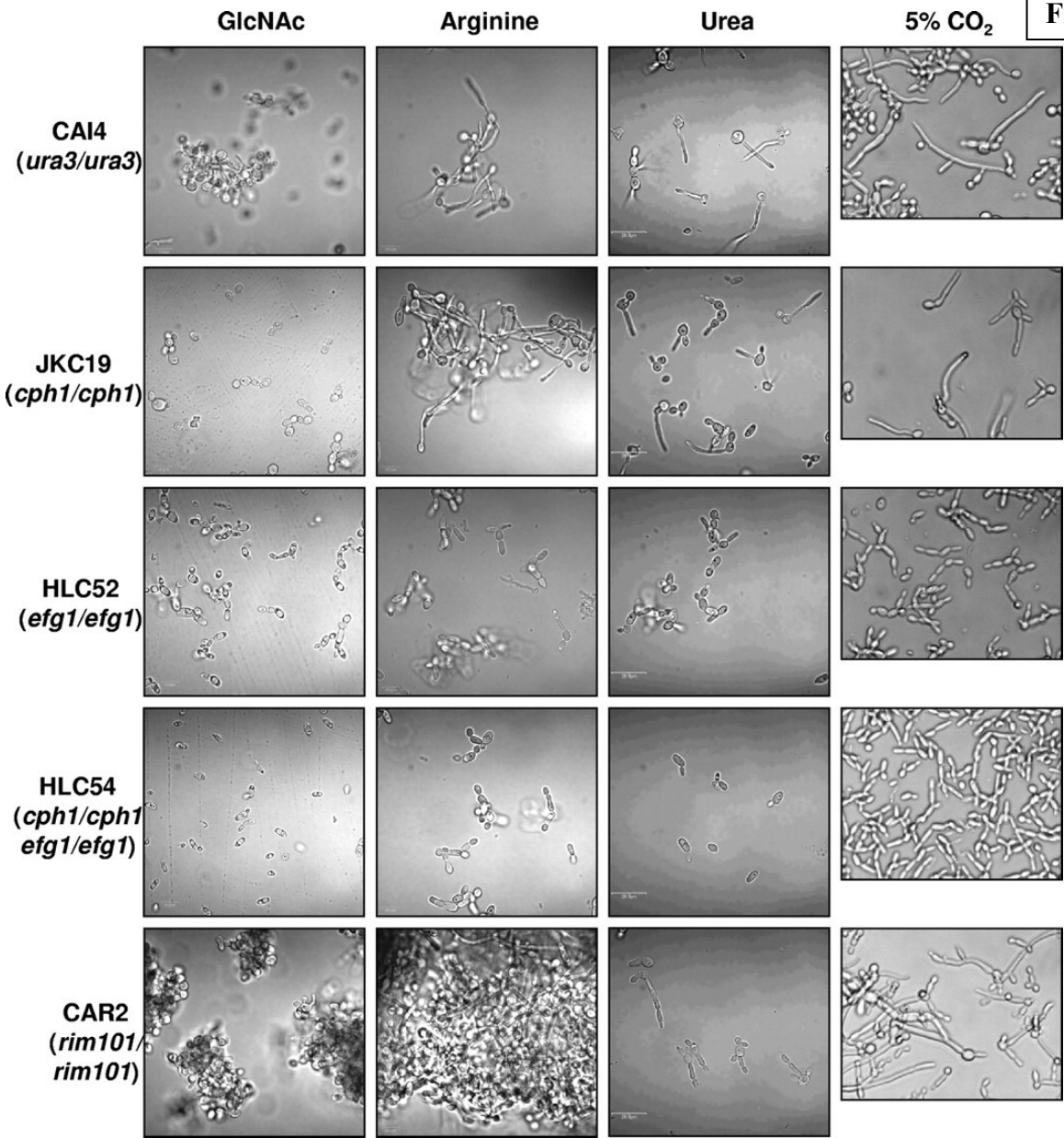


Figure 4-3

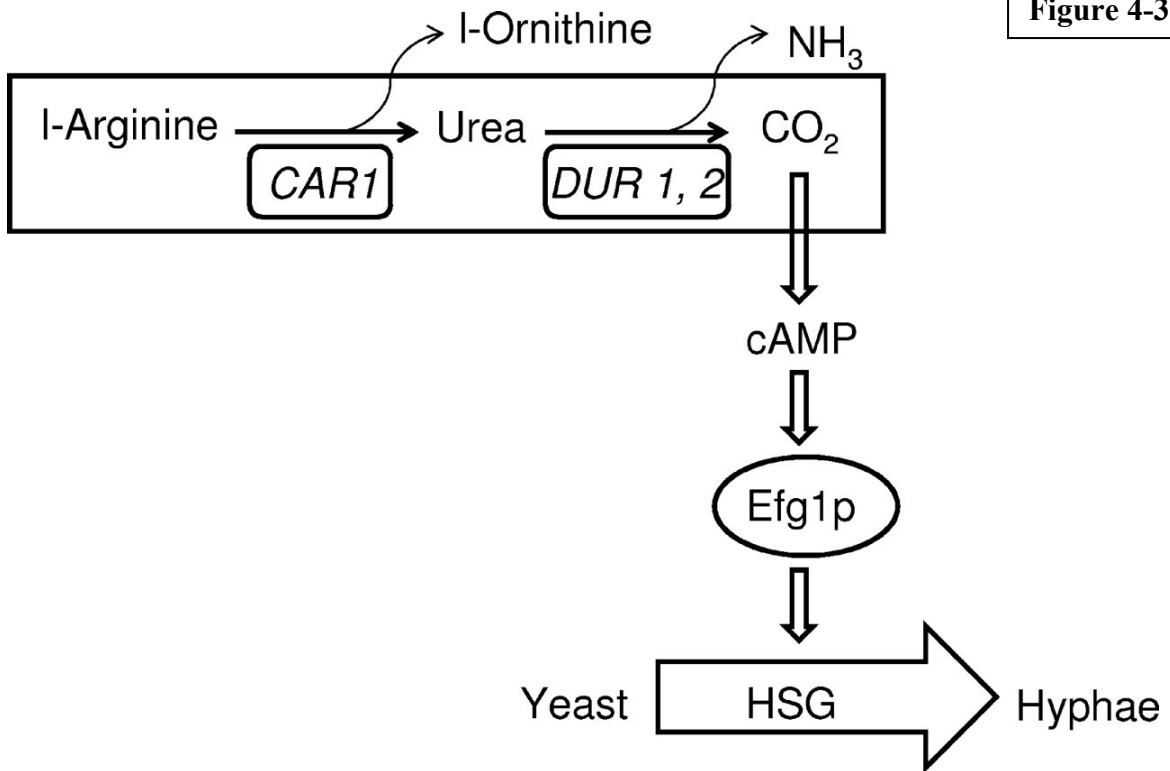


Figure 4-4

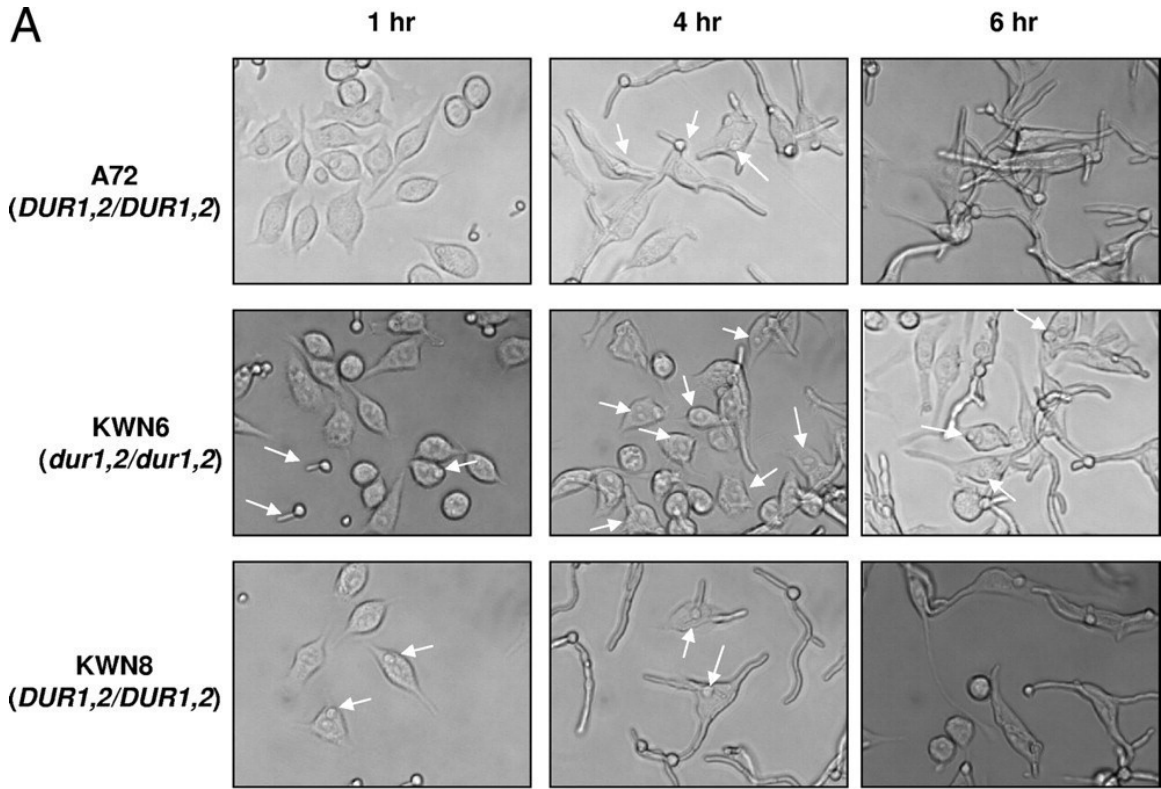


Figure 4-4

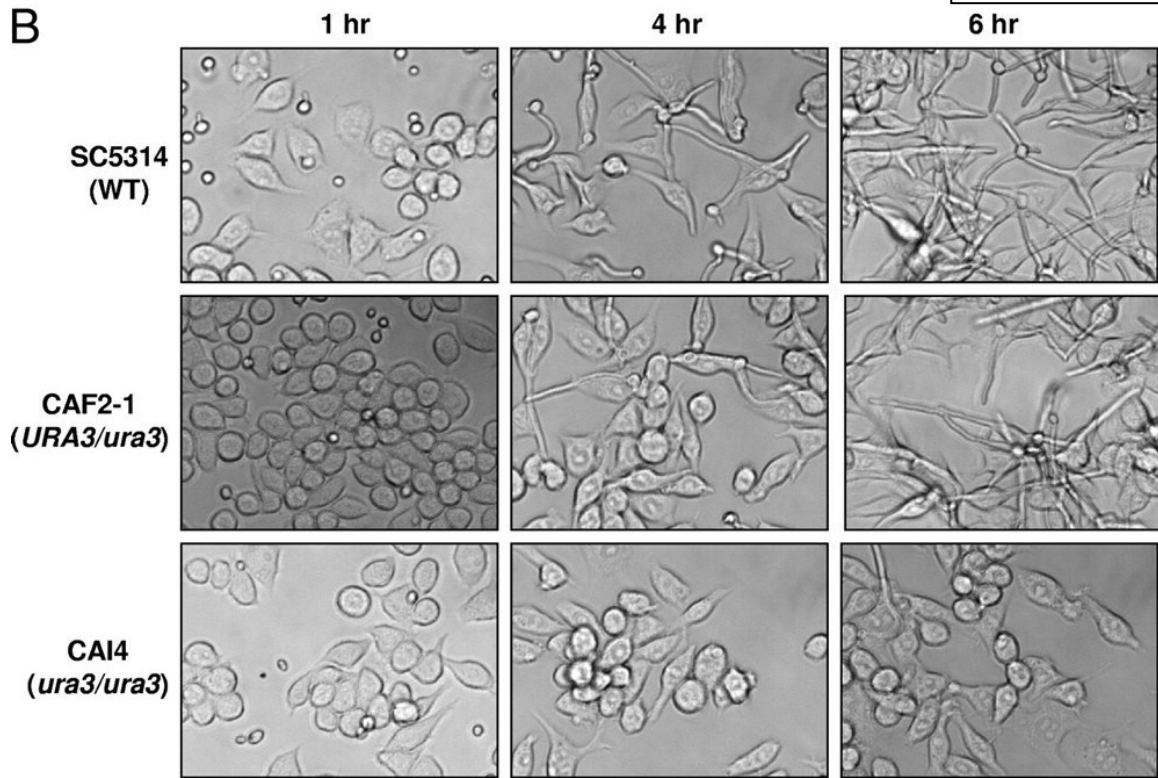


Figure 4-4

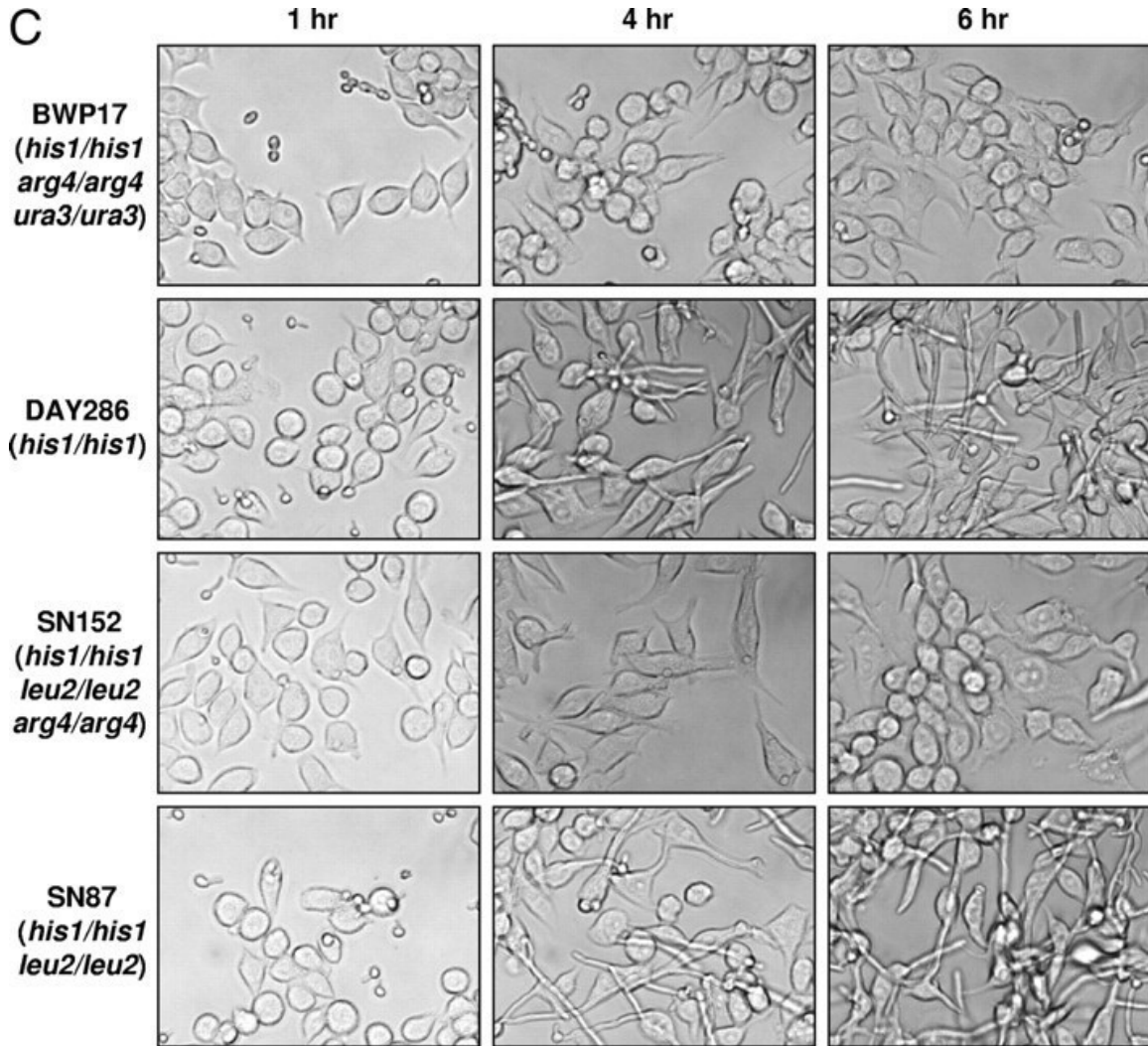


Figure 4-4

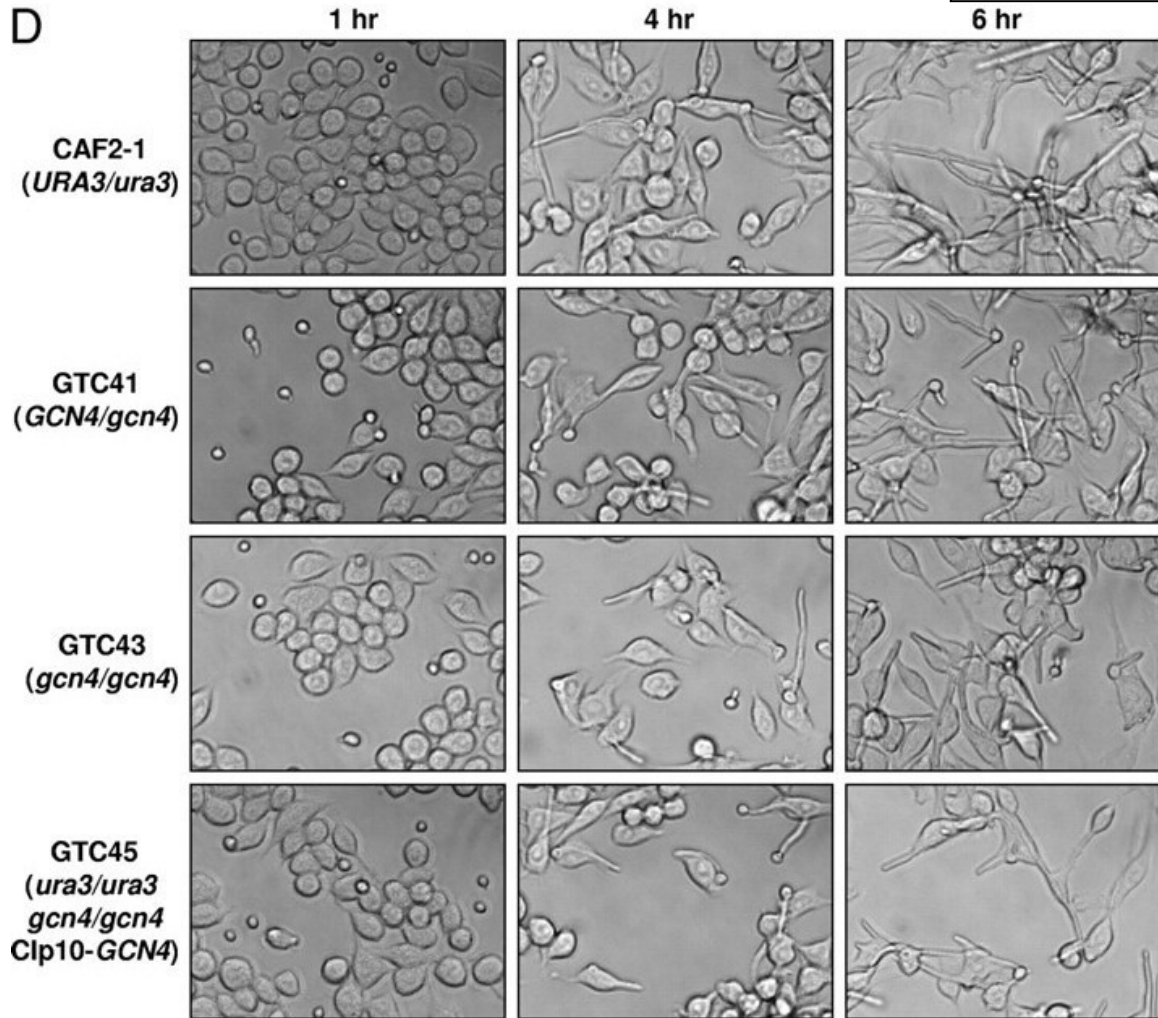


Figure 4-S1

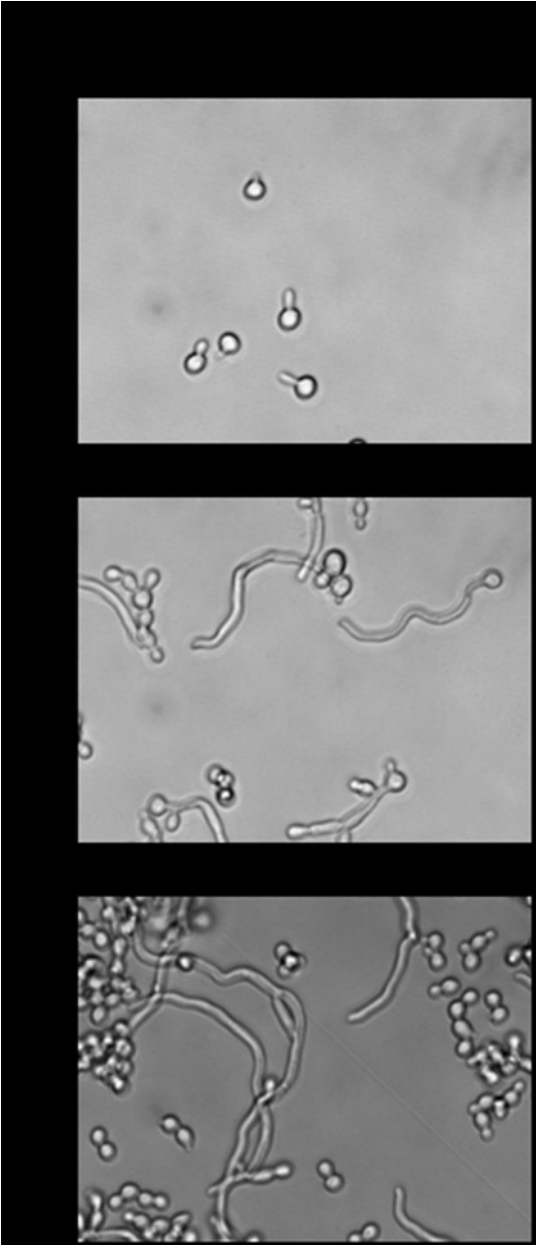


Figure 4-S2

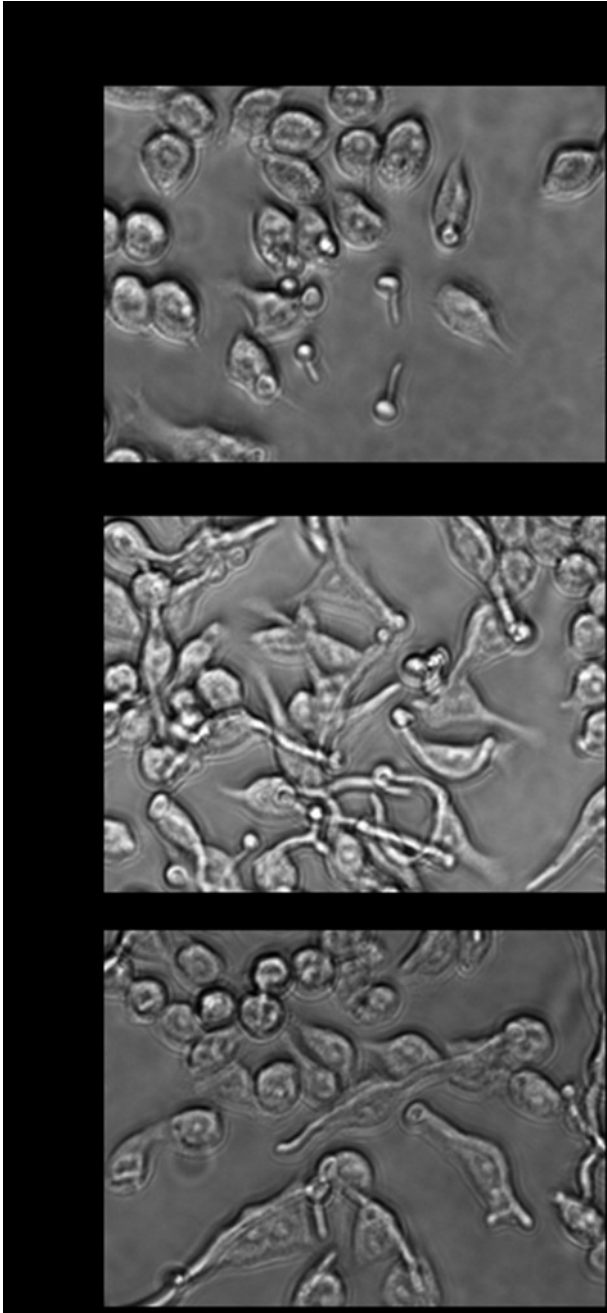
