University of Nebraska - Lincoln
DigitalCommons@University of Nebraska - Lincoln

Dissertations and Theses in Biological Sciences | Biological Sciences, School of

11-2016

The Roles of Biotin in Candida Albicans Physiology

Nur Ras Aini Ahmad Hussin
University of Nebraska-Lincoln, nurrasaini@yahoo.com

Follow this and additional works at: http://digitalcommons.unl.edu/bioscidiss

Part of the Biology Commons, Cell and Developmental Biology Commons, Microbial Physiology Commons, and the Pathogenic Microbiology Commons

Ahmad Hussin, Nur Ras Aini, "The Roles of Biotin in Candida Albicans Physiology" (2016). *Dissertations and Theses in Biological Sciences*. 89.
http://digitalcommons.unl.edu/bioscidiss/89

This Article is brought to you for free and open access by the Biological Sciences, School of at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in Dissertations and Theses in Biological Sciences by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.

THE ROLES OF BIOTIN IN *CANDIDA ALBICANS* PHYSIOLOGY

By

Nur Ras Aini Ahmad Hussin

A THESIS

Presented to the Faculty of

The Graduate College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Master of Science

Major: Biological Sciences

Under the Supervision of Professor Kenneth W. Nickerson

Lincoln, Nebraska

November, 2016

Biotin Auxotrophy and Biotin Enhanced Germ Tube Formation in *Candida albicans*

Nur Ras Aini Ahmad Hussin, M.S.

University of Nebraska, 2016

Advisor: Kenneth W. Nickerson

Due to the increased number of immunocompromised patients, infections by *Candida albicans* have significantly increased in recent years. *C. albicans* transition from yeast to germ tubes is an essential factor for virulence. In this study we noted that Lee's medium, commonly used to induce filamentation, contained 500-fold more biotin than needed for growth. Thus, we investigated the effects of excess biotin on growth rate and filamentation by *C. albicans* in different media. At 37 °C, excess biotin (4 µM) enhanced germ tube formation (GTF) ca. 10-fold in both Lee's medium and a defined glucose-proline medium, and ca. 4-fold in 1% serum. Desthiobiotin, KAPA, and lipoic acid also stimulated GTF and are able to fulfill the biotin auxotrophy requirement. The mechanism by which excess biotin enhances GTF is still unknown except to note that equivalent levels of biotin are needed to create an internal supply of stored biotin. Biotin did not restore filamentation for any of the four known filamentation defective mutants tested. We also found that biotin auxotrophy is not temperature dependent nor influenced by the presence of 5% CO_2. Biotin starvation upregulated the biotin biosynthetic genes *BIO2, BIO3*, and *BIO4* by 11-, 1500-, and 150-fold, respectively, and BIO2p is predicted to be mitochondrion-localized. Based on our findings, we suggest that biotin has two roles in the physiology of *C. albicans*, one as an enzymatic cofactor and another as a morphological regulator.

ACKNOWLEDGEMENT

I was fortunate to be given the opportunity to continue my graduate program at the University of Nebraska-Lincoln, under Dr. Kenneth Nickerson's supervision. I started my journey in the United State as a transfer student from Malaysia finished my bachelor degree at UNL in 2013. In Fall 2012, I began working in Dr. Nickerson's lab and had the chance to do research under Dr. Sahar Hasim's guidance. My passion for Science and doing research had grown since, so I decided to enroll in the UNL Master Program for Biological Sciences. With that, I am very grateful to Dr. Nickerson and Dr. Hasim.

I would like to take this time to thank my advisor, Dr. Nickerson, for the opportunity given to me and for his guidance over these past few years. I was able to develop as a graduate student and researcher due to Dr. Nickerson's advice. He showed me many ways to think positively in constructing experiments and thinking critically. Without his advices, I will not be able to move further.

I started my Master Project in 2014, focusing on biotin roles in *Candida albicans*. This project was a continuation from other lab members, Dr. Swetha Tati, Dr. Sahar Hasim, Jessica Owens, and Ruvini Pathirana. In regards to this, I want to thank my committee members Dr. Wayne Riekhof and Dr. Mark Wilson for their constructive advice, time spent, and patience while overlooking my progress. They had also allowed us to use their lab facility/equipment, which had been really helpful. Without my committee, I would have had a difficult time completing my project, and I am really grateful for having both Dr. Riekhof and Dr. Wilson as my committee. As mentioned, without the other lab members involved in this project, it would not have been published,

as it was a teamwork effort. I want to personally thank each one of them for their contributions to this project. I want to thank the current lab members, Ruvini Pathirana and Maya Khasin, and undergraduates that had been supportive. To Ruvini Pathirana, I am thankful as she had given many insights to help me develop skills in doing research and learn the importance of teamwork. She had been a role model to me on many aspects of my journey as a graduate student. She willingly shared a lot of her experiences and guide me throughout these years. To Maya Khasin, I am grateful to have had a lab colleague that was willing to share her wisdom with me. I also want to mention the undergraduates that joined our team for their commitment to the lab: Ryan Mikus and Cindy Galdamez. I am glad that I was able to work with this team.

Furthermore, I am grateful towards members from Dr. Atkin's lab (Dr. Krista Patefield, Dr. Jessica Hargarten, Dr. Esther Choi), Dr. Riekhof's Lab (Dr. Surabhi Naik, Jithesh Vijayan, Jackie), Dr. Harris' lab (Lakshmi Verra and Jyothi Kumar), and also towards Dr. Weishu Fan, Sunil Kumar, Amit Kumar, Bhanwar Lal Puniya. They had made my days spent in Beadle Center fun and eventful. I had several opportunities to learn from them. Not to forget, I am also grateful to have supportive and understanding friends; Suzana Fernandes, Amanda Libbie, Charlene Gan and Annabel Olson. All of these wonderful people had been my inspiration and they had been really supportive. Last but not least, my thanks are given to my families back in Sabah, Malaysia and my partner, Brian Kleinknecht, for their continuous support and understanding. They had been my very backbone and motivation to move forward. As a gratitude, I want to thank

the Sabah Foundation, Malaysia for supporting me financially throughout my course of study.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS ……..iii

TABLE OF CONTENTS ………………………………………………………...vi

LIST OF FIGURES…………………………………………………………………..ix

LIST OF TABLES ……………………………………………………………………..x

CHAPTER 1: Introduction ……………………………………………………….11

CHAPTER 2: Biotin auxotrophy in *Candida albicans* ……………………………12

Abstract…………………………………………………………………….....20

Introduction……………………………………………………………………21

Methods………………………………………………………………………..23

Results…………………………………………………………………………27

Discussion……………………………………………………………………..30

Figure legends…………………………………………………………………32

Figures…………………………………………………………………………34

Tables …………………………………………………………………………39

CHAPTER 3: High biotin concentration enhances hyphal growth in *Candida albicans* ……………………………………………………………………...........41

Abstract…………………………………………………………………………..42

Introduction………………………………………………………………………43

Methods…………………………………………………………………………..44

Results……………………………………………………………………………46

Discussion………………………………………………………………………..49

Figure legends……………………………………………………………………52

Figures……………………………………………………………………………55

CHAPTER 4: Screening *Saccharomyces cerevisiae* Tet-promoters yeast collection (yTHC) and Future directions ……………………………………………………63

Introduction……………………………………………………...……………64

Methods…………………………………………………………………………..66

Results……………………………………………………………………………67

Discussion………………………………………………………………………..68

Future Aims ……………………………………………………………….…………..69

Tables ……………………………………………………………………………71

APPENDIX ……………………………………………………………………73

REFERENCES………………………………………………………………...78

LIST OF FIGURES

Figure 2.1 Biotin auxotrophy is not temperature-dependent in *C. albicans* **…………….34**

Figure 2.2 Biotin concentration does not influence growth under CO2. **………………..35**

Figure 2.3 *C. albicans* has an internal reservoir for biotin unlike *S. cerevisiae.* **………..36**

Figure 2.4 Biotin starvation activates biotin biosynthesis pathway, and proposed biotin biosynthesis in *C. albicans* was made based on mTPs prediction **………………………38**

Figure 3.1 Biotin enhances hyphal formation in *C. albicans.* **……………………………..55**

Figure 3.2 Biotin biosynthesis precursors and Lipoic acid enhances hyphal formation in *C. albicans***……………………………………………………………………………….56**

Figure 3.3 Biotin does not enhance hyphal formation under 0.5 mM N-acetyl glucosamine (GlcNAc). **……………………………………………………………………………57**

Figure 3.4 Biotin enhances hyphal formation at the same rate with lower phosphate concentration in GPP media**…………………………………………………………..58**

Figure 3.5 Urea amidolyase is required for biotin to enhance hyphal formation**……….59**

Figure 3.6 Hyphal associated transcription factors are required for biotin enhancing hyphal formation**……………………………………………………………………………….60**

Figure 3.7 Biotin effect on GTF of two different strains: SC5314 and published previously, 6713. **……………………………………………………………………………………..62**

Supplementary Figure 4.1 ……………………………………………………………..72

LIST OF TABLES

Table 2.1. List of primers used for gene expression. Amplification was done using *CDC36* as the reference genes……………………………………………………………………39

Table 2.2. Influence of biotin and 5% CO_2 on colony diameter[1] and colony weight after 5 days………………………………………………………………………………………40

Table 4.1 Five strains were identified to growth when supplemeneted with high concentration of biotin/ lipoic acid ……………………………………………………..71

CHAPTER 1

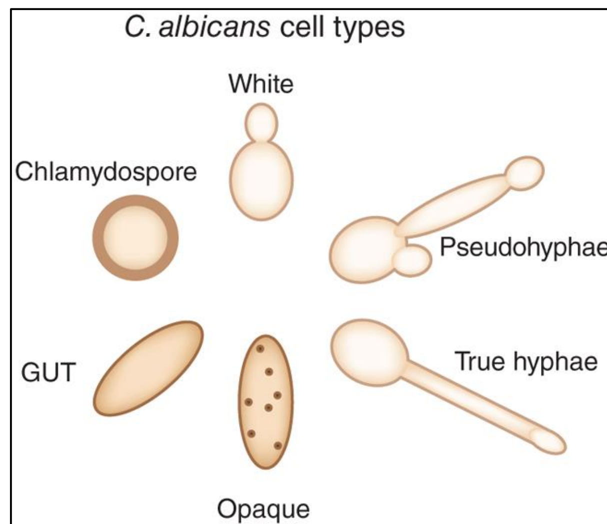
Introduction

Candida albicans is a polymorphic opportunistic pathogen that is a part of normal microbial flora of most humans. Immunocompromised patients are at high risk for a candida infection, also known as candidiasis. Candidiasis can be classified according to the site of infection: oropharyngeal/esophageal (OPC), genital/vulvovaginal (VVC), and invasive candidiasis. Invasive candidiasis or candidemia is the most dangerous as it has the highest mortality rate among the other type of candida infection [2]. It is also the fourth most common cause of nosocomial bloodstream infections among hospitalized patients in the United States [2].

Candidiasis can be treated by several types of drugs. The most common drugs used are Fluconazole, other Azole drugs, Caspofungin, and Amphotericin B. These drugs can be used alone or in combination depending on the type of candida infection. A significant reason to study *C. albicans* despite the available choice of treatments is the increase of drug-resistant [2]. Development of new drugs targeting different aspects of yeast physiology therefore is imperative for successful control of candidiasis.

2. Dimorphism in *C. albicans*

Since *C. albicans* pathogenicity is widely studied based on its morphology, it is best to focus on the factors that can cause *C. albicans* morphological changes. *C. albicans* is known to be a polymorphic fungus, as defined by its ability to form various cell types: yeasts (white phase), hyphae, pseudohyphae, GUT (commensalism-related), opaque (mating cell types) and chlamydospores [3].



Yeast cells are often distinguished by round single cells, which grow through budding resulting in two asymmetrical mother and daughter cells. This budding pattern found on *C. albicans* yeast cells is similar to *S. cerevisiae* [4]. *C. albicans* is also known as a dimorphic fungus, which refers to the reversible switching between yeast to hyphal forms. Hyphal formation is initiated through the formation of germ-tubes, which will be referred to as GTF. Additionally, hyphal formation is a distinct character of most pathogenic fungi. Germ tubes usually form after induction of initial bud site selection,

and theygrow as elongated filaments which are separated by septae [5]. Dimorphism is one of the key virulence factors that could lead to candidiasis.