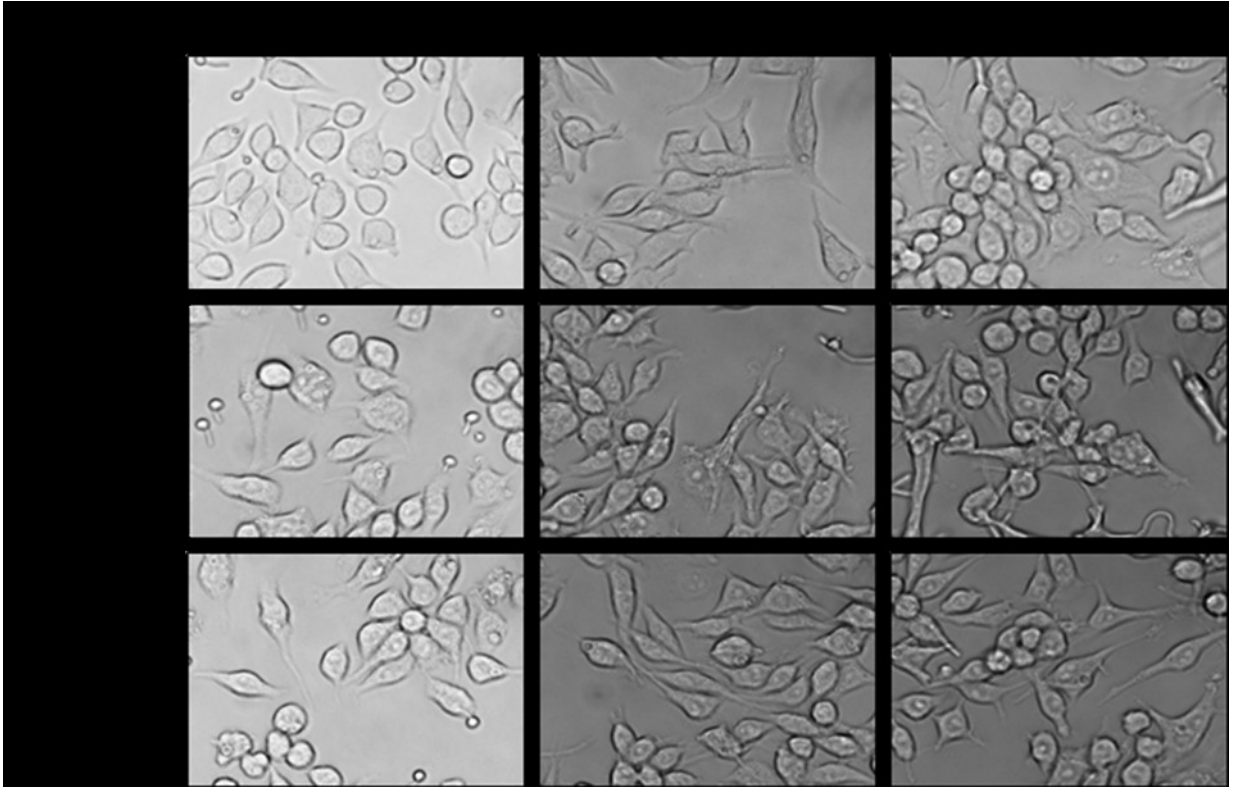


Figure 4-S3



CHAPTER 5

***Candida albicans* Activates Th17-Inducing Cytokines in Murine Macrophage RAW264.7 Line**

Reference:

Ghosh, S., N. Howe, K. Volk, K. W. Nickerson, and T. M. Petro. *Candida albicans* through beta-glucan and farnesol stimulates expression of Th17-inducing cytokines in the murine RAW264.7 macrophage cell line. Immunology letters. Submitted.