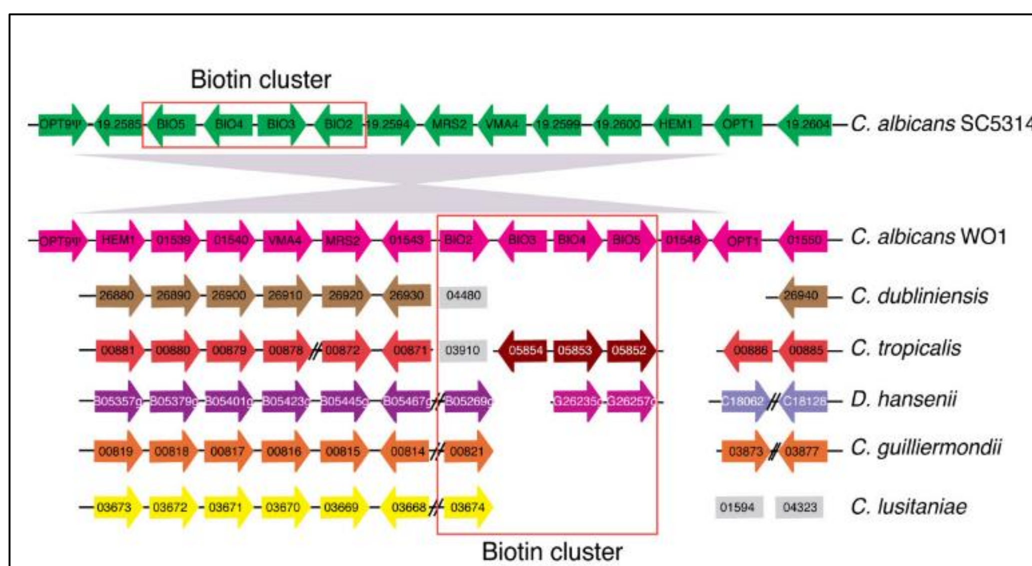
Hyphal triggers are environmental triggers which lead to *C. albicans* hyphal formation. They act through several signal transduction pathways. These environmental triggers include growth at 37°C, the presence of hyphal inducers such N-acetylglucosamine (GlcNAc), non-acid pH, high CO_2, and many more [6-9]. There are several specific media used to induce hyphal growth in *C. albicans,* such as Lee's media, serum, differentiation media containing GlcNAc, and RPMI [9]. Comparison among these media shows that some of them contain high concentrations of biotin; eg. Lee's medium has 40x more biotin than needed for growth [7]. In this regards, we will look into the role of biotin in inducing hyphal formation with the aim of trying to solve the underlying mechanism. An important note for this section is the terminology distinguishing between a hyphal inducer (the sole trigger for hyphae) versus an enhancer (adding to the effect of a hyphal trigger). Biotin is categorized as an enhancer, not an inducer.

3. Biotin auxotrophy in *C. albicans*

Biotin, also known as vitamin H or B7, is a small (244 Da) micronutrient. Although it is an important growth cofactor, most eukaryotes are unable to synthesize biotin de novo, and thus need to scavenge biotin from their environment. In yeasts, like *S. cerevisiae* and *C. albicans,* the biotin requirement is well documented [10-12].

Protein biotinylation is catalyzed in yeasts by a biotin protein ligase (BPL1), which is a homolog of holocarboxylase synthetase (HLCS) in human. BPL is not yet fully characterized in *C. albicans*, but comparing it to a closely related species, *S. cerevisiae,* Pendini et al [13] found an intriguing insight. In their work [13], they found that both *S. cerevisiae* and *C. albicans* SC5314 contain a single BPL gene with 39% identity at the protein level. However, whereas CaBPL used acetyl CoA carboxylase (ACC) and pyruvate carboxylase (PC) as their substrates, this enzyme in *S. cerevisiae* achieved biotinylation of six biotin-dependent enzymes [14]: two isoforms of ACC, two isoforms of PC, urea amidolyase and Arc1p, a tRNA binding protein. Most of these biotin dependent enzymes are crucial for metabolic processes. The two isoforms of ACC are dependent on the localization of the enzymes, where the cytoplasmic ACC acts as the rate-limiting step in fatty acid synthesis and the mitochondrial ACC participates in the synthesis of the cofactor lipoic acid [15, 16]. PC is important as an anaplerotic step in glucose metabolism, where the expression of both isoforms is dependent on the growth medium and carbon sources [17]. For both *C. albicans* and *S. cerevisiae,* urea amidolyase, catalyzes the degradation of urea when urea is the sole nitrogen source [18]. And lastly, Arc1p, was shown to integrate biotin even though biotin is not required for its function [19]. Arc1p is also unusual in that it does not have the usual MKM biotin attachment site. Hasim et al (2013) [20] suggested that Arc1p serves as biotin storage in *S. cerevisiae,* similar to likely storage role of biotinylated histones in *C. albicans.*

Whilst *S. cerevisiae* possess z partial biotin synthesis pathway, it will be unable to grow without biotin, desthiobiotin, or KAPA in the media. The biotin synthesis pathway has been lost from a common ancestor of *S. cerevisiae* and *C. albicans,* and was rebuilt from horizontal gene transfer and gene duplication together with neofunctionalization [11]. Intriguingly, Hall and Dietrich [11] stated that one of the *S. cerevisiae* strain, S288c was able to grow with limited biotin supplemented when both *BIO1* and *BIO6* were introduced into this strain, where *BIO6* can be a duplication of *BIO3 [21]*. The *BIO* gene clusters for *C. albicans* and several other candida species can be seen in the figure below [21]:



For this chapter, we will explore the biotin auxotrophy of *C. albicans* as compared to *S. cerevisiae* through several different analyses such as population studies successive transfers in biotin-free media, protein biotinylation profiles, and peptides localization prediction. Based on these findings, we will better understand biotin utilization in *C. albicans.*

4. Biotin Transport System

As a biotin auxotroph, *S. cerevisiae* as well as *C. albicans* must acquire biotin from their environment. Thus, it will be necessary to have a sophisticated biotin uptake system. According to Rogers and Lichstein [22], *S. cerevisiae's* biotin transport has a high affinity for biotin with an apparent *km* of 3.23 × 10−7 M, and it is a glucose-stimulated, energy-dependent uptake mechanism. In the same study [22], it was shown that the internal biotin concentration can exceed the concentration in the medium by >1000-fold. In terms of the regulation, high levels of external biotin reduced the rate of biotin uptake and the *p*-nitrophenyl ester of biotin caused an irreversible inhibition of the uptake mechanism due to the covalent modification [23]. In 1999, Stolz et al [23] characterized the biotin transport system in *S. cerevisiae* as Vitamin H-Transporter 1 *(VHT1)*. Unfortunately, the biotin transport mechanism in *C. albicans* is has yet to be elucidated, although there is an uncharacterized putative ortholog, *orf19.2397* to the *VHT1* of both *S. cerevisiae* and *S. pombe* [35].

Biotin Auxotrophy and Biotin Enhanced Germ Tube Formation in Candida albicans

Nur Ahmad Hussin,[1] Ruvini U. Pathirana,[1] Sahar Hasim,[1,2] Swetha Tati,[1,3] Jessica A. Scheib-Owens,[1] and Kenneth W. Nickerson[1]*

[1]School of Biological Sciences,

University of Nebraska, Lincoln, NE 68588, USA;

[2]Department of Microbiology,

University of Tennessee, Knoxville, TN 37996, USA

[3]Forrobin Inc.,

Buffalo, NY 14214, USA

*Correspondence: Kenneth W. Nickerson

Email: knickerson1@unl.edu

Tel.: +1-402-472-2253

Acknowledgement: This work was spported by Ann L. Kelsall and the Farnesol and Candida albicans Research Fund, University of Nebraska-Foundation

CHAPTER 2

Biotin auxotrophy in *Candida albicans*

Abstract

The polymorphic fungi, *Candida albicans* requires biotin for growth like many other organisms. Unlike bacteria, *C. albicans* and related species have a partial biotin biosynthetic pathway and thus require precursors to synthesize biotin. The biotin auxotrophic requirement can be fulfilled by desthiobiotin, KAPA and several biotin analogues. In this study, we had further investigated the biotin auxotrophy in *C. albicans* by diluting the external biotin availability (population study) and ran preliminary gene expression analysis. Previous research has not yet shown the availability of biotin storage mechanism. It is crucial to understanding the function of biotin metabolism and how it can lead to morphological changes. Based on our findings, biotin auxotrophy is not temperature dependent nor it can be compensated by the presence of carbon dioxide. These statements were simply due to the reason that auxotrophic can be influenced by temperature and substrate availability. We had also found that under biotin starvation, upregulation of the biotin biosynthetic genes *BIO2, BIO3*, and *BIO4* were increased by 11-, 1500-, and 150-fold, respectively. This meant that the cells did not synthesize enough biotin to sustain their growth. Additionally, using peptide prediction analysis allows us to illustrate a proposal of the biotin biosynthetic pathway with BIO2p being localized in the mitochondrion.

Introduction

Biotin biosynthetic pathway has been well studied as it is an important growth cofactor [24]. However, there are still limitation in synthesizing biotin for some microbes and higher vertebrates as they need to incorporate biotin from an external source. Specifically looking at the interactions of biotin with yeasts, it was dated back to the discovery of biotin by Kögl and Tönnis [25]. They identified biotin as a growth factor obtained from eggs and yeast which promoted the growth of all baker's and distiller's yeasts tested, as well as a large number of other yeasts. Subsequently it was shown that *S. cerevisiae* needs biotin or the biotin analogs D-desthiobiotin, biocytin, or biotin-D-sulphoxide [26].

The last four steps of biotin synthesis are typically conserved (pimeloyl-CoA to biotin) as found in *C. albicans* and closely related species [27]. As such, the biotin auxotrophy for most yeasts can be fulfilled by intermediates of biotin synthesis [28]. Presumably the pathway was lost in a distant ancestor and then rebuilt by horizontal gene transfer, gene duplication, and neofunctionalization [11]. Thus, Phalip et al [29] found that *S. cerevisiae* required an external supply of 7-keto-8-amino-pelargonic acid (KAPA), 7,8-diamino-pelargonic acid (DAPA), desthiobiotin, or biotin although two earlier precursors, pimelic acid and pimeloyl-CoA, would not suffice. *C. albicans* is also a biotin auxotroph [12] and Firestone and Koser [30] found that this biotin auxotrophy could be fulfilled by biotin, desthiobiotin, biocytin, or oxybiotin.

Our previous work on biotinylated histones in *C. albicans* [20] showed that the growth rate for *C. albicans* under biotin deprivation for three growth cycles, or as we refer in this study: population, were decreasing. Even so, the remarkable mark from this

finding was the ability of *C. albicans* to grow over several populations, as compared *Saccharomyces cerevisiae* that cannot restore biotin. The presence of an internal biotin reservoir, possibly biotinylated proteins (including histones [20]) was indicated by the same decreasing growth rate when neutravidin is added in the medium as it is a strong biotin-binding protein.

The present paper studies the role of biotin in *C. albicans* growth. Although been proven as a biotin auxotroph, further investigation is necessary to confirm the auxotrophic requirement as *C. albicans* has the potential of storing biotin. Thus, we tested *C. albicans* SC5314 for its requirement of biotin under several conditions; different temperature, carbon dioxide and minimal defined media supplemented with different biotin concentration.

Methods

C. albicans strains and growth conditions

C. albicans strain SC5314 was obtained from the American type culture collection, Rockville, MD and strain A72 was obtained from Prof. Patrick A. Sullivan, at Otago Univ., NZ. Cells were grown into several types of media; GSB (a minimal defined glucose-salts-biotin medium), a defined glucose-ammonium-salts medium of Yamaguchi [31]), and GPP [32, 33]. When the cells reach OD600 of 1 (Spectra Max Plus microplate reader (Molecular Devices, Sunnyvale, CA)), it will be harvested by centrifugation at 4,200 for 5 mins, washed three times with 50 ml of wash buffer (3.5 g KH2PO4, 4.1 g K2HPO4 in 1 L H2O, pH 6.5), and stored at 4°C for further use.

Analysis of Biotin Auxotrophy

C. albicans cells were grown at $1x10^6$ cells per ml in media supplemented with or without biotin (0 to 100 nM) in 250 ml flasks. The cells for the first population were grown at 25°C, 30°C, 37°C, or 42°C with shaking (225 rpm). After 30 hours, the cells were harvested and washed with distilled water. These cells were then used as inoculum ($1x10^6$ cells per ml) and grown into fresh media supplemented with or without biotin (0 or 100 nM). Again, these cells will be grown with the same condition as previously mentioned, and counted as the second population. At the end of 30 hours, for each population, the cell viability will be determined through staining with 0.05 % methylene blue [34]. This was done in order to determine whether to continue growing the next

population (ie, third or fourth population). For each population, 5 ml samples were taken every 5 or 6 hours, analyzed microscopically, and prepared for dry-weight measurement.

The dry-weight measurement was done using 5 ml samples that been washed twice with distilled water (before drying). After measuring the initial weight of the weighing dish, the washed samples were dried in the drying oven for 6-8 hours at 150°C. Then, the samples were cooled in the desiccator. After constant measurement of the dry-weight of each sample, the data were recorded.

Effects of CO_2 on Biotin Auxotrophy

Biotin deficient inocula were prepared by growing *C. albicans* SC5314 in 50 ml of biotin free glucose-salts [35] medium pH 5.6 for 25-30 hours at 30°C. The cells were harvested, washed three times with distilled water or KP buffer, and stored at 4°C prior to use. The GS plates were prepared with Noble agar supplemented with 0, 5, 50, or 4000 nM biotin. Noble Agar was chosen to minimize the contaminating biotin found in agar. YPD agar plates were used as controls. The inoculum was diluted to 6×10^2 cells per ml so that a 100 µl cell suspension gave approximately 60 CFU/ plate after they had been spread with glass beads. Triplicate plates were incubated 4 to 5 days under four environmental conditions: (i) 30°C, (ii) 37°C, (iii) 30°C with 5% CO_2, and (iv) 37°C with 5% CO_2. Both 5% CO_2 cultures were incubated in a dual chamber VWR 18401R incubator (VWR Scientific, Radnor, PA, USA). After 5 days all colony diameters were measured and averaged. Three colonies per plate were scraped off with a spatula and subjected to dry-weighed.