Abstract

Candida albicans is an opportunistic fungal pathogen that causes candidiasis in immune-compromised patients. After being phagocytized by macrophages, *C. albicans* switches from yeast to hyphae and escapes from macrophage by four to six hours. However, within that time macrophages respond to beta-glucans of *C. albicans* through TLR2 and express cytokines that induce development of the Th17 subset of T cells, which is required for effective immunity to *C. albicans*. The purpose of this study was to examine the cytokine response of macrophages, if any, within three hours after phagocytosis of *C. albicans*. The murine macrophage line RAW264.7 was challenged with wild type live *C. albicans* or *C. albicans* that were heat killed, mutants defective in forming germ tubes, mutants defective in farnesol production, or a clinical isolate that produces farnesoic acid instead of farnesol. Expression of the Th17-inducing cytokines, IL-6, TGF- β , and IL-23p40/p19, was evaluated by qRT-PCR. All viable strains except the isolate that produces farnesoic acid induced expression of IL-6 up to 1000-fold. Hyphae formation did not influence IL-6 expression but it did lower IL-23p19/p40 and TGF- β expression significantly. To understand the components in *C. albicans* responsible for expression of Th17-inducing cytokines, RAW264.7 cells were incubated with farnesol, farnesoic acid, zymosan, heat killed *C. albicans*, or combinations thereof. High expression of IL-6 also occurred when macrophages were stimulated with zymosan, a TLR2 agonist, and farnesol together. Farnesol alone had little effect on IL-6 induction; however zymosan alone induced IL-6. *C. albicans* or farnesol plus zymosan increased TLR2 expression in RAW264.7 cells. Our results suggest that macrophages challenged

with *C. albicans* quickly express robust levels of IL-6 and to a lesser extent IL-23 p19/p40, mostly due to responses to farnesol and zymosan together.

Key words: IL-6, IL23p19/p40, TGF- β , TLR2, *Candida albicans*, farnesol, zymosan.

Introduction

Candida albicans is a commensal fungus that colonizes the human oral cavity and intestine. It is polymorphic in that it converts between the yeast, hyphal, and pseudohyphal forms (1). Individuals with healthy immune systems limit *Candida* growth at mucosal sites. In contrast, a compromised adaptive immune system often leads to mucocandidiasis, oral thrush, or systemic candidiasis with significant mortality (3, 5). Key virulence factors leading to mucosal or systemic candidiasis include the following: i) morphogenesis – yeast to hyphae switching; ii) phenotypic switching, e.g. white – opaque switching; iii) epithelial adhesion; iv) production of extracellular enzymes, e.g. phospholipase B and aspartyl proteases; and v) production of farnesol (21-23). Farnesol was first identified as a quorum sensing molecule (QSM) in that it blocked the yeast to hypha conversion by *C. albicans* (11). Later we showed that farnesol also acted as a virulence factor (21-23). At that time we created a knockout mutation in *DPP3*, the gene encoding a phosphatase which converts farnesyl pyrophosphate to farnesol. This mutant KWN2 (*dpp3/dpp3*) produced six times less farnesol and was ca. 4.2 times less pathogenic to mice than its parent (22).

Since that time we have been interested in farnesol's mode of action as a virulence factor. This mode of action appears to be distinct from farnesol's mode of action as a QSM, i.e. blocking hyphal development. As a step towards deciphering

farnesol's mode of action, we showed that blood from mice pretreated with farnesol had significantly reduced levels of the critical Th1 cytokines IFN- γ and IL-12, accompanied by elevated IL-5 levels (23). The present paper takes a further step in elucidating farnesol's mode of action. These experiments are done with the same *C. albicans*/murine macrophage system that we used to show the importance of arginine biosynthesis by *C. albicans* (8). Following ingestion by macrophages, wild type *C. albicans* turn on arginine biosynthesis, and then metabolize that arginine via arginase (*CARI*) and urea amidolyase (*DURI,2*) to activate the yeast to hyphae switch and escape from the macrophage. Wild type *C. albicans* escaped within 4 – 6 hours whereas both arginine auxotrophs and *dur1,2/dur1,2* mutants were unable to form hyphae or escape (8). A critical unanswered question concerns the extent to which macrophages are able to send cytokine signals before they are killed.

Macrophages produce cytokines, some of which direct CD4⁺ T cell differentiation to a phenotype that promotes effective immunity to *C. albicans*. Macrophage IL-12 directs T cell differentiation to the Th1 subset that produces IFN- γ . Production of IFN- γ activates macrophages. We have previously shown that farnesol decreases expression of IL-12 from macrophages (22). Alternately macrophage IL-23, IL-6, and TGF- β induce a different T cell subset, Th17 (2), which produces IL-17 that is required for resistance against mucosal (3) and systemic candidiasis (13). Production of IL-17 leads to accumulation of neutrophils (13), which phagocytize and kill both yeast and hyphal versions of *C. albicans* (28). However, it is unclear if macrophages that have phagocytized *C. albicans* express these Th17 eliciting cytokines, IL-6, TGF- β , and IL-23 prior to their death.

In order to respond to potential pathogens by making cytokines, macrophages express Toll-like receptors (TLRs) that bind to a series of distinctive pathogen associated molecular patterns (PAMPs) (14, 16). Cell surface TLR2 responds to the beta-glucans (9) and phospholipomannans (18) of *C. albicans* while TLR4 responds to *C. albicans* alpha-mannans (29). However, TLR2 is not alone in its response. Dectin-1 associates with TLR2 to recognize the beta glucans (9) while galectin-3 associates with TLR2 to recognize the beta 1,2 mannosides (17). Therefore TLR2 is a key macrophage cell surface molecule for responses to *C. albicans*.

To examine whether post-phagocytosis hyphae formation and farnesol production influence the ability of macrophages to express Th17-inducing cytokines, we have taken a genetic and molecular approach. In this report we used five strains of *C. albicans*, each defective in either hyphal formation or farnesol production, to determine the impact of these fungal virulence factors on induction of IL-6, IL-23 p19/p40, and TGF- β post-phagocytosis. We show that phagocytosis of *C. albicans* by macrophages rapidly induced IL-6 and increased TLR2 expression regardless of hyphae formation or production of farnesol. However, hyphae formation decreased IL-23p19/p40 induction. By using farnesol, farnesoic acid, and zymosan (a beta-glucan and TLR2 agonist) alone and in combination, we found that farnesol and zymosan act synergistically to induce IL-6 and IL-23p19/p40 but not TGF- β .

Methods

Strains, media, growth conditions and chemicals

The *C. albicans* wild type SC5314 and strain SN152 (*URA3/ura3::imm434 his1/his1 arg4/arg4 leu2/leu2 IRO1/iro1::imm436*) (25) were obtained from Dr. Alexander Johnson, University of California at San Francisco. *C. albicans* 10231 was obtained from the American Type Culture Collection (Rockville, MD). The construction of KWN2 (*dpp3::C.d.HIS1/ dpp3::C.m.LEU2, his1/his1, leu2/leu2, arg4/arg4*) and KWN4 (*dpp3::DPP3/ dpp3::DPP3, his1/his1, leu2/leu2, arg4/arg4*) was described previously (22).

C. albicans strains were grown and maintained in YPD medium (10 g of yeast extract, 5 g of peptone and 20 g of glucose per liter) at 30°C, while auxotrophic mutants were grown in YPD supplemented with 40 µg/ml of required amino acid. Resting phase cells were grown overnight in YPD at 30°C, washed 3 times with 50 mM potassium phosphate buffer, and stored in the same buffer. These cells were used for co-culture with murine macrophage RAW264.7 line. Heat killed cells were prepared by heating the resting phase cells at 60 °C for 2 h. This temperature regime was chosen to be sure the *C. albicans* cells were still intact particles, suitable for phagocytosis. Cell death was confirmed by spreading the heat killed cells on YPD plates; there was no growth following incubation at 30 °C for 24 hours (Data not shown). The murine RAW264.7 macrophage-like cell line was grown in complete culture medium (500 ml of Dulbecco's Modified Eagle's Medium + 50 ml of Fetal Bovine Serum + 0.55 ml of 50 mg/ml Gentamycin) at 37°C in the presence of 5% CO₂.

The trans, trans - farnesol was purchased from Sigma-Aldrich Chemicals, (E, E) – farnesoic acid from Echelon Biosciences Inc., Salt Lake City, UT, and zymosan from

Invivogen, San Diego, CA. Farnesol, farnesoic acid, and zymosan were dissolved in methanol to make stock solutions prior to use.

Challenge of macrophages with *C. albicans* or their components

One day prior to the experiment, RAW264.7 cells that reached confluence in culture media were collected, washed, and counted with a hemacytometer. 10^5 cells per well were plated in culture media in 12-well plates and grown overnight in 5% CO₂ at 37 °C to allow adherence to the surface. On day zero the non-adherent cells were removed from the plates by aspiration and fresh pre-warmed complete culture medium was added. Resting phase *C. albicans* cells were washed with phosphate-buffered saline and their concentrations were measured using a Spectronic 20 spectrophotometer. 4×10^5 cells were added to each well (4:1 *C. albicans* : macrophage ratio), and the plates were incubated for 1 h, 2 h, or 3 h in 5% CO₂ at 37°C. For the challenge of macrophages with various cell components, the RAW264.7 line was treated with: a) 5 μM of farnesol; b) 250 μM of farnesoic acid; c) 25 μg/ml of zymosan; d) 5 μM of farnesol and 25 μg/ml of zymosan; e) 250 μM of farnesoic acid and 25 μg/ml of zymosan; f) 5 μM of farnesol and 4×10^5 heat killed SC5314 cells; or g) 250 μM of farnesoic acid and 4×10^5 heat killed SC5314 cells. After 1, 2, and 3 h culture media was removed and 400 μl of lysing buffer (5Prime PerfectPure RNA Cell and Tissue, RNA isolation kit) was added to the wells and the sample was transferred into tubes and frozen at -80 °C.

RNA isolation, cDNA, and RT-PCR

RNA was extracted using the PerfectPure RNA isolation kit of 5PRIME, Inc. (Gaithersburg, MD) according to the manufacturer's instructions. cDNAs were prepared from 0.3 μg of RNA. Quantitative real-time PCR (qRT-PCR) was carried out in the

presence of 1 mM dNTPs, 2 μ M MgCl₂, 2U Taq DNA polymerase, 1 μ M of each primer and 1 μ L of cDNA in 25 μ L. For IL-6, TGF- β , p19, p40, and GAPDH qRT-PCR was performed with the Platinum-SYBR Green I-UDG-quantitative PCR SuperMix (Invitrogen) and the following primers: IL-23 p19: 5'-GCTGGATTGCAGAGCAGTAATA-3'/5'-GCATGCAGAGATTCCGAGAGAG-3' (124 bp); p40: 5'-ATGGCCATGTGGGAGCTGGAG-3'/5'-TTTGGTGCTTCACACTTCAGG-3' (335 bp); TGF- β : 5'-TACTGCCGCTTCTGCTCCCAC T-3'/ 5'-GATGGCTTCGATGCGCTTCCGT-3' (124 bp); IL-6: 5'-ATGAAGTTCCTCTCTGCAAGAGACT-3'/ 5'-CACTAGGTTTGCCGAGTAGATCTC-3' (638 bp); and GAPDH: 5'-TTGTCAGCAATGCATCCTGCAC-3'/ 5'-ACAGCTTCCAGAGGGGC CATC-3' (149 bp). Quantitative RT-PCR reactions were run on an ABI Prism 7000 thermal cycler in which 1 μ L of cDNA was incubated at 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. Cycle thresholds (Ct) were normalized to Ct for GAPDH for each cDNA and expressed by fold increase using the formula: $2^{-\Delta\Delta C_t}$. For TLR PCRs, all reactions were 30 cycles of 1 min at 94°C, 2 min at 60°C, and 2 min at 72°C and then the PCR products were applied to an ethidium bromide 1.8% agarose gel. The sense/antisense primers used for PCR analyses of TLRs and GAPDH were as follows: TLR1: 5'-GACTAAACCAAATTCCTCATC-3'/5'-GTTGTTTGCAAGGGTAGGTCCT-3' (149 bp); TLR2: 5'-TCTAAAGTCGATCCGCGACAT-3'/5'-TACCCAGCTCGCTCACTACGT-3' (344 bp); TLR3: 5'-TTGTCTTCTGCACGAACCTG-3'/5'-CGCAACGCAAGGATTTTATT-3' (204 bp); TLR4: 5'-CAAGAACAT AGATCTGAGCTTCAACCC-3'/ 5'-GCTCTC

CAATAGGGAAGCTTTCTAGAG-3'; TLR5: 5'-ATGGCATGTCAACTTGACTTGC-3' / 5'-CAGGAGCCTCTCAGTGGTAGTA-3' (168 bp); TLR6: 5'-ATGGTAAAGTCCCTCTGG GATA-3' / 5'-CATGAGTAAGGTTTCCTGTTTGA-3' (168 bp); TLR7: 5'-CCCTT ACCATCAACCACATACC-3'/5'-TACACACATTGGCTTTGGACCC-3' (125 bp); TLR8: 5'-ATGCCCCCTCAGTCATGGATTC-3' / 5'-TTGACGATGGTTGCATTCTGCA-3' (150 bp); TLR9: 5'-ATGGTTCTCCGTCGAAGGACTC-3'/5'-CAGGAACAGCCAATTGCAGTCC-3' (149 bp); TLR11: 5'-GCCAAGGATGGAAAGACATCA-3' / 5'-CCGAGGTACAG AATGGGATGTA-3' (167 bp); and GAPDH: 5'-TTGTCAGCAATGCATCCTGCAC-3' / 5'-ACAGCTTCCAGAGGGGCCATC-3' (149 bp).

Mouse Inflammatory Cytokines and Receptors PCR Array

The mouse inflammatory cytokines and receptors were assayed using a qRT-PCR based kit, RT² *Profiler* PCR Array product # PAMM011 from SA Biosciences, Frederick MD. This array contains 84 genes involved in mediating immune cascade reactions during inflammation. The chemokines, cytokines, and interleukins involved in the inflammatory response are represented as well as their receptors. Untreated, live resting phase SC5314 cells, and heat killed SC5314 cells (MOI 4:1) were prepared as described above and co-incubated with RAW264.7 line for 3 h whereupon mRNA was extracted as described above and cDNAs were prepared from 0.3 µg of RNA. 1 µl of cDNA was added to each well of the 96 well plate of RT² *Profiler* PCR Array. Quantitative PCR reactions were run on an ABI Prism 7000 thermal cycler at 50°C for 2 min, and 95°C for 10 min, followed

by 40 cycles of 95°C for 15 s, and 60°C for 30 s. Cycle thresholds (Ct) were normalized to Ct for GAPDH for each cDNA and expressed by fold increase using the formula: $2^{-\Delta\Delta Ct}$.

Results

Mouse Inflammatory Cytokines and Receptors PCR Array

C. albicans is known to induce several inflammatory cytokines upon recognition of *C. albicans* PAMPs by at least four TLRs (TLR2, TLR4, TLR6, and TLR9) (24). We have used a PCR based array kit, RT² Profiler PCR Array product # PAMM011 from SA Biosciences, to identify the genes of RAW264.7 line that were up or down regulated when challenged with resting phase live or heat killed SC5314 cells for 3 hours. They were compared with untreated RAW264.7 line and only those displaying ≥ 3 -fold changes were taken for analysis. This quick scan revealed that within 3 hours after phagocytosis viable wild type SC5314 induces IL-1 β (1278 fold), IL-1 α (584 fold), and TNF- α (47 fold), which are important for pro-inflammatory responses, and activates IL-10 (38 fold), which is critical for anti-inflammatory response compared with untreated RAW264.7 line. Another important Th17-inducing cytokine, TGF- β was also induced 3.5 fold when RAW264.7 cells were challenged with live SC5314 for 3 h. We also observed that in general the cytokine responses are higher when live *C. albicans* cells were used compared with heat killed cells. Heat killed *C. albicans* cells induced IL-1 β (37 fold), IL-1 α (15 fold), TNF- α (3 fold), and IL-10 (2.5 fold). These results agree with previous observations reported by other groups (24) and suggest that both pro-inflammatory and anti-inflammatory responses are induced during host-fungus interaction.

Role of yeast-mycelia dimorphism in induction of Th17 – inducing cytokines by *C. albicans*.

Since there is no report so far indicating how Th17 develop following *C. albicans* challenge to the macrophages, we decided to identify the cellular components in *C. albicans* responsible for Th17 inducing cytokines, IL-6, IL-23 p19/p40, and TGF- β (2). The response of RAW264.7 cells to five strains of *C. albicans* was explored. Expression of IL-6, TGF- β , and IL-23 p19/p40 by RAW264.7 cells was followed by qRT-PCR for three hours after challenge with five strains of *C. albicans* (Fig. 5-1). This time frame was chosen because in this system wild type *C. albicans* cells form germ tubes and escape 4 to 6 hours after ingestion (8). The question is: Do macrophages signal other parts of the immune system by way of cytokines before they are killed? Fig. 5-1 shows that wild type *C. albicans* strain SC5314 rapidly causes macrophages to express IL-6 but not TGF- β or IL-23 p19/p40. Note that the IL-6 scale is logarithmic and thus expression of IL-6 mRNA increased almost 1000-fold by 3 hours. IL-6 expression in response to heat killed SC5314 was substantially less (Fig. 5-1). Likewise *C. albicans* SN152, KWN2, and KWN4, which are defective in arginine production and thus are also defective in germ tube formation (GTF) and therefore unable to escape from macrophages (8), induced ~1000-fold increase in IL-6 from RAW264.7 cells (Fig. 5-1). Thus GTF and hyphal growth are not necessary for increased IL-6 expression. These results are consistent with the notion that macrophages phagocytizing *C. albicans* can express substantial amounts of a critical cytokine, IL-6, that is necessary for Th17 development before they are killed by escaping *C. albicans* (2, 19). Furthermore, whether or not the *C. albicans* express GTF after phagocytosis does not affect the level of IL-6 expression within 3 h.

Role of farnesol in Th17-inducing cytokine expression.

Four strains of *C. albicans* were chosen to determine whether farnesol production influenced cytokine production, either positively or negatively. Strain 10231 is a wild type strain, capable of GTF and escape from macrophages (Data not shown), but is unusual because it produces farnesoic acid (26) rather than farnesol (12). KWN2 (*dpp3::C.d.HIS1/ dpp3::C.m.LEU2, his1/his1, leu2/leu2, arg4/arg4*), which was made from SN152 (22), is a *dpp3/dpp3* mutant defective in the conversion of farnesyl pyrophosphate to farnesol. It produces only 15% as much farnesol as its parent SN152 (22). Both *DPP3* and farnesol production were reconstituted in KWN4 (*dpp3::DPP3/ dpp3::DPP3, his1/his1, leu2/leu2, arg4/arg4*) (22). Fig. 5-1 shows that strains SN152, KWN2, and KWN4 all increased IL-6 expression ca. 1000-fold. In contrast, strain 10231 and heat killed SC5314 elicited only background levels of IL-6, TGF- β , and IL-23 p19/p40 (Fig. 5-1). Therefore farnesol may play a role in IL-6 expression by macrophages. Presumably the 15% farnesol produced by KWN2 (22) is sufficient for whatever role farnesol has within the macrophage because the IL-6 expression levels by SN152, KWN2, and KWN4 were equivalent (Fig. 5-1).

In contrast to the expression of IL-6, expression of TGF- β and IL-23 p19/p40 was not as dramatic (Fig. 5-1). SN152, KWN2, and KWN4, which secrete farnesol (22) but do not switch from yeast to hyphae inside macrophages (8), stimulated IL-23 p40 one hour after challenge in amounts proportional to their farnesol production levels (22). However, this expression declined to background levels 2-3 hours after challenge (Fig. 5-1). Furthermore, two strains that produce hyphae inside macrophages, SC5314 and 10231, failed to induce IL23 p40 at any time after challenge. One explanation for these

observations is that stimulation of IL-23 p40 expression requires molecules present on the yeast form of *C. albicans* that are not present on the hyphal form. Similarly, expression of IL-23p19 was induced by the *C. albicans* strains that produced farnesol but not hyphae (Fig. 5-1). In contrast to IL-6 and IL-23, expression of TGF- β was only induced at 3 hours and only by strain KWN4 (Fig. 5-1), which produces twice as much farnesol as its SN152 parent (22). Therefore, farnesol may play a role in the expression of IL-23 and TGF- β by macrophages.

Synergistic effect of farnesol and zymosan on Th-17 inducing cytokines.

The results so far suggest that farnesol and perhaps another component of *C. albicans* cells induces IL-6 and IL-23 expression by macrophages. Zymosan, a beta-glucan, is a likely candidate for a fungal molecule which could induce cytokines from macrophages. As a β -1,3-glucan, it is a major cell wall component of both *Saccharomyces cerevisiae* and *C. albicans*, known to stimulate cytokine expression by acting as a TLR2 agonist (27). Accordingly, we determined which combinations of zymosan with farnesol or farnesoic acid induced high levels of IL-6, TGF- β , and IL-23p19/p40 expression by RAW264.7 line (Fig. 4-2). Zymosan (25 μ g/ml), farnesol (5 μ M), or farnesoic acid (250 μ M) by themselves failed to stimulate expression of IL-6, TGF- β , or IL-23 (Fig. 5-2). However, the combination of zymosan and farnesol induced significant expression of IL-6 and IL-23p19/p40, but not TGF- β . For IL-6 (Fig. 5-2), this combination of two cellular components was similar to the level of IL-6 mRNA expression induced by SC5314, SN152, KWN2, and KWN4 (Fig. 5-1). Farnesoic acid and zymosan also stimulated IL-6 and IL-23 p19/p40 even though strain 10231 failed to

do so. It is likely that strain 10231 excretes very low levels of farnesoic acid after phagocytosis in the first three hours.

Role of TLRs for recognition of *C. albicans* and its components

Macrophages can phagocytize, kill, and respond to microbes by producing cytokines following activation of signaling pathways linked to TLRs. The results so far suggest that farnesol and zymosan, a component of the cell wall in *C. albicans*, synergistically induce IL-6 and IL-23 expression from macrophages. Farnesol or zymosan alone did not induce macrophage cytokines. It is possible that farnesol acts by increasing expression of TLR2 sufficiently so that it is capable of responding to zymosan. To determine if farnesol with or without zymosan modulated expression of TLRs by RAW264.7 cells, TLR expression was evaluated by RT-PCR (Fig. 5-3). Unstimulated RAW264.7 cells expressed detectable levels of TLR1, 2, 4, 5, 6, 7, 8, 9, and 11 (Fig. 5-3). In contrast RAW264.7 cells challenged with live SC5314 exhibited decreased expression of TLR4, 5, 8, 9, 11 but increased expression of TLR2 within 3h (Fig. 5-3). Farnesol or zymosan alone or the combination of farnesol and zymosan decreased expression of TLR5, 8, and 11. In contrast, the combination of farnesol and zymosan increased expression of TLR2, 4 and 9. Therefore, the combination of farnesol and zymosan, a known TLR2 agonist, increased expression of TLR2, TLR4 and TLR9 (Fig. 5-3), most likely increasing the ability of RAW264.7 line to respond to zymosan.

Discussion

We have shown that RAW264.7 murine macrophages express IL-6 and IL-23, two Th17-inducing cytokines, within 3 hours of phagocytizing the fungus *C. albicans*.

This rapid time frame is essential for macrophages' participation in development of adaptive immunity because we previously showed (8) that macrophages which have phagocytized *C. albicans* die within 4 – 6 hours because the fungal pathogen converts to hyphae inside the phagosome, pierces the phagosomic and cytoplasmic membranes, and escapes. Therefore, macrophages that phagocytize *C. albicans*, despite their inability to kill this fungal pathogen, can potentially transmit cytokine signals that contribute to the development of effective anti-fungal adaptive immunity. The cytokines which are expressed by macrophages, IL-6 and IL-23, are essential for the induction and maintenance of Th17 (2, 15), which in turn secretes IL17 and IL-22 (20). The Th17 subset of T cells is critical for a successful immune response to *C. albicans* infection (13).

It is also clear from this report that two molecular components of *C. albicans* are responsible for stimulating these cytokines. One of the molecular components is farnesol, a quorum sensing molecule excreted by *C. albicans* (11), and the other component is zymosan, a β -1,3-glucan from the yeast cell wall. Zymosan signals macrophages through TLR2 acting in collaboration with dectin-1 (9) or TLR6 (10). Because TLR2 is known to dimerize with TLR1 (10), TLR6 (10), dectin-1 (9), and galectin-3 (17), future experiments will need to sort out which of the dimer partners of TLR2 participate in the heightened expression of macrophage cytokines IL-6 and IL-23 following phagocytosis. Previous reports have shown that zymosan (7) and farnesol (6) acting separately can induce expression of IL-6. Indeed, the innate immune response to zymosan can also induce aseptic shock, multiorgan failure, and death in the host (5), probably due to excess production of zymosan-induced IL-6 (4). However, we show here

that farnesol and zymosan acting together induce greater expression of IL-6 and IL-23 by macrophages than either of those two factors alone. Therefore the combination of zymosan and farnesol induce macrophages to make the combination of cytokines necessary for development of Th17, a T cell subset essential for immunity to *C. albicans* (3, 13). Further studies will be needed to understand the receptor and signaling system activated for the macrophage response to farnesol. We suggest farnesol is a molecule which associates with or enhances the effect of *C. albicans* PAMP such as beta-glucan.

While IL-6 is important in murine induction of Th17, IL-1 β is also a critical macrophage cytokine for development of Th17 in humans. Viable and heat-killed SC5314 induced IL-1 β 1278- and 37-fold, respectively within 3 hours after phagocytosis. These results suggest that human macrophages phagocytizing *C. albicans* will also express the additional Th17-inducing cytokine, IL-1 β . In general, heat killed *C. albicans* induced far less expression of cytokines compared to the live wild type *C. albicans* cells. Heat killed cells, which are likely to be phagocytized by macrophages, are inactive metabolically and therefore cannot produce farnesol or hyphae. In contrast, live *C. albicans*, which are phagocytized by macrophages, switch their morphology from yeast to hyphae, produce farnesol, kill the macrophage, and escape from it. Neither *C. albicans* 10231 nor heat-killed SC5314 caused increased IL-6 expression (Fig. 5-1). Therefore, active farnesol production by *C. albicans* after phagocytosis is likely necessary for high IL-6 expression from macrophages. Similarly, hyphae formation in the phagosome is not required but does not prevent cytokine expression since equivalent levels of IL-6 were stimulated by the wild type SC5314 and the three arginine auxotrophs, which do not produce hyphae.

Our long term goal is to discern the role of farnesol in pathogenicity. In doing so we must reconcile three quite different modes of action for farnesol. Farnesol was first discovered as a quorum sensing molecule (QSM) for *C. albicans* (11). That is, *in vitro* *C. albicans* produced and excreted farnesol, and when the farnesol concentration exceeded a threshold level it prevented the yeast to hypha switch. Next, in the present paper we found that farnesol, together with zymosan, acted as a signal to elicit production of IL-6 and IL-23. Both of these activities suggest that farnesol production should, if anything, decrease virulence. However, whole animal studies showed that farnesol acts instead as a virulence factor. *C. albicans* mutants which produced 6-fold less farnesol were ca. 4-fold less virulent (22) and wild type *C. albicans* which had been treated with sublethal levels of fluconazole, thus producing 8-12 times more farnesol (12, 21), were ca. 6-fold more virulent (21). It is possible that the high level of inflammatory cytokines induced by farnesol in synergism with beta-glucan *in vitro* is lethal during systemic candidiasis. Alternatively, we found that farnesol significantly reduced the mouse serum levels of IFN- γ and IL-12 during systemic candidiasis (23), which could prove to enhance virulence. Further study will be needed to connect these observations. For now farnesol's exact mode of action as a virulence factor remains elusive.

C. albicans that is disseminated in healthy mice through the tail vein clear *C. albicans* systemic infection within 7 days even with 10^5 inoculum size (21). The time frame suggests that innate immunity is not sufficient for complete control of *C. albicans* infection and emphasizes the importance of T cell responses for successful control of Candida infection. This dependence on T cell development is the reason we see systemic candidiasis results in death in AIDS patients and those who are undergoing

chemotherapy, both of which have compromised T cell responses. In many cases the current drugs available in the market are unable to treat such patients because of the drug resistance and biofilm development by *C. albicans*. So it will be extremely valuable to develop therapies to enhance the appropriate T cell response for effective immunity to *C. albicans*.

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Figure Legends

Figure 5-1. RAW264.7 cells express IL-6 (A), TGF- β (B), IL-23 p40 (C) and IL-23 p19 (D) in response to challenge by *C. albicans*. Real-time qRT-PCR of IL-6 (A), TGF- β (B), IL23 p40 (C), and IL-23 p19 (D) mRNA in RAW264.7 cells after 1, 2, and 3 h. A total of 1×10^5 RAW264.7 cells was challenged with phosphate buffered saline (PBS) (control) or challenged with 4×10^5 (MOI 4:1) cells of SC5314 (wild type), or Heat Killed SC5314 (HKSC5314), or 10231 (wild type clinical isolate) – secrete farnesoic acid instead of farnesol; or SN152 (*URA3/ura3::imm434 his1/his1 arg4/arg4 leu2/leu2 IRO1/iro1::imm436*) – defective in germ tube formation inside macrophage and secrete farnesol similar to the wild type; or KWN2 (*dpp3::C.d.HIS1/ dpp3::C.m.LEU2, his1/his1, leu2/leu2, arg4/arg4*) – defective in germ tube formation inside macrophage and secrete six times less farnesol compared to the wild type; or KWN4 (*dpp3::DPP3/ dpp3::DPP3, his1/his1, leu2/leu2, arg4/arg4*) – defective in germ tube formation inside macrophage and secrete two times more farnesol compared to the wild type; dissolved in phosphate buffered saline (PBS) for 1, 2, and 3 h. Bar graphs represent mean \pm SEM of three independent experiments compared with GAPDH as positive control. *, Indicates that the mean is significantly different from control, $p < 0.05$.