Immunofluorescence Stain

C. albicans SC5314 were grown in SC media supplemented with or without biotin, and two populations were collected. Staining protocol were followed as described in Madsen et al [36], with the exception of cell fixation using 100 microliters of 0.4% paraformaldehyde. The incubation period with 0.4% paraformaldehyde was 15 mins at room temperature. Samples was then washed with KPO_4 buffer before storing in 4°C for further use. For biotin detection, Alexa 549-conjugated streptavidin (Life Technologies) was used, while standard DAPI staining (nucleic acid detection) used as control. Microscopy imaging were captured by using EVOS FL Microscopy located in Dr. Riekhof's lab (Magnification was set to 40X).

Gene Expression Analysis

A preculture of *C. albicans* grown in synthetic complete (SC) medium was reinoculated into SC medium and treated with or without biotin until they reached mid log phase. The cells were harvested and total RNA were extracted using a phenol extraction method as described by Kohrer & Domdey [37]and then DNase treated with Turbo DNA-free kit (Invitrogen/Thermo Fischer Scientific) according to the manufacturer instructions. Complementary DNA was prepared using first strand cDNA using SuperScript® III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen™) according to the manufacturer's recommendation using oligo dT primers. Based on 1:1 ratio production of

cDNA, 100 ng of resulting sample was subjected to quantitative PCR using iQ™ SYBR® Green Supermix in a Biorad iCycler iQ real time PCR detection system. Each cDNA preparation was normalized using *CaCDC36* as an internal control. The primers used for genes under study, *BPL1*, *BIO2*, *BIO3*, *BIO4*, *BIO32* and *CDC36* in this study are listed in Table 3.1. Quantitative RT-PCR data were normalized in two steps as described previously [37] and analyzed using two-way ANOVA with the post Bonferroni comparison test using GraphPad Prism Version 5.02, GraphPad Software (San Diego, California, USA).

Mitochondrial targeting peptides (mTPs) predictions

mTPs predictions on *C. albicans BPL1*, *BIO2*, *BIO3*, *BIO4*, *BIO32* genes were performed by using PSORT II [38] server (http://psort.hgc.jp/form2.html), MitoFates [39] server (http://mitf.cbrc.jp/MitoFates/cgi-bin/top.cgi), TPpred 2.0 [40] server (http://tppred2.biocomp.unibo.it/tppred2/default/index) and TargetP [41] server (http://www.cbs.dtu.dk/services/TargetP/). Default settings were used for all four predictors.

Results

Biotin auxotrophy is not temperature-dependent in *C. albicans.*

C. albicans is a biotin auxotroph [45] and biotin is supplemented routinely to all defined growth media for *C. albicans*. Addition of $\geq$ 100 nM biotin, desthiobiotin, and KAPA were enough to fulfill *C. albicans* biotin auxotrophy but not with pimelic acid. some vitamin requirements in bacteria and fungi are temperature dependent [22]. However, we found that the biotin auxotrophy in *C. albicans* is not temperature dependent; biotin was required for growth at all temperatures from 20 to 42°C (Fig. 2.1).

Biotin concentration does not influence growth under CO_2.

To test the whether there is a correlation of having CO_2 as either their substrate or product for all the biotin-containing enzymes [42], and thus investigating whether CO_2 will be able to fulfill the biotin auxotrophy, *C. albicans* SC5314 was grown under 5% carbon dioxide. GS agar plates containing four levels of biotin (0-4 µM) along with YPD control plates were incubated under four conditions (30°C and 37°C both with and without 5% CO_2). Colony diameter and dry weight were recorded after 5 days (Table 2.2). On these defined agar plates, the colonies were barely visible without the addition of biotin (Table 2.2 and Fig. 2.2). However, with biotin, the colony diameters and colony weights were slightly smaller than those observed on the YPD plates. Significantly, the colony diameters and mass were not statistically different for biotin supplemented plates contained 5, 50, or 4000 nM biotin. 5% CO_2 did not increase the colony diameter or mass except in one case (37°C + CO_2) where the colony diameters with biotin (7 $\pm$ 0.3 mm)

were twice as large (Table 2.2) because the colonies were predominantly filamentous and appeared spread. The filamentous nature of the colonies was determined by both microscopy and the wrinkled appearance of the colonies. Wrinkled colonies were observed for the 37°C + CO_2 grown cells because 5-10% CO_2 also triggers GTF [43, 44]. Finally, the short spikes as seen emerging from the colonies on YPD at 30°C + CO_2 (Fig 2.2) were slightly filamentous when observed by microscopy. This phenomenon could be due to the influence of 5% CO_2.

***C. albicans* has an internal reservoir for biotin.**

Unlike *S. cerevisiae, C. albicans* are able to grow for several populations when starved with biotin (Fig 2.3 a & b). One possible explanation is that *C. albicans* has an internal reservoir for biotin through protein biotinylation (Fig 2.3 c). Also noted that smaller proteins were not biotinylated when biotin is limited, which could be the targeted for biotin reservation. It was shown that *BPL1* gene was upregulated (Fig 2.3 d), probably in order to scavenge biotin from biotinylated proteins, and used it for growth.

PYC and ACC are >120 kDa and >200 kDa respectively.

Biotin biosynthesis activated during biotin starvation; another possible explanation on why *C. albicans* were able to grow for several populations.

Regulation of the biotin biosynthetic genes in response to biotin starvation was explored in SC medium with and without 2µg/L biotin (Fig. 2.4). RT-PCR showed that the upregulation of *BIO2, BIO3,* and *BIO4* increased 11-, 1500-, and 150-fold respectively in

the biotin starved cells, while *BIO32* remained effectively unchanged. Upregulation of these genes indicate that biotin is a necessity for cell growth [45], even though the production of biotin is not enough to maintain cell growth. The fact that *BIO32* remained unchanged is consistent with the suggestion by Fitzpatrick et al [21] that *BIO32* is more likely to be involved in arginine or glutamate metabolism then in biotin synthesis.

Discussion

This study shown that *C. albicans,* a biotin auxotroph, possess internal reserves for biotin without biotin supplementation. When biotin was diluted out from the media, *C. albicans* were able to either scavenge its internal reserves of biotin or synthesize biotin enough to allow extensional growth. In our finding [46], *C. albicans* has the ability to accumulate internal, covalently attached biotin. Based on the western blot analysis (Fig 2.3), we also have concluded that when biotin is abundant, most of the protein biotinylation occurs on smaller proteins, including histone as shown by Hasim et al [20]. These smaller proteins however were not biotinylated when biotin is limiting, implying that the biotin could be extracted from these proteins and channeled to growth. Upregulation of *BPL1* under biotin starvation can also be used as an estimation of the cells scavenging biotin from this internal reserves. Another important aspect of *BPL1* upregulation (Fig 2.3) is that BPL disruption affect metabolic pathways such as fatty acid biosynthesis and gluconeogenesis [13]. Hence, under biotin limiting, it is sensible for the cells to have accessible BPL. As mentioned previously, larger proteins got biotinylated which includes important metabolic enzymes such as pyruvate carboxylase and acetyl CoA carboxylase. The biotin domains of these enzymes were characterized as substrates for BPL [13].

C. albicans have a partial biotin biosynthesis (Fig. 2.4), and it was found to be upregulated under biotin starvation. However, there was not enough of biotin synthesized to compensate the growth requirement. According to Fitzpatrick et al., [21] *C. albicans* SC5314 and WO-1 contain distinct biotin clusters including *BIO2, BIO3, BIO4*, and *BIO5*. Subcellular localization analysis showed that most of the *BIO* genes (Fig. 2.4) are

predicted to be outside of the mitochondrion except for *BIO2*. It is presumably due to evolutionary conservation, *BIO2* has to remain mitochondrial as found in *A. thaliana* [47], where the biotin sulfur atom is obtained from an iron/sulfur cluster protein. *C. albicans* does not contain *BIO1*, a pimeloyl-CoA synthase, and *BIO6*, the alternate method of synthesizing KAPA that are found in biotin prototrophic Sake yeasts [27]. The implication is that biotin or its precursors, desthiobiotin and KAPA are available in the blood or another location where infection is occurring. According to Zakikhany et al [48], both *BIO2* and *BIO4* were upregulated during infection for ca. 2.5-fold. Similarly, *BIO2* was upregulated ca. 107-fold on shifting from low iron to high iron growth conditions [49]. The necessity for *C. albicans* to upregulate its biotin biosynthetic pathway could be related to an adaptive mechanism in the host.

Our next goal will be to investigate the relationship of biotin employment for growth and hyphal formation. Analysis on morphological and environmental regulators such as *HAP43, NGT1*, and etc., are necessary to unravel biotin utilization when cells are exposed to stress. Proteomic, together with mutational analyses of biotinylated proteins, will help to elucidate the exceptional specificity of this protein modification.

Figure Legends

Figure 2.1 Biotin auxotrophy is not temperature-dependent in *C. albicans*.

Wild type *C. albicans* SC5314 cells were grown in GPP at **(a)** 25°C, **(b)** 30°C, **(c)** 37°C and **(d)** 42 for 30 hours. Cells were then reinoculated into the same media and grown under the same condition as previously mentioned. The dry-weight from second population GPP were collected every 6 hours**.** Cells were treated in either with or without 100 nM biotin for all conditions. Values shown are the average of triplicate experiments.

Figure 2.2 Biotin concentration does not influence growth under CO2.

Wild type C. albicans SC5314 cells were grown in YPD 2% agar and GS-Biotin 2% agar at 30°C, 37°C, 30°C + 5% CO_2, and 37°C + CO_2 for 4 to 5 days**.** Noted the GS media contains 0 nM, 5 nM, 50 nM, 4 µM of biotin. Different magnifications were used to show colony morphologies. Colony sizes and weights of triplicate experiments' average + SD were shown in **Table** 2.2.

Figure 2.3 *C. albicans* has an internal reservoir for biotin unlike *S. cerevisiae.*

Wild type *C. albicans* SC5314 cells were grown in **(a)** SC and Lee's medium at 30°C for 30 hours. **(b)** Cells were treated with Alexa 549 streptavidin antibody to show biotin content of the first population and second population grown in SC medium. *S. cerevisiae* is used as reference [36] **(c)** Streptavidin-HRP western blot analysis of the biotinylated protein content from cells that were grown in YPD, GSB and SC media either without biotin or with 2 µg/l biotin. **(d)** Relative expression of *C. albicans BPL1* in SC medium either without biotin or with 2 µg/l biotin. Normalization was done by using

housekeeping gene, *CDC36*. Data shown are the average of triplicate experiments and displayed as Mean + SE.

Figure 2.4 Biotin starvation activates biotin biosynthesis pathway, and proposed biotin biosynthesis in *C. albicans* was made based on mTPs prediction

(**a**) Relative expression of *C. albicans BIO2, BIO3, BIO4*, and *BIO32* in SC medium under biotin starvation. Normalization was done by using housekeeping gene, *CDC36*. Data shown are the average of triplicate experiments and displayed as Mean + SE. **(b)** Based on four online-based software, mitochondrial targeting peptides (mTPs) predictions for *BIO32, BIO3, BIO4, BIO2* were used as guideline in generating this model. This model assumes that biotin biosynthesis occurs in the cytoplasm except for the conversion of desthiobiotin to biotin by *BIO2* which occurs in the mitochondrion. Biotin, desthiobiotin and KAPA (8-amino-7-oxononanoate) could be taken up by two different transporters, which are *VHT1* and *HNM3/ BIO5*, respectively. Note that the mitochondrial transporters for biotin uptake, desthiobiotin uptake, and biotin secretion may be different manifestations of the same transport system. This mitochondrion model is based on different roles of biotin and desthiobiotin in growth and filamentation.

Figures

Figure 2.1 Biotin auxotrophy is not temperature-dependent in *C. albicans*.