Figure 5-2. RAW264.7 cytokine expression in response to the challenge by yeast cell components. Real-time qRT-PCR of IL-6 (A), TGF- β (B), IL23 p40 (C), and IL-23 p19 (D) in RAW264.7 cells. A total of 1×10^5 RAW264.7 cells was challenged with 5 μ l of methanol (control); or farnesol (5 μ M); or farnesoic acid (250 μ M); or zymosan (25 μ g/ml); or farnesol (5 μ M) and zymosan (25 μ g/ml) together; or farnesoic acid (250 μ M)

and zymosan (25 µg/ml) together; or Heat Killed SC5314 (4×10^5) and farnesol (5 µM) together; or Heat Killed SC5314 (4×10^5) and farnesoic acid (250 µM) together; dissolved in 5 µl of methanol for 3h. Bar graphs represent mean \pm SEM of three independent experiments compared with GAPDH as positive control. *, Indicates that the mean is significantly different from control, $p < 0.05$. FOH: farnesol; FCOOH: farnesoic acid; Zymo: zymosan; HK: heat-killed SC5314 cells.

Figure 5-3. Expression of TLRs in RAW264.7 cells. Total RNA was isolated from RAW264.7 cells. RT-PCR for TLR1, 2, 3, 4, 5, 6, 7, 8, 9, 11 and GAPDH (G) was performed as outlined in Methods section. After 30 cycles of PCR, cDNA was electrophoretically separated on ethidium bromide 1.8% agarose gel.

Figure 5-1

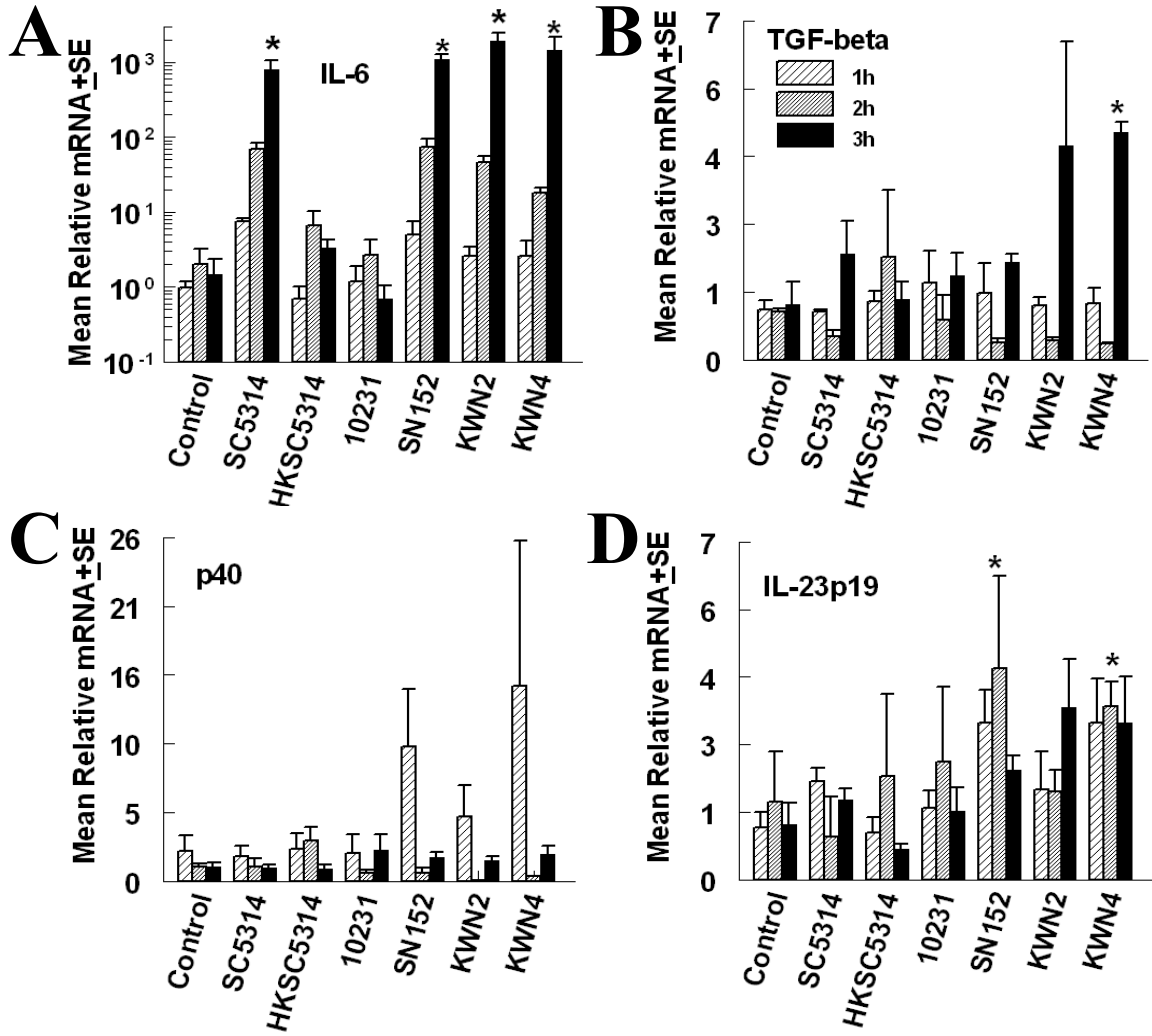


Figure 5-2

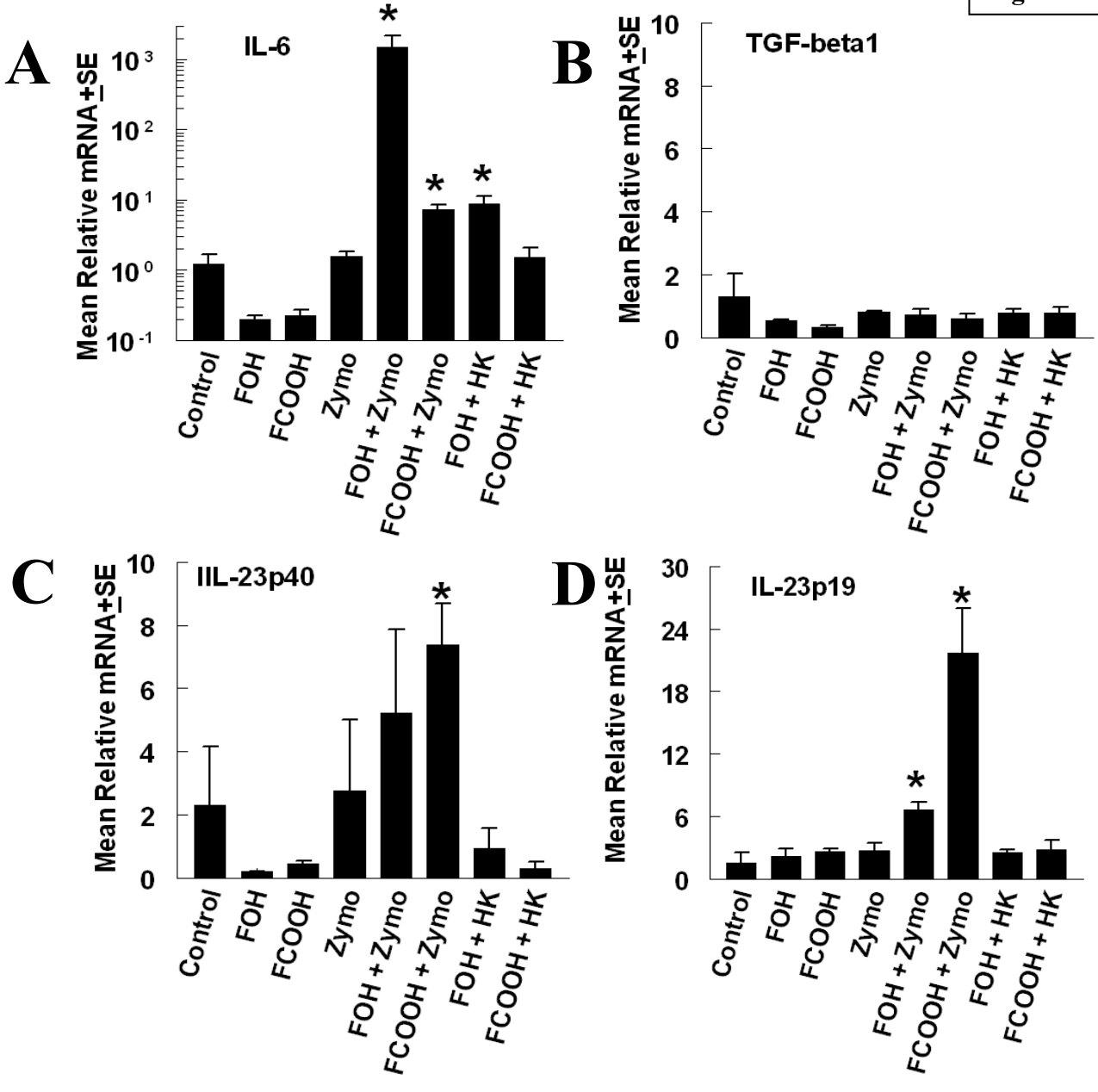
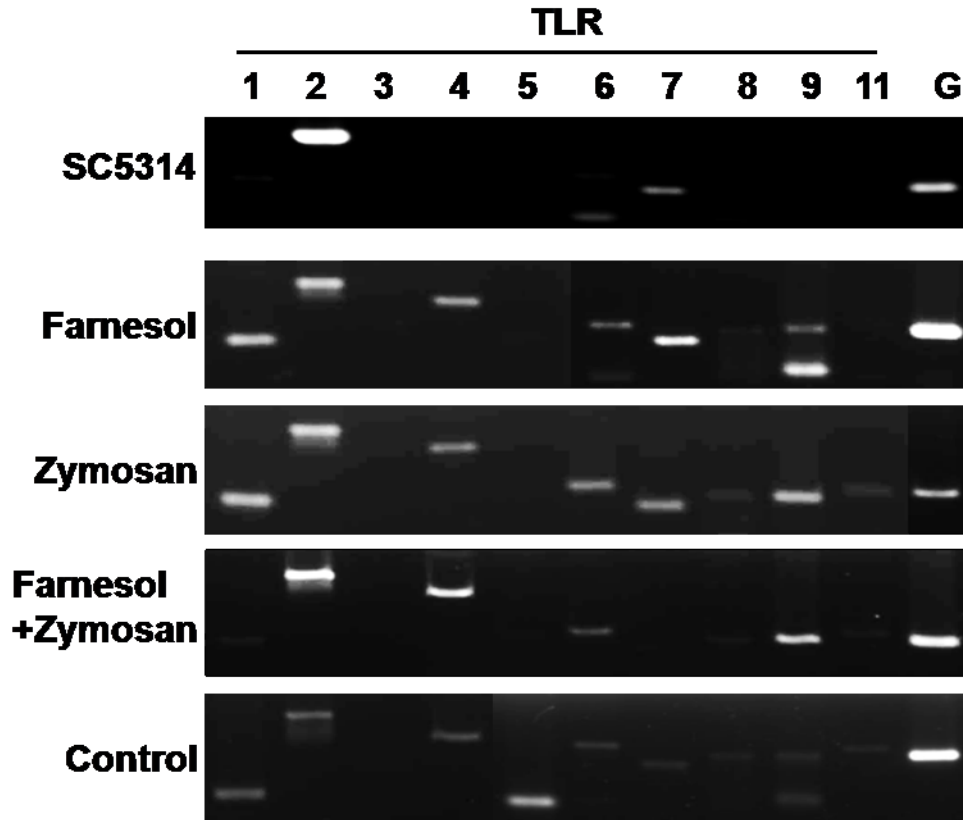


Figure 5-3



CHAPTER 6

Future Direction

This chapter contains data from four studies which are “works in progress”, i.e. not yet ready to be fully fledged manuscripts.

6.1. The role of aromatic alcohols in morphogenesis in *C. albicans*.

Introduction

Our studies have shown critical regulation of aromatic amino acid metabolism by different transcription activators in *C. albicans* (4). Aromatic amino acid catabolism is regulated by Aro80p, Gln3p, Gat1p, and Gcn4p, as well as Rim101p and the pH pathway. In case of poor nitrogen conditions these amino acids are utilized by Ehrlich's fusel oil pathway (4), in which transamination, followed by an irreversible decarboxylation, and then reduction, produces aromatic alcohols which are secreted outside the cells (4). Like other fungi and yeasts, *C. albicans* cannot utilize the carbon skeleton of aromatic amino acids (4). In our previous studies we have quantified the production of aromatic alcohols produced by *C. albicans* cells grown in different physiological conditions (4). In that study we reported that the production of these aromatic alcohols was not influenced much by the growth temperature (30 °C vs. 37 °C), but was elevated under anaerobic conditions compared to aerobic conditions or when the precursor compounds (phenylalanine, tyrosine, or tryptophan) were included in the growth media. Conversely, production was greatly reduced in the presence of ammonia (even in the presence of other poor nitrogen sources) (4).

One previous study suggests that tyrosol, one of the aromatic alcohols, can reduce the lag phase and accelerate germ tube formation in dilute cultures of *C. albicans* (3). In that study tyrosol failed to stimulate hypha formation at higher cell densities or in yeast inducing conditions (3). Another study by the same group suggested that phenethyl alcohol and tryptophol, but not tyrosol, stimulated morphogenesis in *S. cerevisiae* cells by inducing the expression of *FLO11* (important for flocculation) through a Tpk2p-

dependent mechanism (2). These studies suggest that these aromatic alcohols can induce filamentation in fungi and yeasts in a species specific manner. We wanted to address this question further if and how these aromatic alcohols can induce hyphal morphogenesis in *C. albicans* and to elucidate the mechanism of actions.