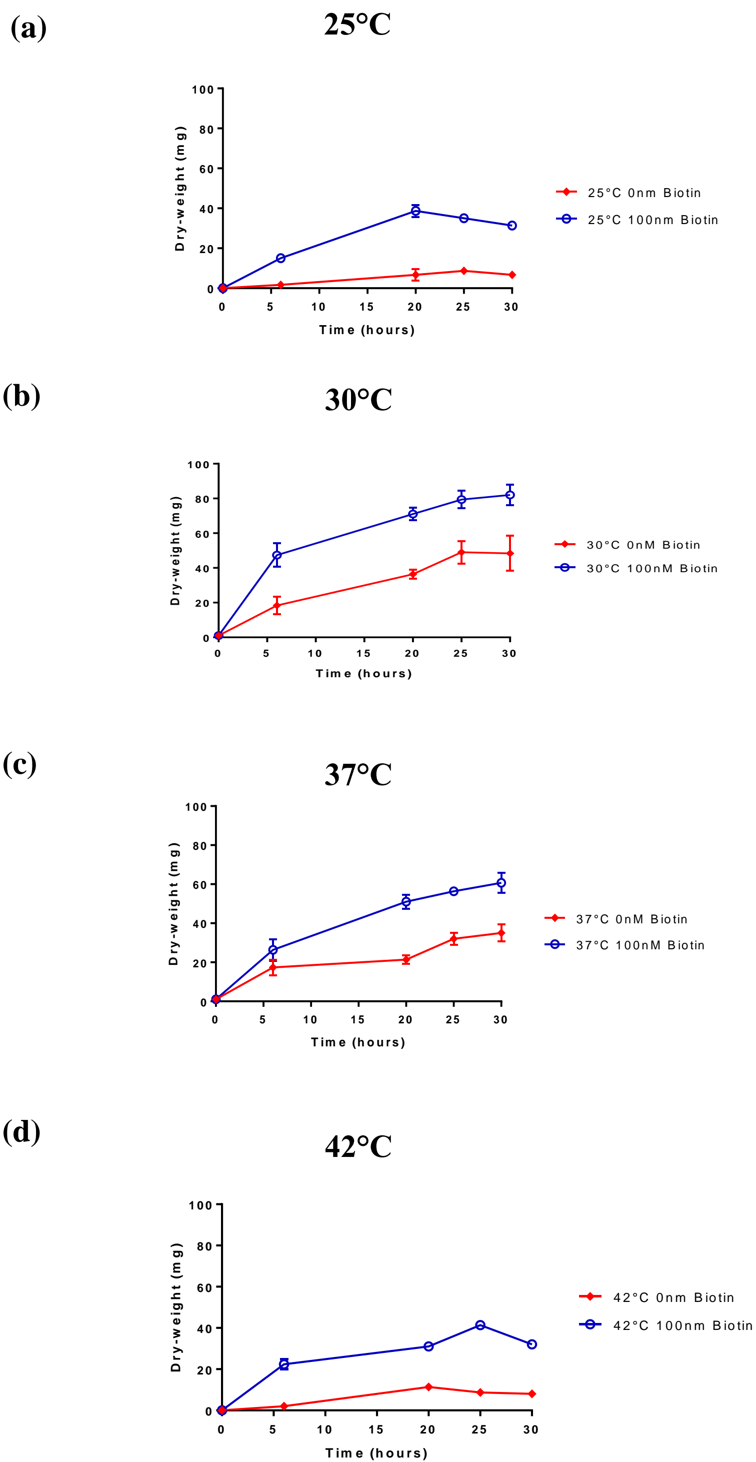
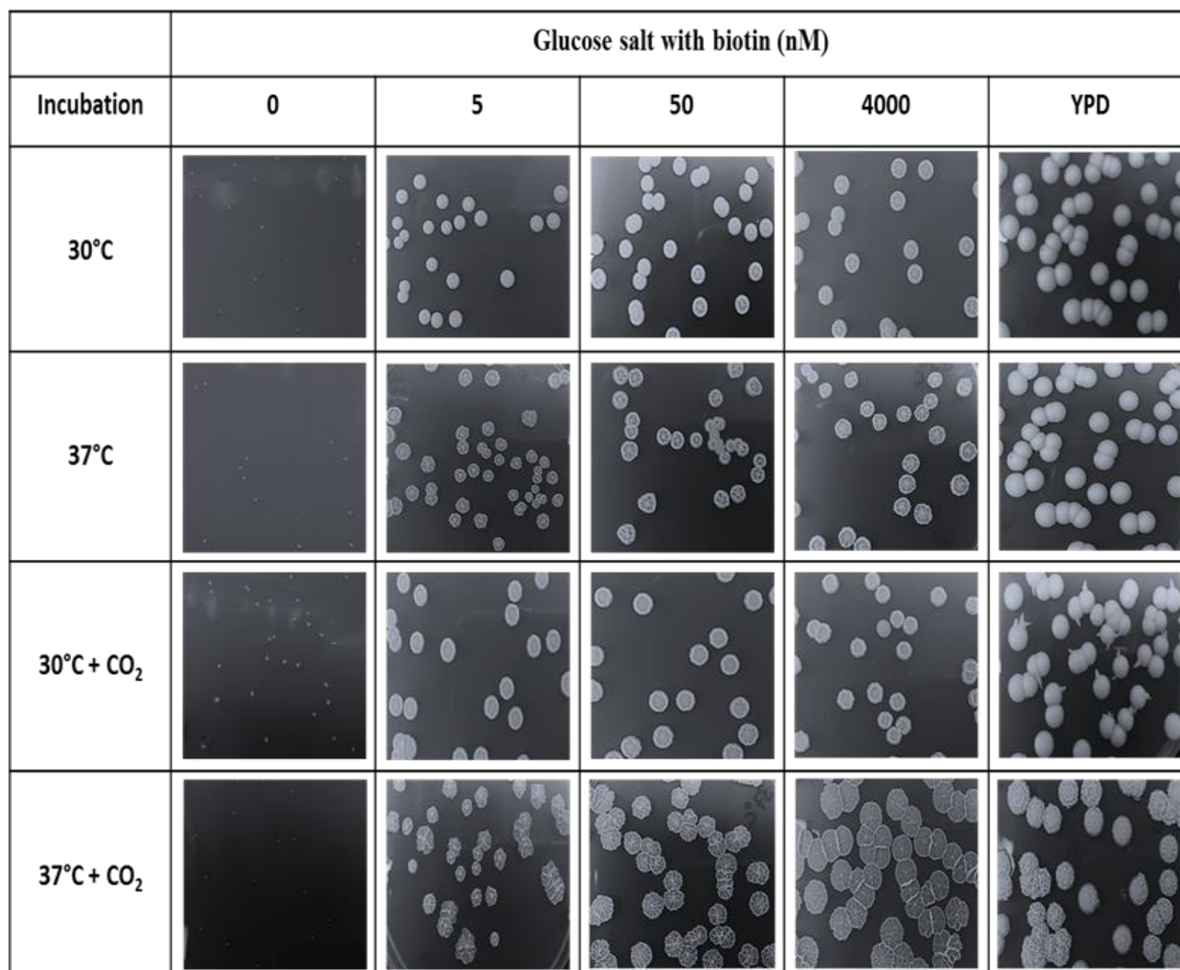


Figure 2.2 Biotin concentration does not influence growth under CO2.



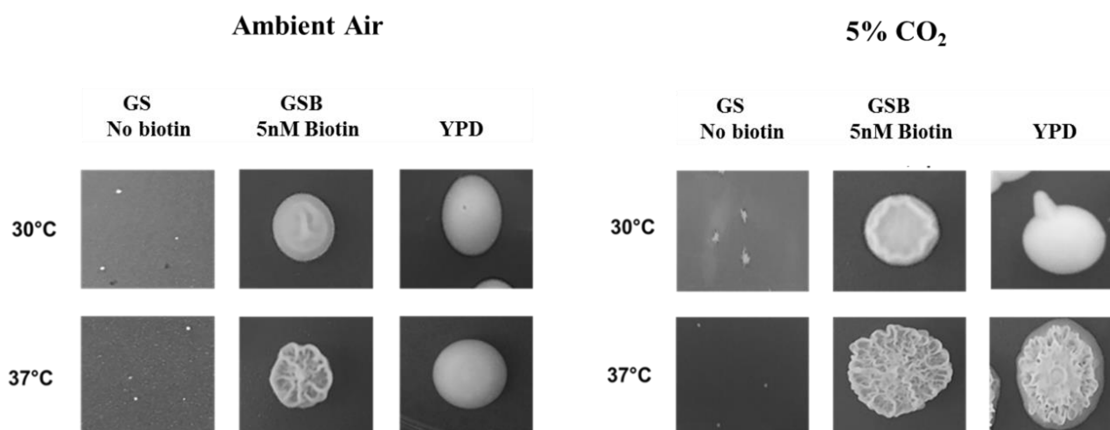
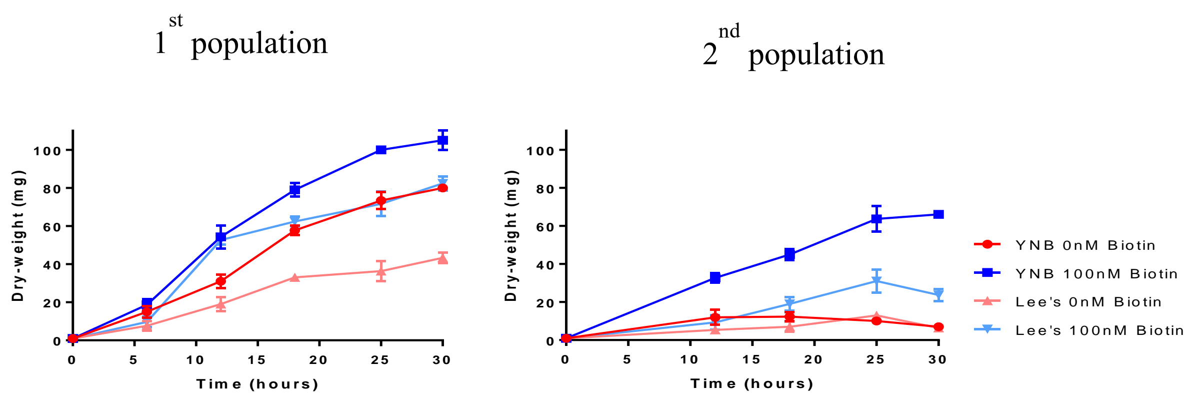
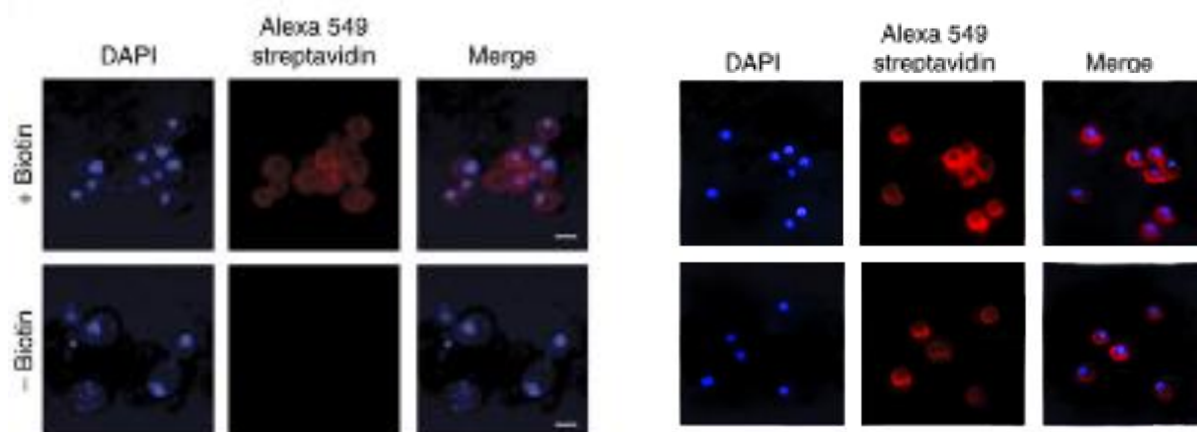


Figure 2.3 *C. albicans* has an internal reservoir for biotin unlike *S. cerevisiae.*

(a)

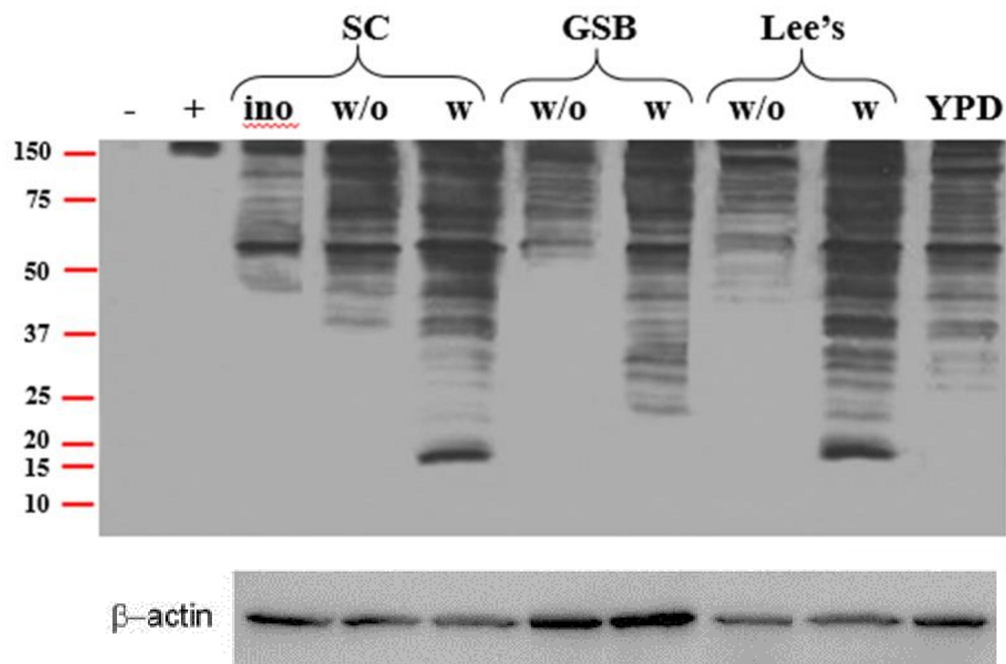


(b) *S. cerevisiae* *C. albicans*



Madsen et al. (2014)

(c)



(d)