Results

Aromatic alcohols induce pseudohyphae in *C. albicans*:

We studied the morphology of *C. albicans* in the presence of exogenous aromatic alcohols. Resting phase cells were inoculated in YPD media at 37 °C. After the cells had reached 0.5 O.D., phenethyl alcohol, tyrosol, or tryptophol were added to a final concentration of 100 µM. After 2 hours the cells were stained with calcofluor white and cell morphology was evaluated by microscopy. Calcofluor white stains the chitin ring and is effective in differentiating hyphae and pseudohyphae (17). In the case of hyphae, the chitin ring appears in the filament whereas in case of pseudohyphae the chitin ring is at the constricted neck (17). We found that 100 µM phenethyl alcohol, tyrosol, or tryptophan induced pseudohyphae (Fig. 6-1). As a control we added 2.5 mM GlcNAc to actively growing cells in YPD at 37 °C and after 2 hours the added GlcNAc had induced hypae (Fig. 6-1).

We next wanted to address which pathways are involved in the induction of pseudohyphae by aromatic alcohols. For this purpose we used mutants defective in the cAMP dependent PKA pathway (*efg1/efg1*), MAP kinase pathway (*cph1/cph1*), or both (*cph1/cph1 efg1/efg1*). These mutant strains along with their parent CAI4 were tested in similar conditions as described above and the morphology was monitored after 2 hours. The induction of hyphae was stimulated with 100 µM tyrosol in the parental strain CAI4

whereas it stayed as budding yeasts in unsupplemented YPD, all at 37 °C (data not shown). These results confirmed our previous observation (Fig. 6-2). We also used 2.5 mM GlcNAc as a positive control for hyphal morphology in *C. albicans* (Fig. 6-2). Each of the mutant strains defective in transcription factors for hyphal morphogenesis failed to produce either hyphae or pseudohyphae (Fig. 6-2), suggesting involvement of both the cAMP dependent PKA kinase and MAP kinase pathways in aromatic alcohol induced morphogenesis.

Farnesol's mode of action is predominant over tyrosol:

Several previous studies from our lab show that farnesol can block germ tube formation at concentrations as low as 5 μ M (8, 12). Thus, we wanted to test if the tyrosol induced morphogenesis can override farnesol's action or *vice versa*. We used mGPP at 37 °C for 4 hours and quantified the percentage of budding yeasts, hyphae and pseudohyphae. In these assay conditions mGPP alone gave 90% hyphal morphogenesis while the addition of 5 μ M farnesol as expected was able to block the yeast to hyphal switch (Fig. 6-3). Increasing amounts of tyrosol (10, 20, 40, 60, and 80 μ M) were used along with different levels of farnesol (5, 10, and 20 μ M) for a farnesol tyrosol competition assay. We found that farnesol's effect was predominant; at all concentrations of farnesol tested, most (>65%) of the *C. albicans* cells grew as yeasts regardless of how much tyrosol was present (Fig. 6-3). However, at higher levels of tyrosol, i.e. 40, 60, and 80 μ M, the percentages of yeast cells were a little bit lower and the percentages of pseudohyphae were a little bit higher (Fig. 6-3). The percentages of pseudohyphae always increased in the presence of high levels of tyrosol, in a small but consistent manner (Fig.

6-3). This set of data confirms our previous observation (Fig. 6-1) that higher levels of the aromatic alcohols induce pseudohyphae in *C. albicans*.

Aromatic alcohols induce pseudohyphae by derepressing Gcn4p:

The accumulation of high levels of fusel alcohols sends a nitrogen starvation signal to *S. cerevisiae* cells, thus inhibiting eukaryotic translation initiation factor 2B (eIF2B) (15) and inhibition of eIF2B derepresses Gcn4p (6). Earlier we showed that as in *S. cerevisiae* the production of fusel alcohols in *C. albicans* is dependent on nitrogen sources. In the presence of preferred nitrogen sources such as ammonia, *C. albicans* cells inhibit the metabolism of aromatic amino acids and we see reduced fusel oil production. Similarly, in the presence of poor nitrogen sources like proline fusel oil secretion is much higher. When a high level of fusel oil is secreted, these alcohols might send a nitrogen starvation signal to the cells that inhibit eIF2B, as was reported earlier in the literature for *S. cerevisiae* (15). This makes sense as the cells will start preparing to go into stationary phase when they run out of nitrogen. The cells will also derepress Gcn4p under nitrogen starvation conditions or in the absence of any individual amino acid (6). We wanted to test if a similar response occurs in *C. albicans* cells.

We tested for the presence of Gcn4p by western blot analysis, using a Gcn4p antibody raised against *S. cerevisiae*. We treated the cells the same as we had for the morphological studies just described. Resting cells were inoculated in YPD at 37 °C and at an O.D. of 0.5 the cells were treated with nothing (control) or 100 µM of phenethyl alcohol, tyrosol, or tryptophol. After 2 hours the cells were harvested and western blot analysis was performed on the total cell lysate. Western analysis revealed that Gcn4p was derepressed when the cells were treated with 100 µM phenethyl alcohol, tyrosol, or

tryptophol (Fig. 6-4 A). To check if the *S. cerevisiae* Gcn4p antibody we used was actually detecting *C. albicans* Gcn4p, we grew CAF2 (*GCN4/GCN4*), GTC41 (*GCN4/gcn4*), and GTC43 (*gcn4/gcn4*) in YPD at 37 °C. When the cell densities reached at 0.5, 40 mM of a histidine analog 3-amino triazole (3AT) was added for 2 hours. Western blot analysis of these cells revealed that the *S. cerevisiae* directed antibodies also detected Gcn4p from *C. albicans* cells since they did not detect anything in the *gcn4/gcn4* mutant GTC43 (Fig. 6-4 B). Also the size of the band (~ 35.3 kDa) appeared at the expected region. Together these data supported the conclusion that Gcn4p is derepressed in the presence of 100 µM aromatic alcohols (Fig. 6-4 A).

To distinguish the intervening steps between aromatic alcohol addition and elevated Gcn4p, we also used an antibody specific for the phosphorylated form of eIF2 α . This antibody recognizes eIF2 α phosphorylated at serine 52 but not the unphosphorylated eIF2 α (16). The eIF2 α is just upstream of and inhibits eIF2B (6) (see Fig. 1-2) which in turn represses Gcn4p. In the presence or absence of aromatic alcohols, phosphorylated eIF2 α was not detected, suggesting that the aromatic alcohols are activating Gcn4p independent of eIF2 α . Thus, the pathway for aromatic alcohol activation in *C. albicans* exactly duplicates what Ashe et al (1) reported for *S. cerevisiae* cells (Fig. 1-2).

Discussion

In this report we demonstrate the role of aromatic alcohols in pseudohyphae development in *C. albicans* cells. At a high concentration of aromatic alcohols (~ 100 µM), *C. albicans* cells induced pseudohyphae by a mechanism that was dependent on both the cAMP/PKA pathway and the MAP kinase pathway. These concentrations of aromatic alcohols are biologically significant in a biofilm where the local micro-

environment will be anaerobic and/or nutritionally poor. We further show that aromatic alcohols induce Gcn4p in an eIF2 α independent manner similar to *S. cerevisiae* cells. Under the assay conditions we employed we did not see any effect of these aromatic alcohols, in terms of pseudohyphal development, at lower concentrations (10 and 20 μ M). The requirement for high levels of aromatic alcohols for pseudohyphal development is consistent with the fact that *C. albicans* cells actually secrete very high levels of the aromatic alcohols. When grown under nitrogen poor conditions, *C. albicans* cells can secrete 1030, 2530, and 660 μ g/g of dry weight of phenethyl alcohol, tyrosol, and tryptophol respectively at 37 °C (4). We can expect similar or higher concentrations of the exogenous aromatic alcohols in local environments such as inside a biofilm. Otherwise, this difference could be cell density dependent, i.e. 10 or 20 μ M of exogenous fusel oil would work at lower cell density but 100 μ M must be required to work at higher cell densities. We grew the cells up to 0.5 O.D. which is high cell density $\sim 1.5 \times 10^7$. Chen et al 2004 found the effect of tyrosol at 20 μ M level and they used much lower cell density, i.e. 10^5 cells/ml.

In case of *C. albicans* cells, Gcn4p is also reported to induce hyphal morphogenesis by interacting with the Ras-cAMP pathway (17). Our observations that, in the presence of aromatic alcohols *C. albicans* cells induce pseudohyphae, as well as derepressing Gcn4p, fit beautifully with the two models (1, 17) and merge them together. Derepression of Gcn4p in the presence of aromatic alcohols also makes sense because when poor nitrogen sources are being used, the buildup of fusel alcohols externally sends a signal to the cells to activate the general amino acid control (GAAC) response by activating/elevating Gcn4p. Gcn4p then will activate amino acid biosynthetic genes. In

case of *C. albicans*, Gcn4p also stimulates pseudohyphal development (17). This is the reason we saw pseudohyphal development when the cells were treated with the aromatic alcohols. The feedback regulation of aromatic amino acid metabolism and its regulation in filamentous morphogenesis are described in fig. 6-5.

It is important that these Gcn4p derepression experiments be repeated in defined GPP medium at a series of phosphate concentrations because Hornby et al, 2004 showed that high (>300 mM) phosphate caused *C. albicans* to grow predominantly as pseudohyphae (7). We expect that western blots using both anti-Gcn4p and anti-eIF2 α -serP52 antibodies would reveal how high phosphate growth media triggers pseudohyphae (7).

6.2. A hypothesis on recruitment of Tup1p with Gcn4p derepression results in morphogenesis in *C. albicans*

In one section of the thesis we described how Gcn4p is derepressed during our GlcNAc induced germ tube assay. In the same assay, addition of amino acids repressed Gcn4p and was accompanied by faster germ tube formation kinetics. We also found that added amino acids in the germ tube assay competed with farnesol. That is, a higher level (50 μ M) of farnesol was needed to block germ tube formation when amino acids (400 μ g/ml) were added to the GlcNAc-induced assay. Confirming this view, 20 μ M farnesol blocked germ tube formation when amino acids were added after 30 minutes, supporting the hypothesis competition between farnesol's inhibition and amino acids' activation of germ tube formation. From the previous study by our group (Kebaara et al, 2008) we know that added farnesol increases the levels of *TUPI* mRNA and Tup1p protein present. In the yeast *S. cerevisiae*, it has been reported that Gcn4p may enhance its own binding

upstream of the amino acid biosynthetic genes by recruiting Tup1p (9). It is very tempting to hypothesize similar circumstances in the opportunistic pathogen *C. albicans*. Therefore, in case of nitrogen starvation when Gcn4p is derepressed, the cells will recruit Tup1p, a general co-repressor, along with more Gcn4p. That will cause accumulation of high level of Tup1p which will bind to the promoter regions of hyphal specific genes as repressor. A high level of Tup1p is also observed in farnesol treated cells. This explains why we observed a competition between farnesol and amino acid treated cells if amino acid treated cells also repress Tup1p.

6.3. Early phase arginine biosynthesis inside the macrophage is regulated by non-sense mediated mRNA decay (NMD) pathway in *C. albicans*

We have reported the role of arginine metabolism in hyphal morphogenesis and its importance as a signal for *C. albicans* cells engulfed inside macrophage (5). To summarize, following phagocytosis by macrophages, *C. albicans* up-regulates arginine biosynthetic genes at the early phase. Arginine can then be broken down by the enzyme Car1p (arginase) to urea and ornithine. Urea is degraded to ammonia and CO₂ by the enzyme Dur1,2p (urea amidolyase). CO₂ then acts as a signal and activates cAMP/PKA pathway to stimulate hyphae. Inside a macrophage this series of events is critical for the *C. albicans* yeast-to-hyphae switch as was explained in chapter four. So, an important question concerns how the arginine biosynthetic genes are up-regulated in a macrophage engulfed *C. albicans* cell at the early phase. Significantly, we found that the arginine biosynthetic genes were not up-regulated by the general amino acid control response (GAAC). This conclusion is based on *gcn4/gcn4* mutants defective in the GAAC response (6), but are not defective in the yeast to hyphae switch inside macrophage. We

also did not expect that this intra-macrophage arginine biosynthesis would be regulated by Gcn4p, which would be expected to activate all the amino acid biosynthetic genes, and not just the arginine biosynthetic genes. How are the arginine biosynthetic genes turned on specifically? There is only one set of arginine biosynthetic genes in the opportunistic fungal pathogen *C. albicans*. So we hypothesize that arginine biosynthesis can be regulated by different transcription factors under different conditions. For example, when the cells experience nitrogen stress in general then the transcription factor Gcn4p will activate all the amino acid biosynthetic genes, including the arginine biosynthetic pathway.

In a special condition such as that experienced by *C. albicans* cells inside macrophage, where only arginine biosynthetic genes are up-regulated, it can be a novel regulatory mechanism or it may be some other activator like Arg82p or the Arg80p-Mcm1p-Arg81p complex which regulates arginine biosynthesis, as occurs in the case of the yeast *S. cerevisiae* (11). Another interesting regulation includes the degradation of mRNA. If mRNA degradation of the transcript for the arginine biosynthetic genes is slow, then we would also expect up-regulation of arginine biosynthesis. From the original transcriptional profiling data (10) we find that at the early phase, i.e. at 1 hour, *NMD3* (*NAM7/UPF1*), *NMD5* (orf 19.4188), and orf 19.5136 were down-regulated 0.30-, 0.35-, and 0.17- fold respectively in *C. albicans* cells that had been phagocytized by macrophages compared to non-phagocytized cells. This set of data suggests that inside the macrophage nonsense mediated mRNA decay (NMD) is shut off. *NMD3*, *NMD5*, and orf 19.5136 are essential components of NMD in *C. albicans*. In case of fission yeast *Schizosaccharomyces pombe*, the *upf1* mutant, which is defective in NMD, is sensitive to

oxidative stress (14). If arginine biosynthetic genes are the targets of NMD, then we can correlate these two sets of data and explain how the arginine biosynthetic genes were up-regulated specifically inside macrophage. We hypothesize that after phagocytosis *C. albicans* cells shut off NMD. Arginine biosynthetic gene transcripts, the normal targets of NMD, are degraded less rapidly and thus they are up-regulated at the initial phase following phagocytosis.