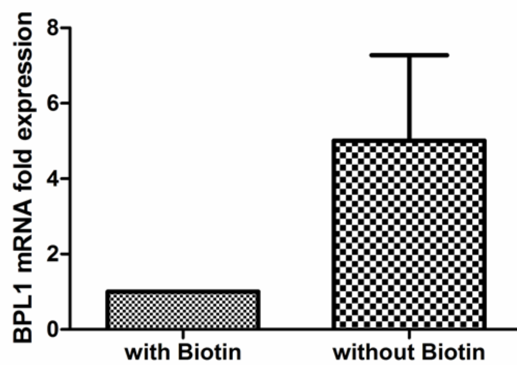
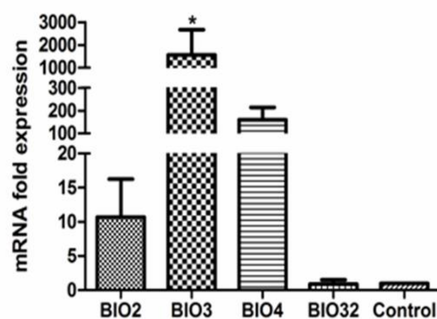
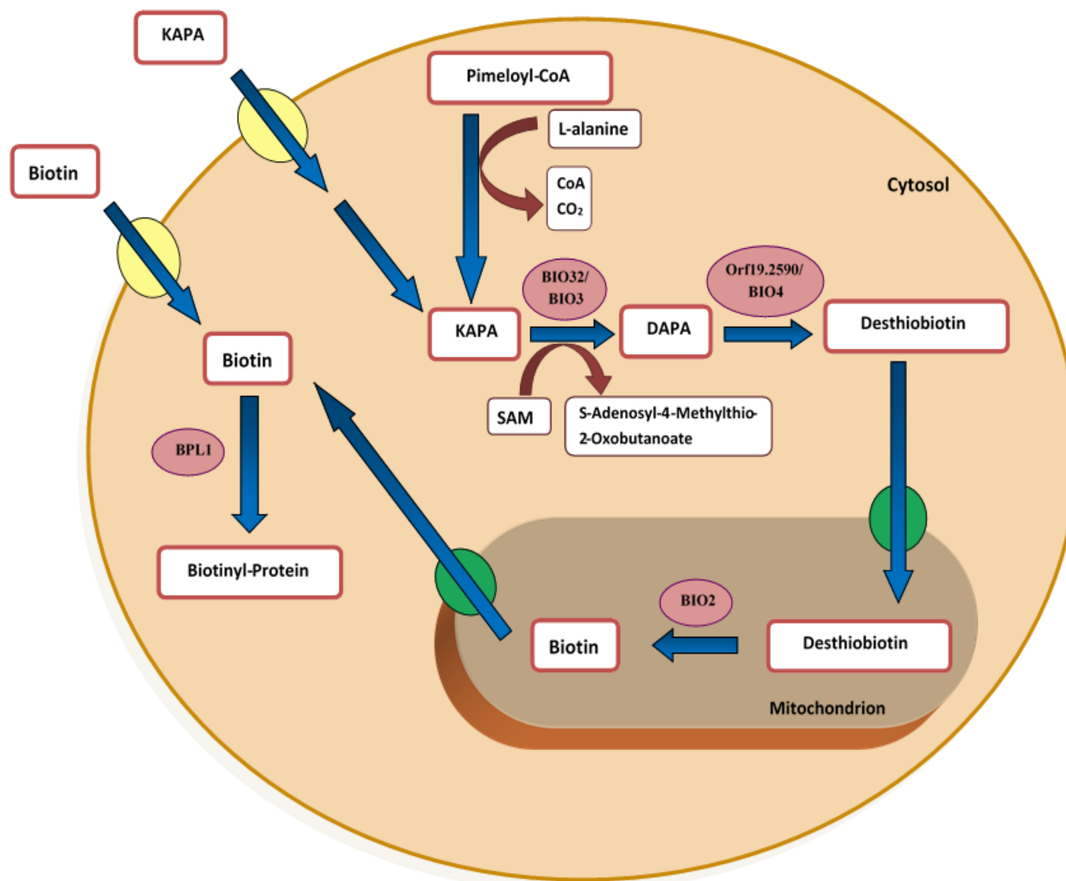


Figure 2.4 Biotin starvation activates biotin biosynthesis pathway, and proposed biotin biosynthesis in *C. albicans* was made based on mTPs prediction

(a)



(b)



Tables

Table 2.1 List of primers used for gene expression. Amplification was done using *CDC36* as the reference genes.

Gene Abbreviation	Primer Sequences (Forward -F and Reverse -R)	Source
BIO2 orf19.2593	F: 5’-GCACCCAGAATCATTGCCAA-3’ R: 5’-ACTGCTCGTGTTCCTTCATG-3’	This study
BIO4 orf19.2590	F: 5’-AGTAGCTCGGAGTGGATTGG-3’ R: 5’-TTAGAATGAGGGATGTTCGCA-3’	This study
BIO32 orf19.3567	F: 5’-GTGGACGAGGATTATTTGGGGAA-3’ R: 5’-TCCGTCTATTGTTCCCTTTCCA-3’	This study
BIO3 orf19.2591	F: 5’-AAACTGGAGCCTGGGAAACT-3’ R: 5’-GGCGAACCCAAACACCTAAA-3’	This study
BPL1 orf19.7645	F: 5’-GTTGAATGAGATCAGACGTGGA-3’ R: 5’-GCCATTGTCAACGTCCACTT-3’	This study
CDC36	F: 5’-GACCGTCCAGTATAAATCCACCAC-3’ R: 5’-TCAAGACGGGCTCCACATTACTAT-3’	Pendrak et al. [38]

Table 2.2 Influence of biotin and 5% CO_2 on colony diameter[1] and colony weight after 5 days.

	Glucose salts with biotin (nM)		
Incubation	**0**	**5, 50, and 4000**	**YPD[2]**
30°C	0.5[3]	4 ± 0.07[1] (0.9)	6 ± 0.51 (1.4)
37°C	0.5[3]	3.5 ± 0.15 (0.9)	6 ± 1.02 (1.4)
30°C + CO_2	1.0[4]	3.5 ± 0.05 (0.8)	5.5 ± 0.45 (1.4)
37°C + CO_2	0.5 [3]	7 ± 1.55 (0.8)	6 ± 0.88 (1.4)

[1] Colony diameter in mm (colony weight in mg). Values are the average of 7 replicates, using 3 biological replicates with 3, 2, and 2 technical replicates, respectively.

[2] Yeast Extract-Peptone-Dextrose (YPD) also serves as the 100% control for plating efficiency. All plates were spread with 100 µL of cells resulting in 60 ± 10 colonies per plate.

[3] Colonies were too small to be scraped off and weighed.

[4] Colonies could not be weighed because they had penetrated into the agar.

CHAPTER 3

High biotin concentration enhances hyphal growth in *Candida albicans*

Abstract

As a common pathogenic fungus in humans, *Candida albicans* morphological characteristics and factors affecting its morphology have been studied intensively. Dimorphic changes between the yeast to hyphal form of *C. albicans* are crucial to its pathogenicity and become the main focus of this study. Comparison between the common media used for inducing hyphal growth show relatively high concentration of biotin greater than needed for cells growth. Thus, we investigate the effect of excess biotin on hyphal formation by *C. albicans* in Lee's medium, Serum and GPP. At 37°C, excess biotin (4µM) enhances germ tube formation (GTF) by 10-fold. We also found that desthiobiotin, 7-keto-8-aminopelargonic acid (KAPA), and lipoic acid also increased the rate of GTF. The mechanism by which 1-4 µM biotin enhances GTF is still unknown except to note that equivalent levels of biotin are needed to create an internal supply of stored biotin and biotinylated histones. To have an idea on the cellular function, we screened several known filamentation defective and obligately filamentous mutants involve in the hyphal signaling pathway. Biotin did not affect any of the filamentous defective mutants, but it did reduce the clumps of filamentous mutants, Δ*rbf1* and Δ*nrg1*. Finally, we found no evidence supporting prior claims that *C. albicans* only forms hyphae at very low biotin (0.1 nM) growth conditions.

Introduction

Candida albicans is a commensal fungus belonging to the skin and mucosal flora and one of the most common human pathogens, where it causes candidiasis in immunocompromised individuals [2]. One of the striking features of *C. albicans* is its ability to switch from budding yeast to filamentous form, which is highly correlated to its pathogenicity. This dimorphic switching is often assessed either in liquid culture or solid medium such as (i) Lee's medium which contains 8 amino acids [7], (ii) serum [45], (iii) buffered N-acetyl-D-glucosamine [50, 51], (iv) L-proline [52], and (v) glucose-phosphate-proline (GPP) [53], all at $\geq$ 37 °C. In addition, anaerobic growth gave hyphae at all temperatures tested from 25° to 37°C [54].

There are several environmental cues involved in the signaling transduction of hyphal formation of *C. albicans,* which in turns activates specific cellular networks [9]. The list of these environmental factors is still incomplete due to the complexity of triggering hyphal growth in *C. albicans.* Odds [45] listed 34 chemical and environmental factors which favored filamentous forms and 25 factors which favored yeast or blastospore forms and suppressed hypha formation. Comparison amongthe media used for inducing hyphal formation reveals that the biotin concentration is relatively high (40 times more than needed for growth) in Lee's medium [7]. As such, one could question whether biotin plays a role in inducing hyphal growth.

Here we tested whether biotin plays a role in triggering the hyphal growth in *C. albicans* by using various amounts of biotin supplemented in some of the common hyphal inducing media: Lee's medium, Serum, GPP and GlcNAc (Differentiation

medium). Clarification of a hyphal trigger which by itself can initiate hyphal development and an enhancer which improves the effectiveness of a hyphal trigger should be noted.

Methods

C. albicans strains and growth conditions

Wild-type *C. albicans* strain SC5314 was obtained from the American type culture collection, Rockville, MD and strain A72 was obtained from Prof. Patrick A. Sullivan, Otago Univ., NZ. CAF2-1 (ura3::imm434/URA3), CAI4 (ura3::imm434/ura3::imm434), JKC19 (ura3::imm434/ura3::imm434, cph1::hisG/cph1::hisG, URA3::hisG), HLC52 (ura3::imm434/ura3::imm434, efg1::hisG/efg1::hisG, URA3::hisG), HLC54 (ura3::imm434/ura3::imm434, cph1::hisG/cph1::hisG, efg1::hisG/efg1::hisG, URA3::hisG) [55] were obtained from Dr. Gerald R. Fink, Whitehead Institute at MIT, Massachusetts. CAI4-Ras1 (Constructed by transformation of DH326 with pLJ57), CAI4-Ras1^{G13V} (Constructed by transformation of DH332 with pDH240), CDH107 (ura3/ura3 ras1::hisG-URA3-hisG/ras1::hisG), ras1/ras1 +RAS1 (ura3::λimm434/ura3::λimm434 ras1::hisG/ras1::hisG:RAS1-URA3), and CR216 (ura3::λimm434/ura3::λimm434 cdc35::hisG-URA3-hisG/cdc35::hisG) [8] were from Prof. Deborah Hogan.

Cells were grown overnight in 250ml flasks with 50ml of either YPD (10 g of yeast extract, 20 g of peptone, and 20 g of glucose per liter) or/and GSB (a minimal defined glucose-salts-biotin medium) media at 30°C shaking at 225 RPM. At stationary

phase, the cells were harvested by centrifugation at 4200 rpm for 5 min, washed three times with 50 ml of wash buffer (3.5g KH_2PO_4, 4.1g K_2HPO_4 in 1L H_2O, pH 6.5), and then stored at 4°C for further use.

Analysis of germ tube formation

C. albicans cells were inoculated at 10^7 cells per ml in prewarmed 25 ml flasks containing 5 ml media supplemented with or without 0-10 µM biotin, desthiobiotin, 7-keto-8-aminopelargonic acid (KAPA), and lipoic acid (LA), and incubated with shaking (225 rpm) at 37°C for 4 hours. Media used were Lee's media [7], 1% serum, and GPP (a defined glucose-phosphate-proline medium of Kulkarni and Nickerson [32]. Every 30 mins or 2 hours, a sample was analyzed microscopically with a total of 100-300 cells counted to determine the percentage of cells that had formed germ tubes. Briefly, germ tube formation (GTF) is the initiation of hyphal elongation. Cells were tested in vitro with 2.5 mM N-acetylglucosamine (GlcNAc) at 37°C to be sure that their germ tube forming ability was close to 100% [53]. The effect of biotin in Lee's medium was also observed in separate experiments conducted with and without L-proline. Data from three independent experiments are presented with their average and standard deviation (std. dev.) values. Desthiobiotin was purchased from Santa Cruz Biotechnology, Santa Cruz CA, while KAPA was from Cayman Chemical Co., Ann Arbor, MI. These chemicals were prepared as 3mg/100ml stock solutions in distilled water and filter sterilized with a 0.2 um Whatman Syringe Filter.

Results

Biotin, desthiobiotin, KAPA and lipoic acid enhances hyphal formation in *C. albicans*

Filamentation-inducing media used to induce germ tube formation in *C. albicans* are diverse in their chemical composition. We examined the effect of high biotin concentration (4µM) in different filamentation media such as Lee's media, 1% serum, GPP (Fig 3.1). We also tested the ability of these cells to form germ tubes by using 2.5 mM GlcNAc in imidazole buffer. After 4 to 5 hours of incubation at 37°C, the cells were analyzed microscopically. We found that cells in media containing 4 µM biotin form germ tubes faster compared to media with 0-1µM biotin (Fig 3.1). Similar observation was found when 4 µM of lipoic acid or two of the biotin biosynthesis precursors, desthiobiotin and KAPA were added to the filamentation inducing media (Fig 3.2).