6.4. Arginine induced biofilm formation is mediated by nitrogen catabolite repression in *C. albicans*

In our previous studies we found arginine induced hyphal morphogenesis in liquid cultures of *C. albicans* [Chapter 4 and (5)]. We also wanted to explore the phenotype of *C. albicans* cells in solid, plate assays. When *C. albicans* wild type SC5314 and control DAY286 cells were grown on YPD or GPP plates at 37 °C for 2 days, they formed smooth colonies (Fig. 6-6 a). But when the nitrogen source was changed to arginine (GPR), then the *C. albicans* cells reproducibly gave rise to wrinkled colonies (Fig. 6-6 a). When we screened Dr. Aaron Mitchell's collection of *C. albicans* mutant strains, most of the mutants formed wrinkled colonies. The top panel of Fig. 6-6 a shows smooth colonies for DAY286 (control strain) on YPD and GPP plates and wrinkled colonies of DAY286 on GPR plates, all at 37 °C. We then screened for: mutants that formed wrinkled colonies on YPD plates or smooth colonies on GPR plates. Fig. 6-6 b and Table 6-1 shows the mutant strains that formed wrinkled colonies on YPD and Fig. 6-6 c and Table 6-2 shows the mutant strains that formed smooth colonies on GPR. Annotated genes or ORFs are taken from Candida Genome Database (CGD) and are shown at the bottom of each photograph. Formation of wrinkled colonies can be associated with the activation of

adhesion genes (*ADH*) and growth as hyphae. The mutants that formed wrinkled colonies in YPD plates are generally defective in that the homozygous mutants have abnormal cell wall, defective hyphal growth, or biofilm formation (Candida Genome Database).

The mutants that did not form wrinkled colonies on GPR plates are shown in Fig. 6-6 c and Table 6-2. Among them we selected the *bcr1/bcr1* strain that is known to be directly related to adhesion (13). Bcr1p is a transcription factor that regulates all the adhesion genes (*ALS*) in *C. albicans* cells. It is also required for biofilm formation. This correlation provides a possible explanation as to why arginine was causing formation of wrinkled colonies. We reason that somehow arginine is required to express the *ALS* genes which are important in adhesion, thereby leading to formation of wrinkled colonies. In this scenario, the *bcr1/bcr1* strain, which is defective in the transcription factor for the *ALS* genes, could not form wrinkled colonies (Fig. 6-6 c).

The adhesion genes are also required in biofilm formation. The expression of *BCR1*, and thereby activation of the *ALS* genes, is needed to form biofilms on surfaces. So we reasoned that if arginine was an important factor to activate adhesion genes by inducing Bcr1p, then arginine should also induce biofilm formation *in vitro*. We used an established method for biofilm formation (13) with the variation that instead of silicon squares we used sterilized human tooth discs to promote biofilms. We found that the biofilm formation was dependent on the nitrogen source provided using wild type SC5314 cells. Ammonia inhibited biofilm formation (Fig. 6-7) whereas proline and arginine promoted biofilm formation. A control, mGPP (GPP + GlcNAc) also induced biofilm formation. These results, along with the plate based colony morphology data, suggest that arginine as well as other poor nitrogen sources activates Bcr1p by inducing

NCR and thereby causing wrinkled colony formation on plates and biofilm formation on tooth discs.

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Figure Legends:**Figure 6-1. Effects of aromatic alcohols in morphogenesis in wild type *C. albicans*.**

The wild type *C. albicans* SC5314 cells were grown in YPD at 37 °C till 0.5 O.D., and then treated with either 2.5 mM N-acetyl glucosamine (GlcNAc), or 100 µM phenethyl alcohol, tyrosol, or tryptophol for 2 hours. The cells were stained with calcofluor white. Photomicrographs (DIC and fluorescent) showing germ tube formation with GlcNAc treated cells whereas pseudohyphae formation with aromatic alcohol treated cells. Calcofluor white stains the chitin ring which appears at the bud neck in pseudohyphae and inside of germ tube in case of true hyphae.

Figure 6-2. Effects of tyrosol in morphogenesis in non-filamentous mutants.

Photomicrographs showing germ tube assays for CAI4 (parent), JKC19 (*cph1/cph1*), HLC52 (*efg1/efg1*), and HLC54 (*cph1/cph1, efg1/efg1*) in the presence of 2.5 mM N-acetyl glucosamine (GlcNAc) (first column) and 100 µM tyrosol (TOH) (second column) at 37°C after 4 hours. Representative photomicrographs are taken in a confocal microscope.

Figure 6-3. Farnesol tyrosol competition in morphogenesis in *C. albicans*. The percentage (%) germ tube bioassay was conducted in GPP+GlcNAc (pH 6.5) at 37°C with 0, 5, 10, and 20 µM farnesol (F) and 0, 10, 20, 40, 60 and 80 µM tyrosol (T). The percentages of yeasts (Y), pseudohyphae (P), and mycelia (M) were calculated after 4 hours. Last bar, 20 µM farnesol still prevented germ tube formation when added 30 minutes after the cells were inoculated. Tyrosol was present from T₀.

Figure 6-4. Derepression of Gcn4p by aromatic alcohols in *C. albicans*. a) SC5314 cells were grown in YPD at 37 °C till 0.5 O.D., and then treated with nothing, 100 µM

tyrosol (TOH), tryptophol (TrpOH), or phenethyl alcohol (PEA) respectively for 2 hours.

b) CAI4 (*GCN4/GCN4*), GTC41 (*GCN4/gcn4*), and GTC43 (*gcn4/gcn4*) strains were grown in YPD at 37 °C till 0.5 O.D., and then treated with 40 mM of 3-amino triazole (3AT) for 2 hours. The presence of Gcn4 protein is shown. Act1 levels were used as loading control.

Figure 6-5. Model for feedback control of amino acid biosynthesis and

morphogenesis by fusel alcohols in *C. albicans*. *C. albicans* can use the aromatic amino

acids tryptophan, phenylalanine and tyrosine as cellular nitrogen sources. This results in the production of tryptophol, phenylethanol and tyrosol, collectively known as fusel oils.

Fusel oil production depends on environmental factors including the availability of aromatic amino acids, ammonia, oxygen level, and alkaline pH (indicated by dotted

lines). Aromatic amino acids stimulate Aro80p, a transcription activator required for full expression of *ARO8* and *ARO9* (encoding aromatic transaminases) and *ARO10* (aromatic

decarboxylase). Accumulated aromatic alcohols can then derepress Gcn4p to activate

amino acid biosynthetic genes. Gcn4p also has a role in hyphal morphogenesis in *CPH1*

and *EFG1* dependent manner. Genes are in boxes; enzymes/proteins are in ellipses. The

scheme is based on our findings, as well as on pathways reported for both *S. cerevisiae*

and *C. albicans* by other groups.

Figure 6-6. Effect of arginine in wrinkled type colony formation in *C. albicans*.

Photomicrographs of A) DAY286 showing smooth colony in YPD, and GPP whereas

wrinkled colony in GPR at 37 °C after two days (control); B) mutants that formed

wrinkled colonies in YPD at 37 °C; and C) mutants that formed smooth colonies in GPR

at 37 °C after screening Aaron Mitchell mutants' collection.

Figure 6-7. Effect of nitrogen sources on biofilm formation *in vitro* by wild type *C. albicans*. *C. albicans* SC5314 cells were grown in YPD overnight at 30°C, diluted to an OD₆₀₀ of 0.5 in 2.0 ml of defined media with varying nitrogen source (GPA, GPP, GPP with GlcNAc, GPR, or R with Glu), and added to a sterile 12-well plate with each well containing an autoclaved tooth disc. The autoclaved tooth disc had been treated with sterile BSA overnight and washed with PBS prior to the biofilm assay. The plate was incubated at 37°C for 90 min with agitation at 150 rpm. Unadhered cells were removed by washing the discs with 2 ml of PBS and transferring them to a fresh 12-well plate with 2 ml of the same nitrogen variable media. This plate was incubated at 37°C for 60 hrs with agitation at 150 rpm and biofilm formation was visualized with concanavalin A-FITC (25 ug/ml, Sigma Chemical, St. Louis, MO). Biofilms were stained for 1 hr in the dark at 37°C with agitation at 150 rpm and examined by confocal scanning laser microscopy (FU5000) (Nobile et al, 2006). *C. albicans* cells are stained green. Observe that the ammonia in GPA inhibited biofilm formation and both arginine and proline promoted biofilm formation.

Table 6-1:

| Mutant strains that formed wrinkled colonies on YPD | | |
|--|----------------------------|---|
| Strain # | CGD information/orf | Closest <i>S. cerevisiae</i> homolog |
| CW-C-7, 8, 9 | orf19.3869 | <i>BBC1</i> |
| C-A-4 | orf19.11410 | <i>AZF1</i> |
| C-C-4 | orf19.3127/ <i>CZF1</i> | <i>UME6</i> |
| C-E-5 | orf19.909/ <i>STP4</i> | <i>STP4</i> |
| D-B-2 | orf19.4662/ <i>RLM1</i> | <i>RLM1</i> |
| 1-A-5 | orf19.6760/ <i>MDS3</i> | <i>PMD1</i> |

Table 6-2:

| Mutant strains that formed smooth colonies on GPR | | |
|--|----------------------------|---|
| Strain # | CGD information/orf | Closest <i>S. cerevisiae</i> homolog |
| C-B-10 | orf19.723/ <i>BCR1</i> | <i>USV1</i> |
| D-A-6 | orf19.1358/ <i>GCN4</i> | <i>GCN4</i> |
| 2-C-8 | orf19.7381/ <i>ZCF37</i> | <i>LYS14</i> |
| 2-G-8, 9 | orf19.6032/ <i>SPE1</i> | <i>SPE1</i> |
| 2-G-4, 5 | orf19.1759/ <i>PHO23</i> | <i>PHO23</i> |
| 3-A-10 | orf19.3818/ <i>GOA1</i> | - |

Figures:

Figure 6-1

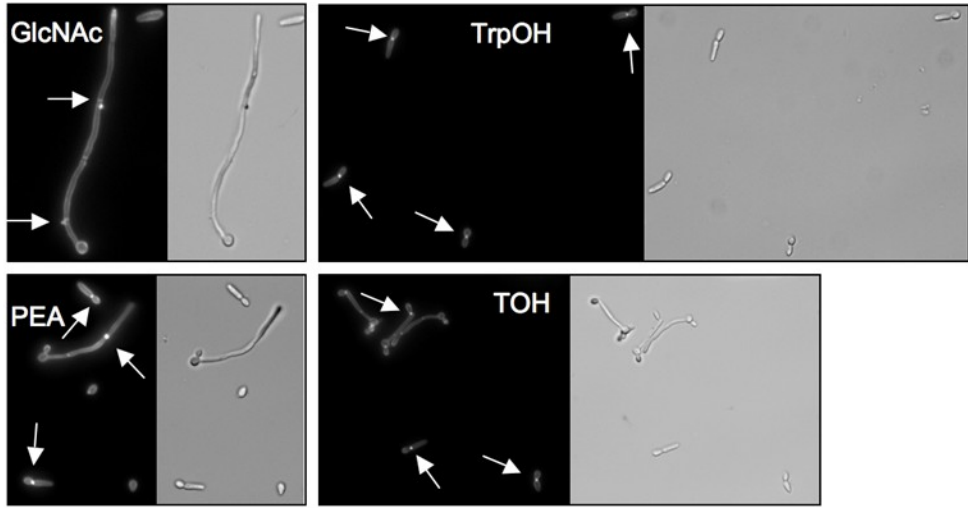


Figure 6-2

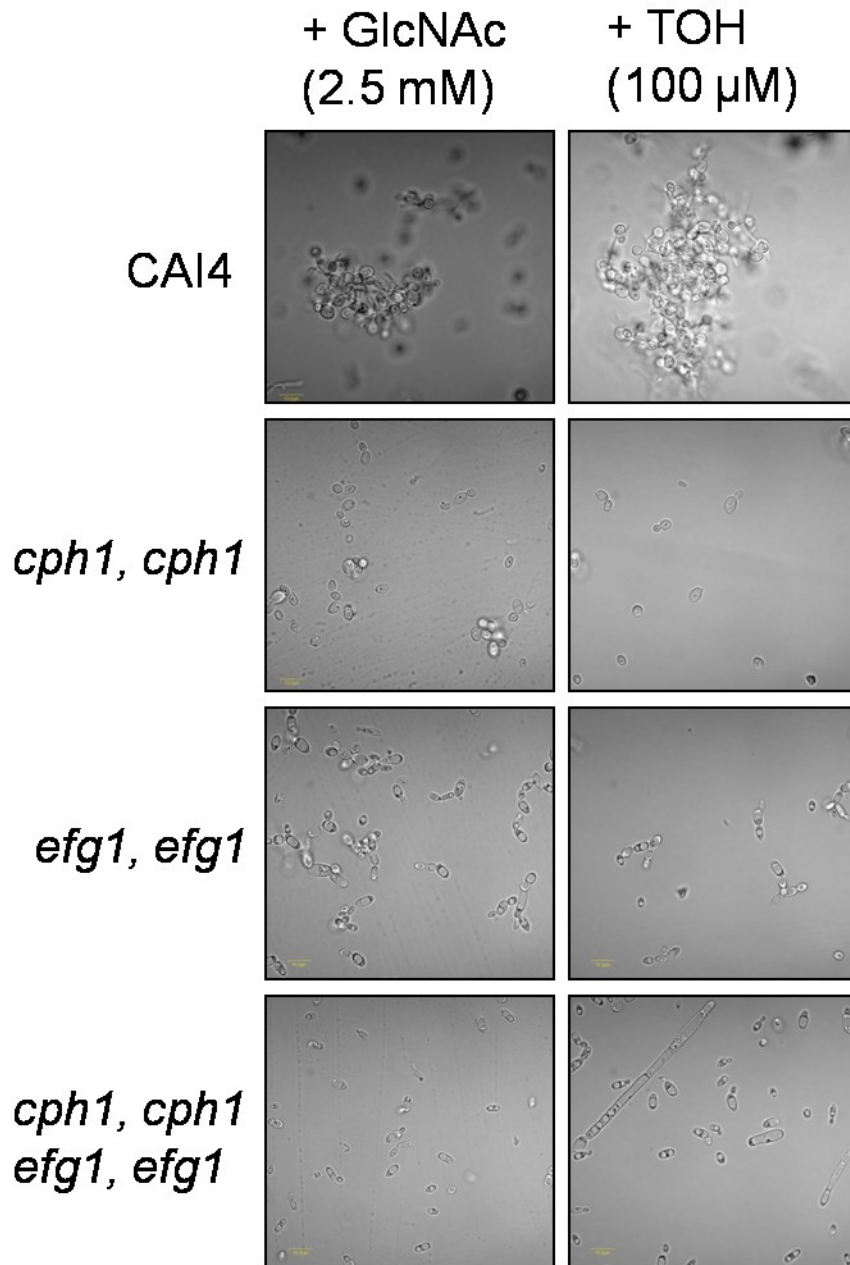


Figure 6-3

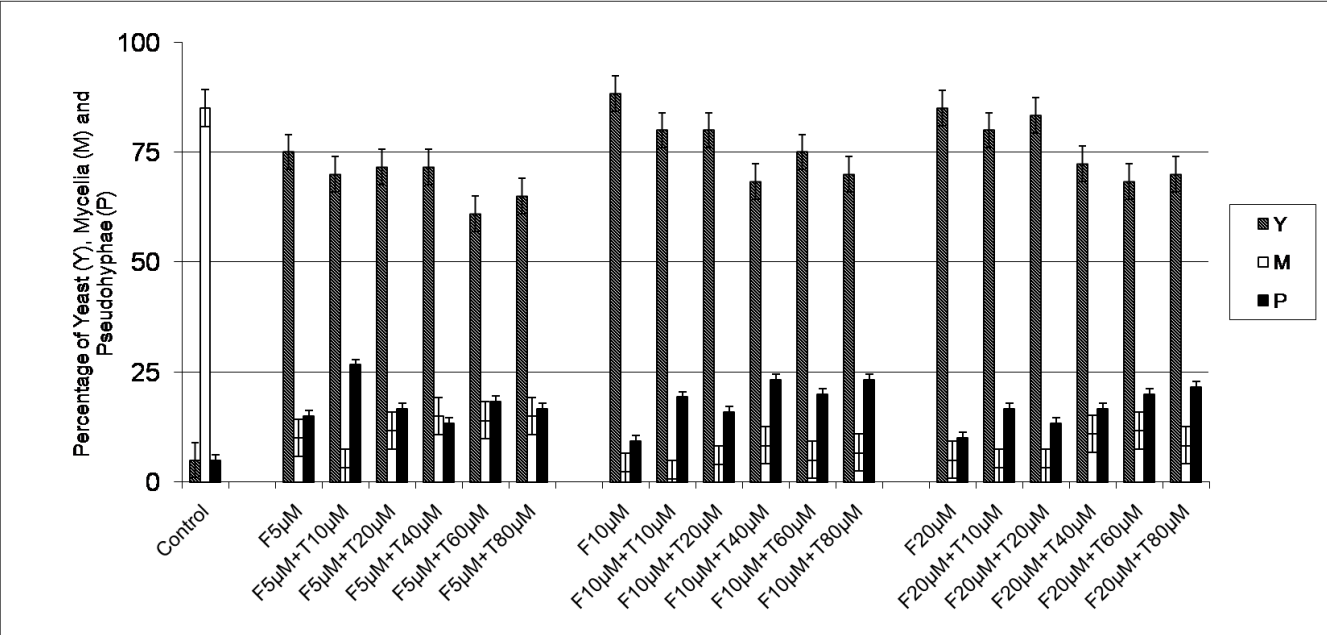


Figure 6-4

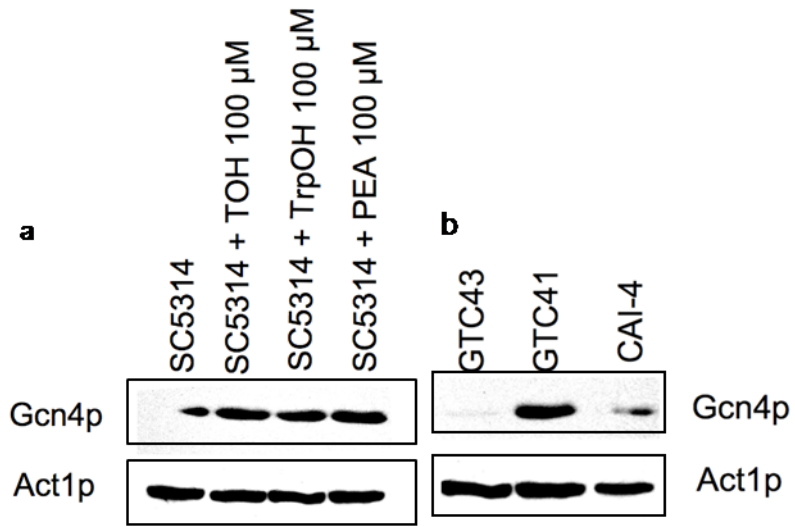


Figure 6-5

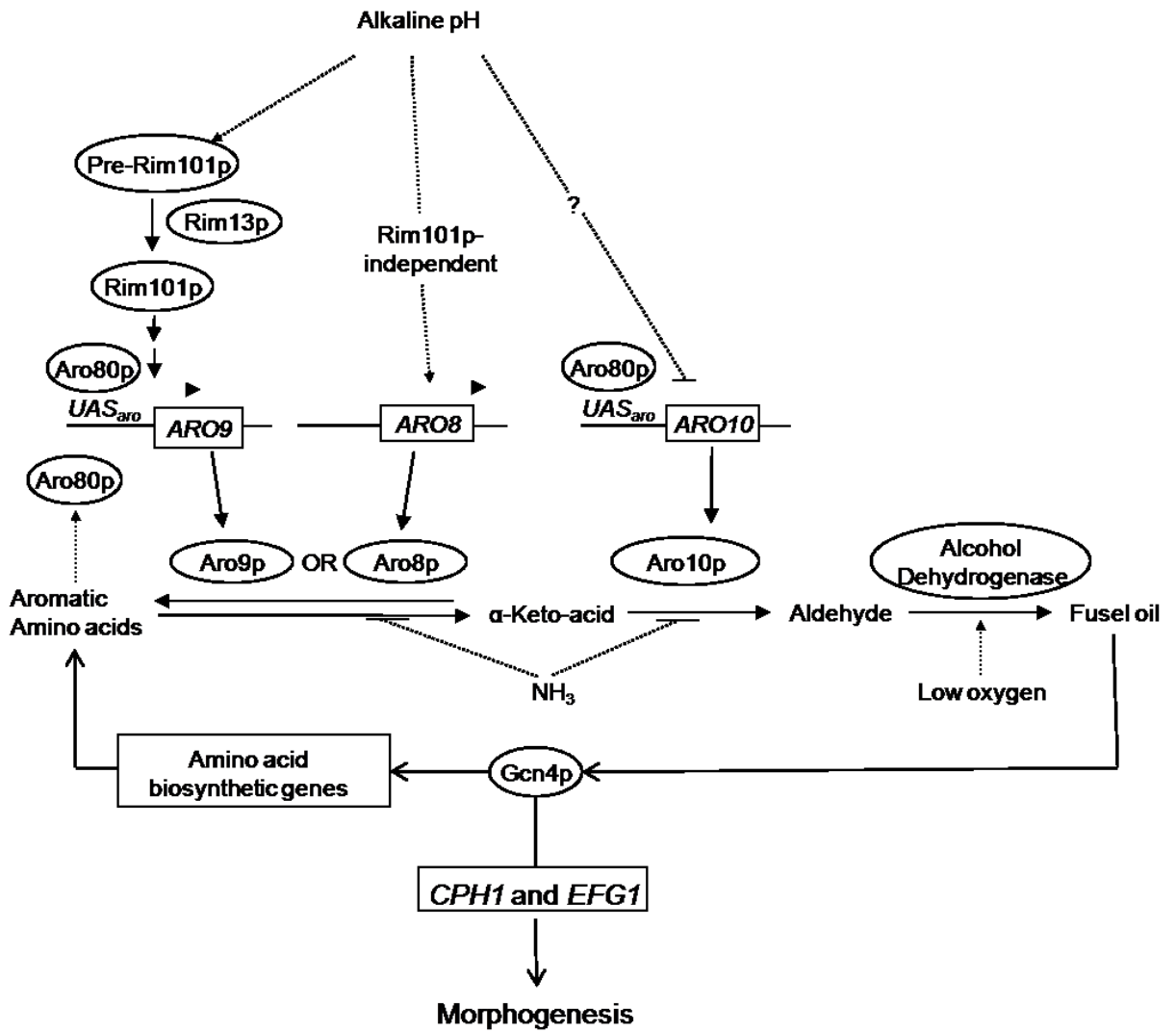


Figure 6-6

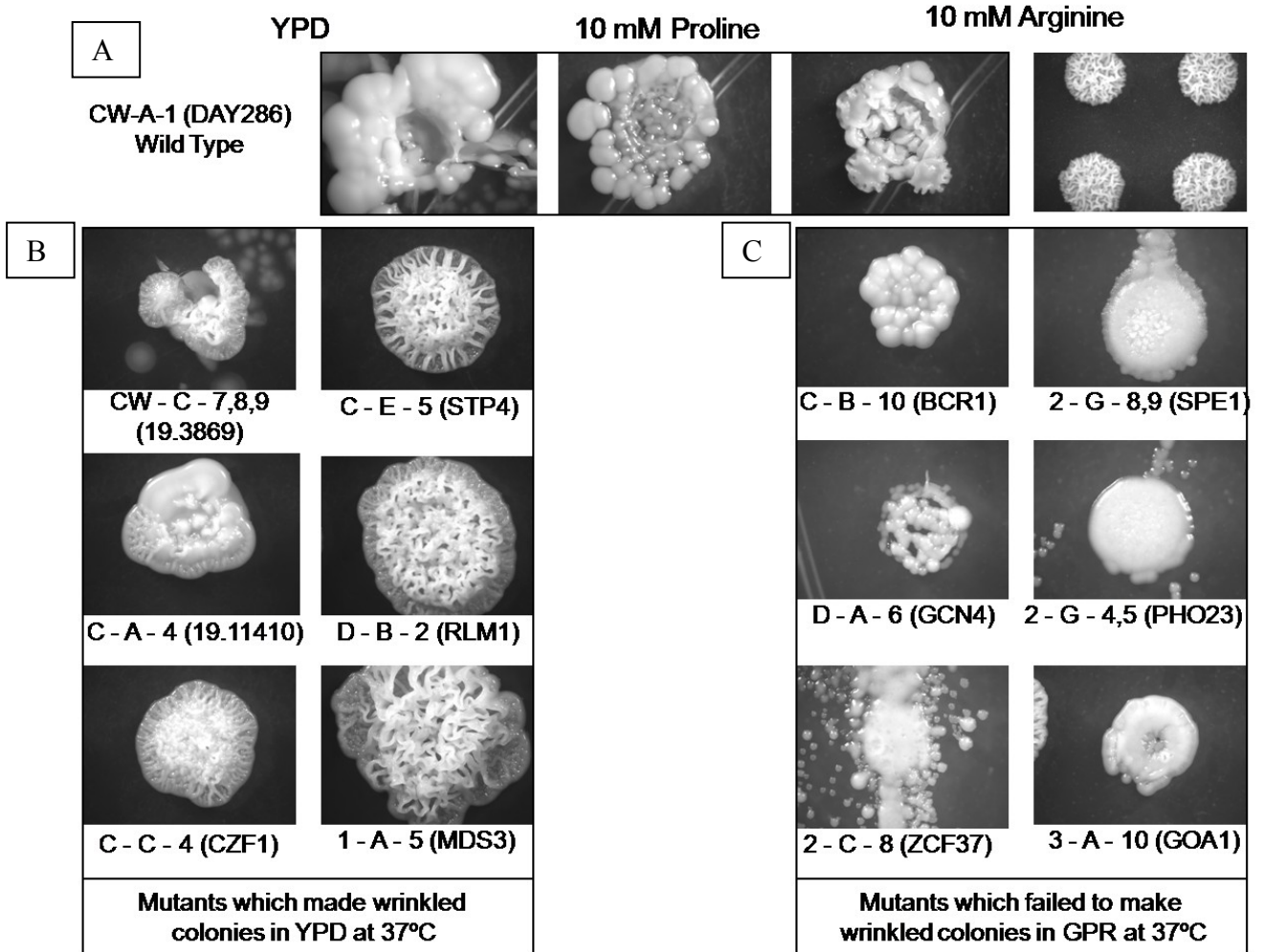
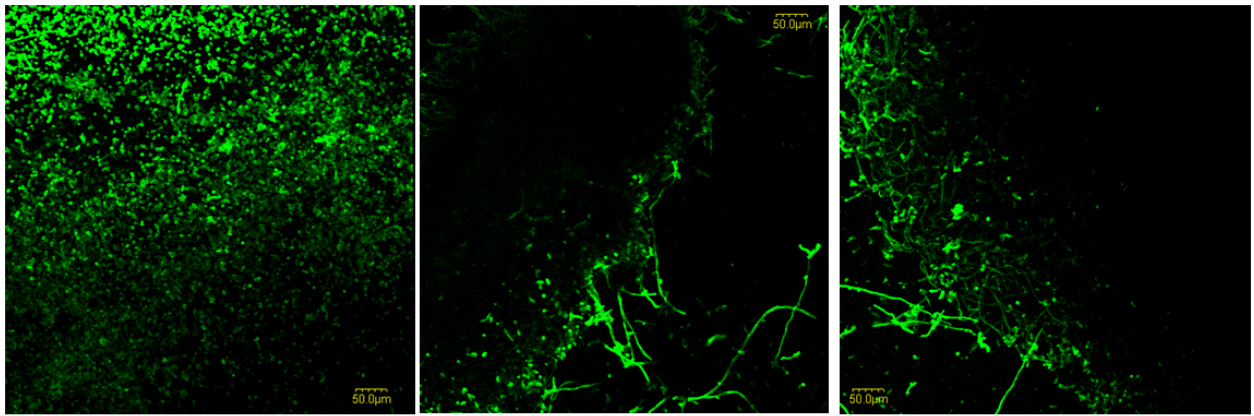


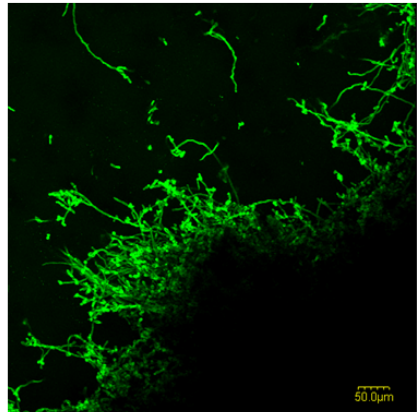
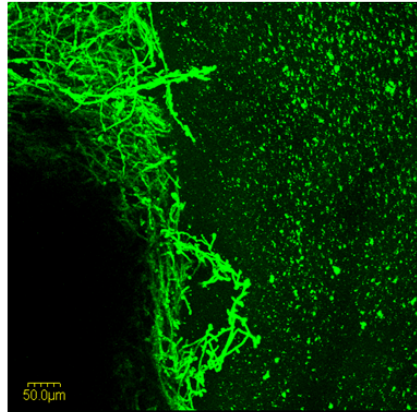
Figure 6-7



GPA

GPP

GPP+GlcNAc



GPR

R+Glu