Biotin does not enhance hyphal formation under 0.5 mM GlcNAc.

It was found that increasing amount of GlcNAc also increases the rate of hyphal growth [6]. With 2.5 mM GlcNAc, high concentration of biotin does not increase the rate of GTF. It is possible GlcNAc increases the rate of GTF fast enough that it masked the effect from biotin. Thus, we lowered the GlcNAc concentration to 0.5 mM and mixed it with 0 or 4 µM biotin and recorded the germ tube formation within 4 hours. We found that there was no significance change on the GTF with 4 µM biotin (Fig 3.3).

Biotin enhances hyphal formation at the same rate with lower phosphate concentration in GPP media.

Phosphate can stimulate pseudohyphae formation when supplemented at a higher concentration, which is $\geq$ 100mM [56]. Thus, we examined if lowering the phosphate concentration in GPP could affect the germ tube formation at 37°C. We found that there are no changes in the rate of germ tube formation with lowered phosphate concentration from ~50 mM to 2 mM or 5 mM (Fig 3.4). However, when the temperature was increased to 42°C, the rate of germ tube formation drops as the rate of pseudohyphae formation increases (data not shown).

Urea amidolyase is required for biotin to enhance hyphal formation.

Suman et al (2009) [57] shown that the DUR1,2 (urea amidolyase) is a biotin dependent enzyme and required for hyphal formation when urea is used as the main nitrogen source. We tested the ability of *dur1,2* knockout mutants (KWN6) to form germ tubes when supplemented with high biotin (4 µM) in two different buffer, 2.6 mM GlcNAc and GPP (L-Proline replaced with 10 mM urea). KWN6 does not form germ tube when urea is used as the sole nitrogen sources (Fig 3.5), indicating that urea amidolyase is required for biotin to enhance the hyphal formation.

Hyphal associated transcription factors are required for biotin enhancing hyphal formation.

As shown previously, biotin enhanced the germ tube formation in *C. albicams*, however, biotin's mode of action is still unknown. One approach to this question is to test a series of hyphal defective and filamentous mutants to see if biotin could restore their hyphal forming ability. Accordingly, we examined the Δ*efg1,* Δ*cph1*, Δ*ras1,* Δ*cdc35,* Δ*nrg1,* Δ*rbf1,* Δ*rfg1* and Δ*tup1* deletion mutants of *C. albicans*. However, none of the hyphal defective mutants are able to form hyphae in 2.5 mM GlcNAc, Lee's media and 1% serum, either with or without 4 μM biotin. These experiments collectively show that high biotin acts before adenylyl cyclase and the two transcription factors for hyphal associated genes, Efg1p and Cph1p. The filamentous mutants were not affected by the biotin added in the same media tested, except for small changes of the clumping patterns of Δ*rbf1* and Δ*nrg1* (Fig 3.6).

Excess biotin effect on *C. albicans* strain 6713

Our data shows that high biotin stimulates GTF seems at variance with the work of Yamaguchi [31, 58, 59], who reported that GTF occurred at low biotin concentration of 0.1 – 0.4 nM. Yamaguchi's (1974) work was considered to be significance as it is cited for providing scientific proof biotin in preventing or curing *C. albicans* infections. The growth conditions mentioned by Yamaguchi [58] were in contradictory in a sense that the same temperature is usually used to achieve GTF. In his work [31, 58] cells of *C. albicans* 6713 were scraped from Sabouraud 2% glucose agar at 37°C and inoculated into

a glucose-ammonium salts medium (pH 5.5) at 29°C or 30°C, even though it is now agreed [45, 60] that an elevated temperature (37°C) is required for all hypha-inducing conditions. We replicated the experimental conditions as described by Yamaguchi [31] in all respects including the inoculum size. However, we found that low biotin is unable to enhance GTF with either *C. albicans* SC5314 or A72 at either 30 or 37°C. We were unable to obtain *C. albicans* 6713 since it is no longer available.

Discussion

This work demonstrates that ≥ 4 μM biotin, 40x more than the concentration needed for growth, enhances hyphal formation of *C. albicans* at 37°C. Even so, this effect only observable in hyphal-inducing media such as Lee's media and 1% Serum. Hyphal enhancement also noticeable when biotin is replaced with the same concentration of desthiobiotin, KAPA and lipoic acid [46]. However, when biotin is mixed with 2.5 mM GlcNAc in imidazole buffer, the rate of hyphal formation was not affected. The rate of hyphal growth induced by GlcNAc are concentration-dependent [6]. Thus, to confirm whether biotin effect on the germ tube formation is not masked by a high GlcNAc concentration, we lowered the GlcNAc concentration down to 0.5 mM. Nonetheless, biotin still did not affect the hyphal formation. This could imply that different signaling pathways are involved when biotin enhances hyphal formation with the present of inducers available in Lee's Media or Serum. In other word, for GlcNAc to induce hyphal growth, it needed to be first transported into the cells [6], which turns on different pathways that are not affected by biotin.

In this regards, we were also decided to screens mutants of morphogenetic regulators such as Δ*efg1,* Δ*cph1,* Δ*efg1/cph1,* Δ*ras1,* Δ*cdc35* (hyphal defective) and Δ*nrg1,* Δ*tup1,* Δ*rbf1,* Δ*rfg1* (filamentous) in respect to high biotin, and we found that with biotin supplemented in the hyphal inducing media, there was no significance differences other than both Δ*rbf1* and Δ*nrg1* formed loose clumps (Fig 3.6). Based on the result, both Efg1p and Cph1p are required for biotin to boost the hyphal growth considering that these are the major regulators [61].

Phosphate thought to have an effect of hyphal formation, so we decided to use lower phosphate concentration to check the ability of biotin in enhancing hyphal formation in GPP medium although we found that there was no difference with the rate of hyphal formation. This show that phosphate concentration in GPP medium does not affect the mycelial development, rather it affecting pseudohyphae development as shown by Hornby (2003) [56]. Hornby (2003) found that higher phosphate concentration will increase the rate of pseudohyphae formation while dropping the rate of mycelia formed by *C. albicans* A72; ie. at 400mM of phosphate, the rate of both pseudohyphae and mycelia will be at 50%. The same pattern was found when we used *C. albicans* SC5314 (data not shown).

In addressing a previous claim from Yamaguchi [31], we were unable to show the increase of GTF with low biotin concentration. It is possible that this phenomenon can only occur with the strain 6713 and not with the other *C. albicans* strains. For instance, *C. albicans* 6713 could have had reduced levels of heatshock protein 90 (Hsp90) because strains engineered with reduced Hsp90 levels form hyphae in serum at 30°C as well as

37°C [62]. Finally, it is also possible that *C. albicans* 6713 would now be reclassified as *C. dubliniensis*, a closely related species known to produce fewer hyphae under all induction conditions tested. These possibilities are not, of course, mutually exclusive.

In conclusion, we found that high biotin enhances germ tube formation in *C. albicans* given the right condition such as the present of hyphal inducer. Even so, when combining GlcNAc and biotin, there was no significance increase of hyphal growth rate. We can also clarify that the major regulators of hyphal growth such as Efg1p and Cph1p are needed for biotin to enhances GTF. However, the underlying mechanism of biotin enhancing GTF still need to be unravel by investigating more into the molecular aspects. For instance, studying the regulation of genes and factors when biotin is present as compared to absent of biotin will help in clarifying the global picture of cellular function.

Figure Legends

Figure 3.1 Biotin enhances hyphal formation in *C. albicans*.

Wild type C. albicans SC5314 cells were grown in YPD and then in GSB, both at 30°C until cells reached stationary phase**.** **(a)** Cells were inoculated in Lee's media with different biotin concentrations (0 nM, 100 nM, and 4 μM) at 37°C for 4 hours. **(b)** Cells were treated in Lee's Media containing 4 μM biotin, either with or without L-proline at 37°C for 4 hours. As a positive control, cells were treated with 2.5 mM N-acetyl glucosamine (GlcNAc) to test for the ability of forming 100% germ tube. **(c)** and **(d)** are tested in GPP and 1% serum, respectively. Observation at every 30 minutes are shown. Values shown are the average of triplicate experiments + SD.

Figure 3.2 Biotin biosynthesis precursors and Lipoic acid enhances hyphal formation in *C. albicans.*

Wild type C. albicans SC5314 cells were grown in YPD and then in GSB, both at 30°C until cells reached stationary phase**.** Cells were inoculated in Lee's media with 4 μM of KAPA (7-keto-8-aminopelargonic acid), desthiobiotin and lipoic acid at 37°C for 4 hours. As a positive control, cells were treated with 2.5 mM N-acetyl glucosamine (GlcNAc) to test for the ability of forming 100% germ tube. Observation at every 30 minutes are shown. Values shown are the average of triplicate experiments + SD.

Figure 3.3 Biotin does not enhance hyphal formation under 0.5 mM N-acetyl glucosamine (GlcNAc).

Wild type C. albicans SC5314 cells were grown in YPD and then in GSB, both at 30°C until cells reached stationary phase**.** Cells were treated in Imidazole with **(a)** 2.6 mM GlcNAc and **(b)** 0.5 mM GlcNac with different biotin concentrations (0 nM, 100 nM, 400 nM, and 4 µM) at 37°C for 4 hours. Observation at every 30 minutes are shown. Values shown are the average of triplicate experiments + SD.

Figure 3.4 Biotin enhances hyphal formation at the same rate with lower phosphate concentration in GPP media.

Wild type C. albicans SC5314 cells were grown in YPD and then in GSB, both at 30°C until cells reached stationary phase**.** Cells were treated in Glucose, Phosphate, Proline (GPP) with 4 µM biotin and different phosphate concentrations (2 mM, 5 mM, and 40 mM) at 37°C for 4 hours. Observation at every 30 minutes are shown. Values shown are the average of triplicate experiments + SD.

Figure 3.5 Urea amidolyase is required for biotin to enhance hyphal formation.

Urea amidolyase (Dur1,2p) is needed for urea utilization, converting urea to CO_2, which signals to hyphal formation [57]. Wild type SC5314, wild-type A72, KWN6 (*dur1,2/dur1,2*) strains were grown in YPD and then in GSB, both at 30°C until cells reached stationary phase**.** Cells were treated in **(a)** Glucose, Phosphate, Proline (GPP) or Glucose, Phosphate, Urea (GPU), also in **(b)** Imidazole buffer containing 2.6 mM

GlcNAc or Urea either with or without 4 μM biotin, incubated at 37°C for 4 hours. Observation at every 2 hours are shown. Values shown are the average of triplicate experiments + SD.

Figure 3.6 Hyphal associated transcription factors are required for biotin enhancing hyphal formation.

Signal transduction involve in hyphal specific genes expression requires several inducers [9]. Biotin enhancing hyphal formation requires several transcription factors such as *RAS1, EFG1,* and *CPH1*. High biotin concentration affects clumping pattern of hyphal corepressors mutants, Δ*nrg1* and Δ*rbf1*. Figure shown clumping pattern of these mutants in GPP media, supplemented without or with 4 μM biotin after 4 hours at 37°C.

Figure 3.7 Biotin effect on GTF of two different strains: SC5314 and published previously, 6713.

Yamaguchi's [58] work shown that mycelial growth can be achieved with lower biotin at 30°C, unlike other claims that hyphal growth are usually dominant at 37°C [60]. As such, we used commonly used *C. albicans* strains SC5314 and A72 using the same set up mentioned in Yamaguchi's work [31, 58, 59]. The figure shown that there was no significant increase of GTF both at 37°C (Fig 2.7) or 30°C (data not shown). The figure also shown that the dry-weight for cells supplemented without or with 0.4 nM and 4 nM biotin.

Figures

Figure 3.1 Biotin enhances hyphal formation in *C. albicans*.

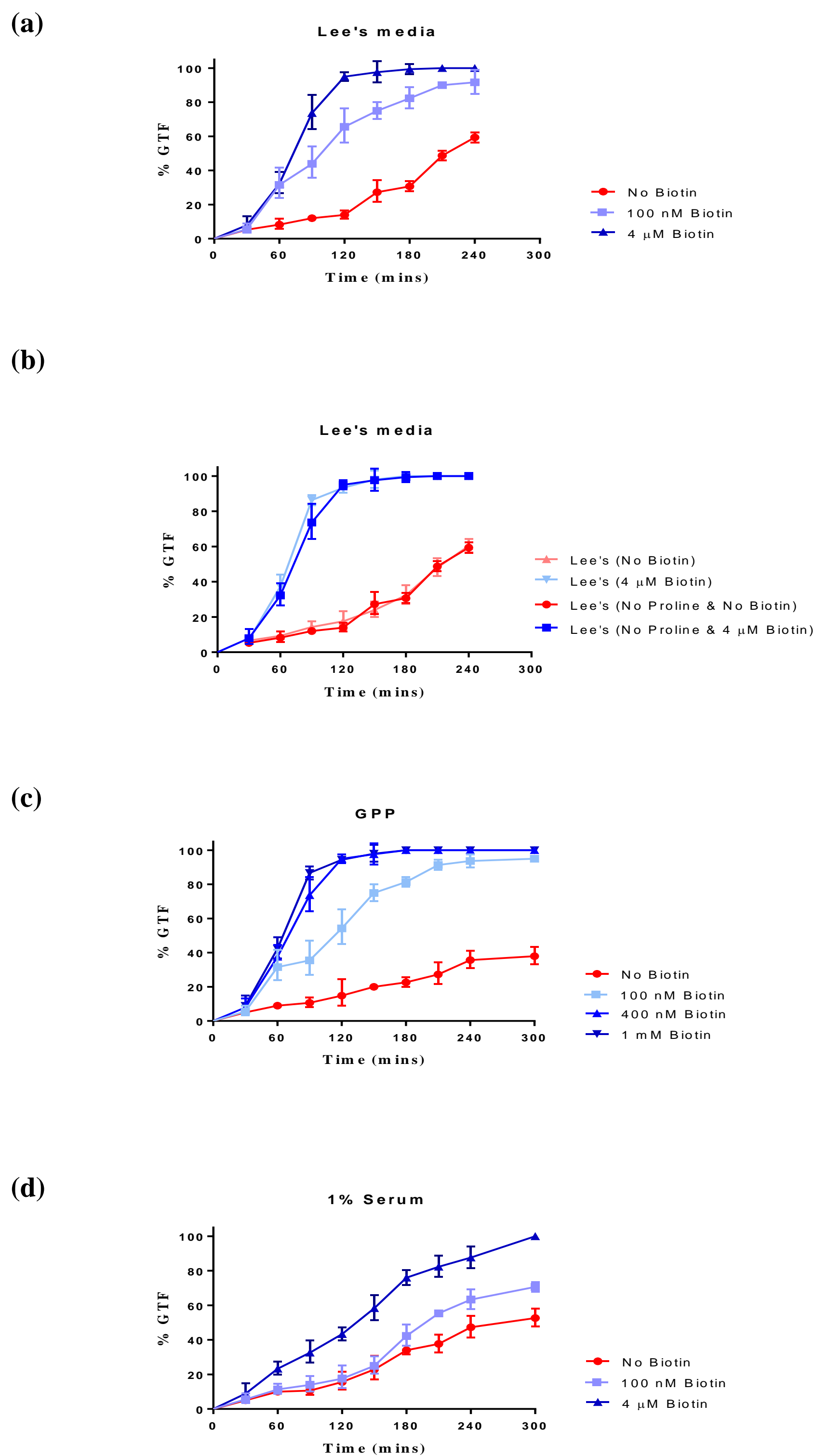
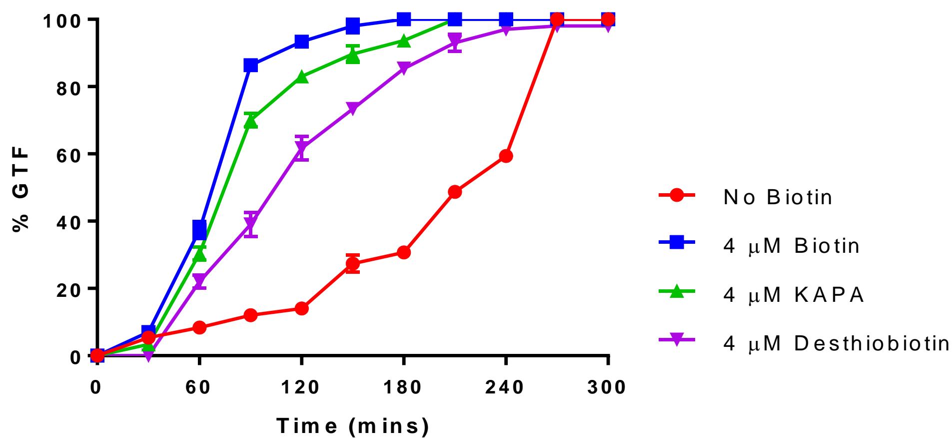


Figure 3.2 Biotin biosynthesis precursors and Lipoic acid enhances hyphal formation in *C. albicans*.



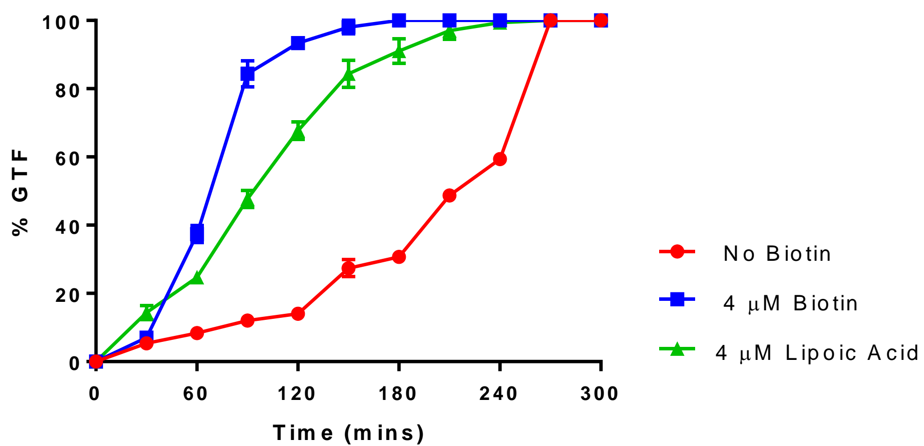
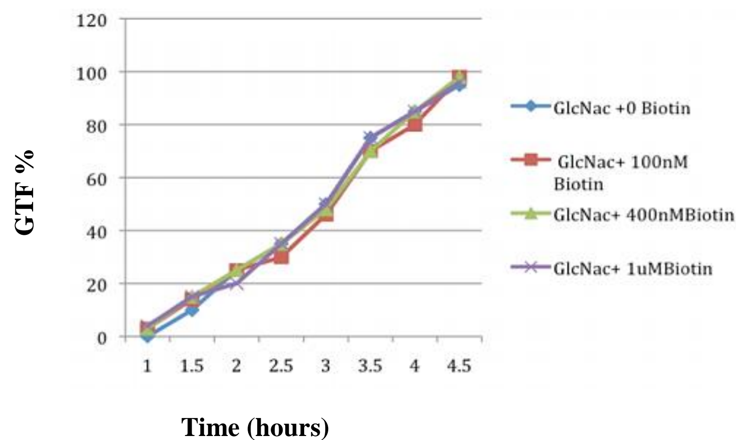


Figure 3.3 Biotin does not enhance hyphal formation under 0.5 mM N-acetyl glucosamine (GlcNAc).

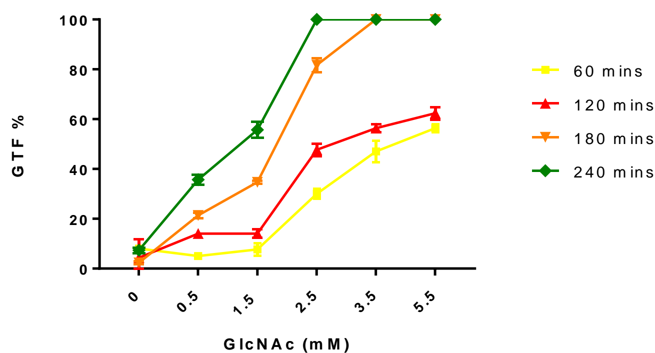
(a)



Time (hours)

Source: Tati, 2010 [1]

(b)



(c)

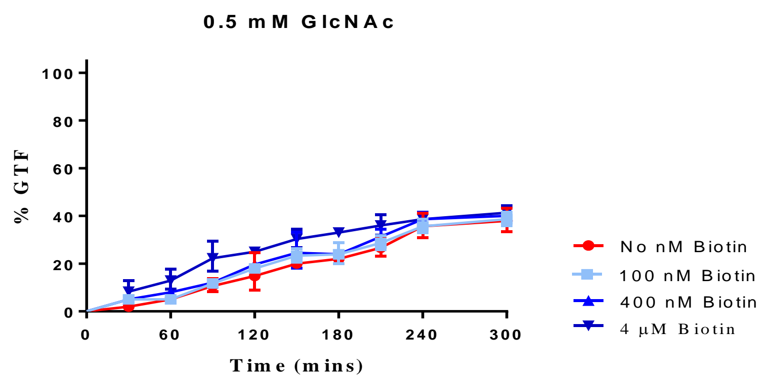


Figure 3.4 Biotin enhances hyphal formation at the same rate with lower phosphate concentration in GPP media.

(a)

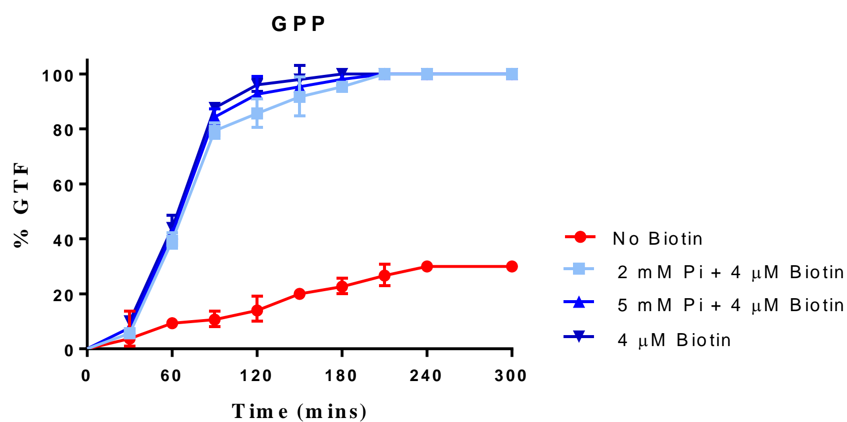
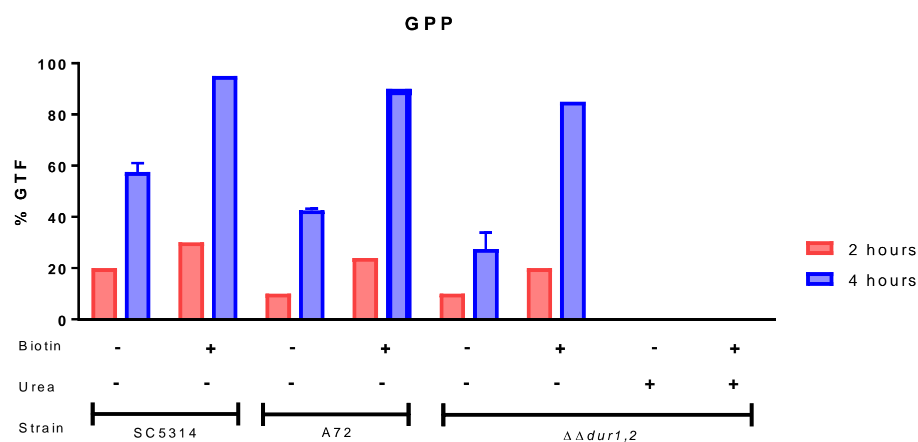


Figure 3.5 Urea amidolyase is required for biotin to enhance hyphal formation.

(a) GPP & GPU media with 4 µM Biotin



(b) 2.5 mM GlcNAc with 4 µM Biotin

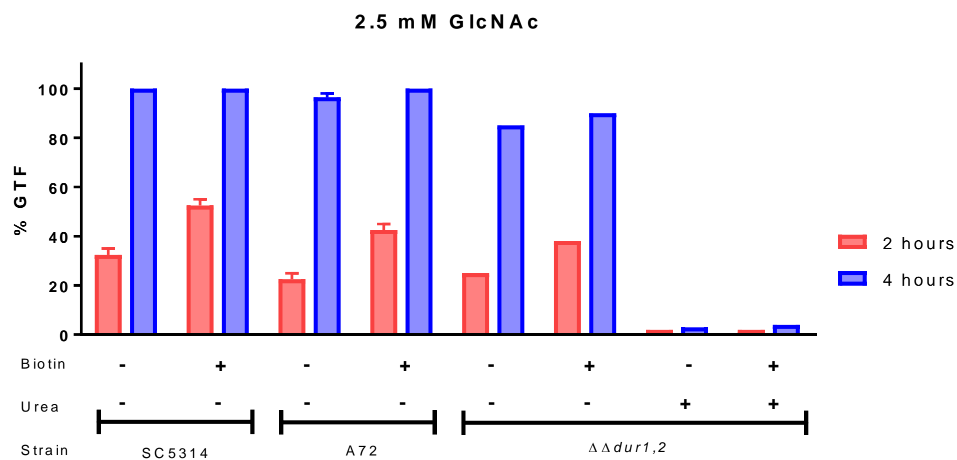


Figure 3.6 Hyphal associated transcription factors are required for biotin enhancing hyphal formation.

Source: Sudbery, 2011 [9]

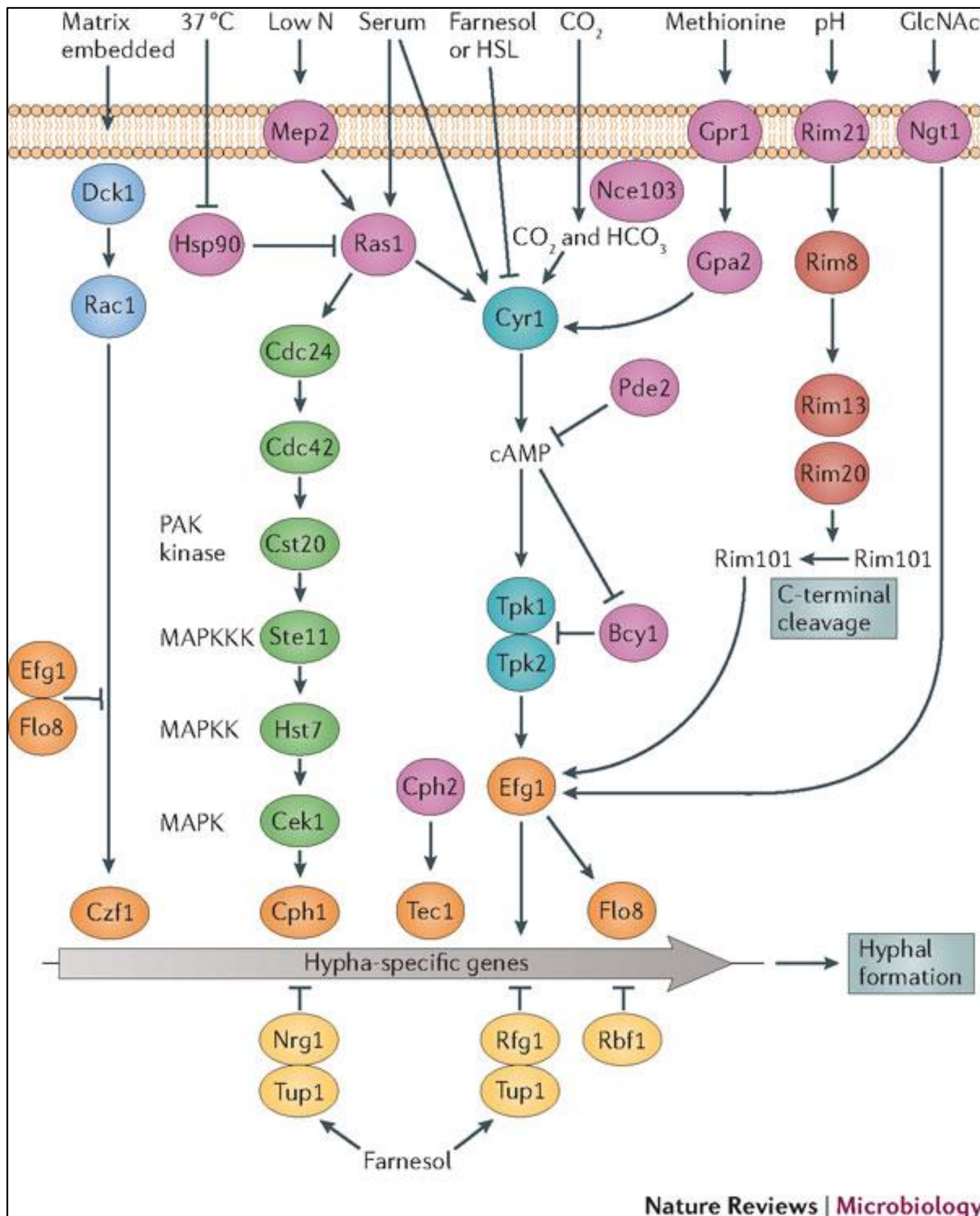


Figure 3.6 Hyphal associated transcription factors are required for biotin enhancing hyphal formation.

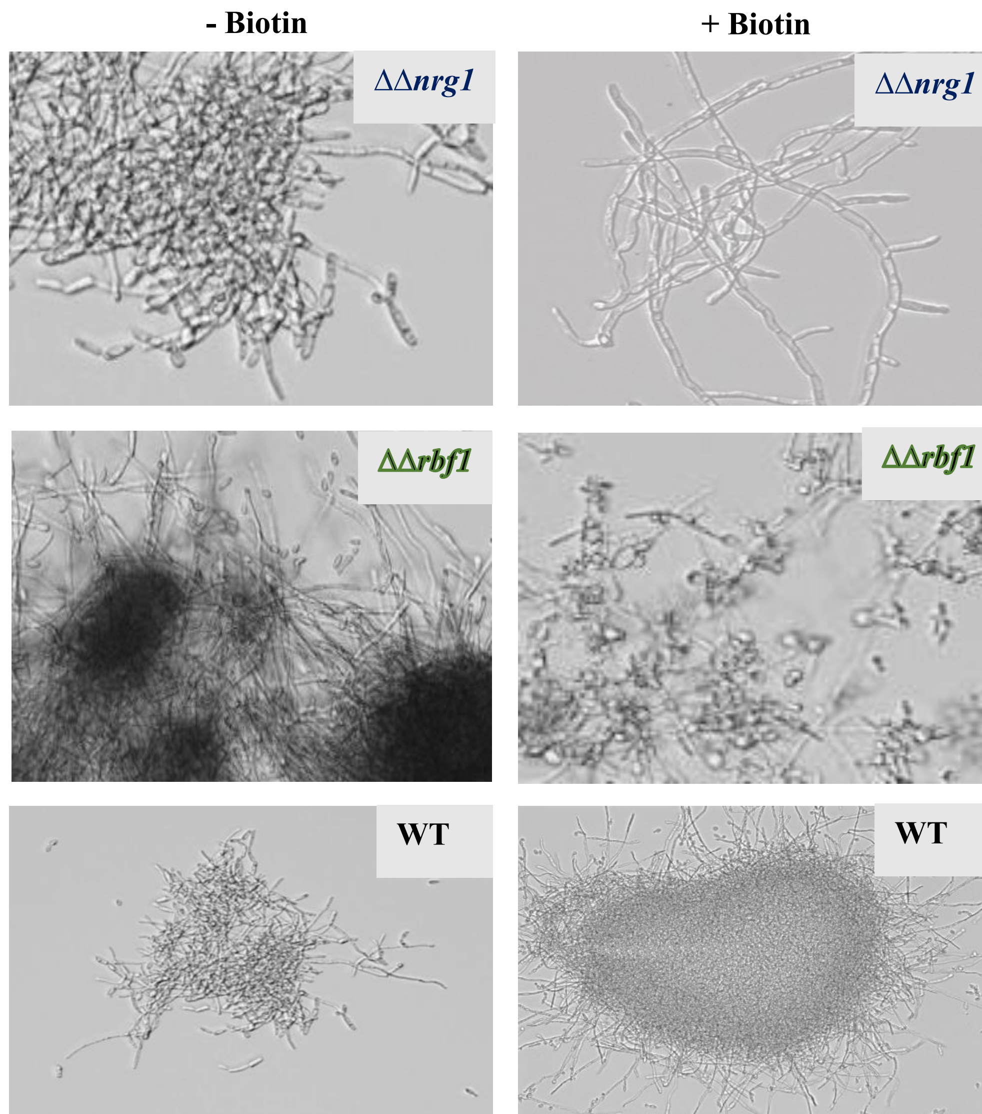
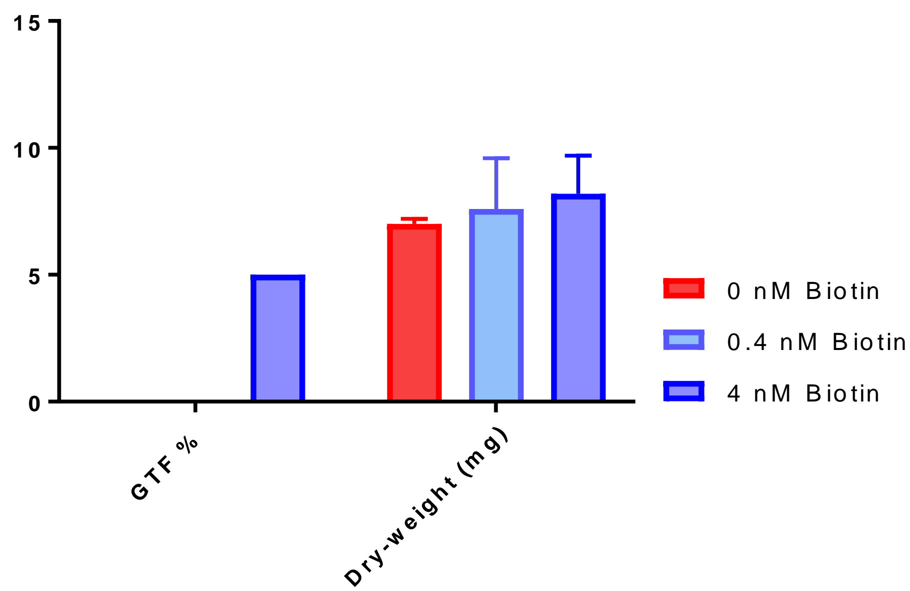


Figure 3.7 Biotin effect on GTF of two different strains: (a) SC5314 and (b) published previously, 6713.



CHAPTER 4

Screening *Saccharomyces cerevisiae* Tet-promoters yeast collection (yTHC) and future directions

Unpublished work

Introduction

Biotin (Vitamin H) is an important cofactor for a set of enzymes involved in metabolic processes. Most microorganisms have the ability to synthesize biotin, while other multicellular eukaryotic organisms, like *C. albicans*, are biotin auxotrophs. In spite of being a biotin auxotroph, *C. albicans* still possesses an incomplete biotin synthesis pathway. However, *C. albicans* requires greater amount of biotin than the amount it can synthesize, and thus, the cells should be able to utilize external biotin. To uptake biotin, the cells need a transport system which can be regulated accordingly. Regulation for the biotin transport can be the same as most transport system, where its activity will be dependent on the substrate availability [10]. Although little is known about *C. albicans* biotin transport, a comparable study can be made through its benign relative, *S. cerevisiae.* The biotin transport system on the cell membrane, VHT1 in yeast, has been identified and characterized [22]. However, there is still meagre evidence showing characteristics of biotin transport on the mitochondrial membrane, despite that it has been known as the final step in biotin synthesis occurs in the mitochondria. In some organisms, protein biotinylation also occurs in the mitochondria [63] which implies that biotin is incorporated into the mitochondria.

The genome-wide yeast deletion library revealed over 1000 protein-coding genes which were essential for the cell viability, although some of the genes function are yet to be defined. The complexity of these essential genes suggested a method of replacing their endogenous promoters with the Tet-promoters, such that the genes would be selectively and progressively turned off by increasing concentrations of doxycycline This collection

is referred as the Tet-promoters yeast collection (yTHC), from the Hughes Laboratory, University of Toronto [64]. It has over 800 essential yeast genes which can be regulated, ie. the gene expression will be turned off by the presence of doxycycline [65]. The mechanism behind the Tet-Off expression system is that doxycycline activate the repressors which then bind to the tetracycline-responsive promoter element [36] and transcription will be inactive.

As such, it will be intriguing to test whether some of these essential genes can be complemented by supplementing the cells with higher biotin/ lipoic acid concentrations. To find our hypothetical mitochondrial transport protein, we will first need to identify the strains that can only grow with excess biotin/ lipoic acid. Comparison analysis will allow us to understand biotin utilization of these strains in relation to specific cell functions. Thus, this experiment could lead us to discover the transport mechanism for biotin in mitochondria. In this study, we will screen yTHC with high concentrations of biotin and lipoic acid (structurally similar to biotin) supplemented in the media.

Methods

The yTHC strains were obtained from Wayne Riekhof's lab and duplicated by using sterilized 96-well replicators into fresh 96-well plate containing 100 µL of YPD media at 30°C for 16 -20 hours. For long term storage, 15% of sterilized glycerol was added to each well of the replica plate and kept at -80°C. SC media (2% agar) were prepared in Omni-Tray plates with the following treatments: i. SC media , ii. SC media with 4 µM biotin, iii. SC media with 4 µM lipoic acid, iv. SC media with 10 µg/ml doxycycline, v. SC media with 10 µg/l doxycycline and 4 µM biotin, vi. SC media with 10 µg/l doxycycline with 4 µM lipoic acid. The 96-well plate grown previously will be used as the inoculum, where the 96-well replicators will be used to stamp the cells from each well simultaneously from a 96-well plate onto the Omni-Tray of SC media. These plates then will be grown at 30°C for 4-5 days and the colony characteristics will be observed. Growth on SC media containing doxycycline and biotin or lipoic acid will be noted. Growth onto an Omni-Tray with SC-media containing the regular amount of biotin (2 µg/l) was used as a control.

To confirm the effect of biotin or lipoic acid on these collections, spot assays for each strain of interest will be conducted by using the same inoculum of the YPD containing 96-well plate. Serial dilutions of the inoculum for each strain will be done accordingly in 96-well plates containing SC media without doxycycline . Afterwards, each dilution of each strain will be transferred into plates containing SC media with the same six types of treatments as mentioned previously by using multichannel pipette.

Colony growth was observed after the third and fifth day. Any growth under SC media containing doxycycline and biotin or lipoic acid will be of interest.

*Supplementary figure 4.1 will be a summary of the methods used.

Results

In the first screening, we found five strains to be growing when supplemented with 10 µg/l doxycycline and high concentration (4 µM) of biotin or lipoic acid: CDC45 (SLD4), SMD3, YOR004W (UTP23), YHC1 (U1-C) and YGL098W (USE1/SLT1), as shown in Table 4. However, further confirmation through spot assay analysis showed that these strains were unable to grow when supplemented with excess biotin or lipoic acid. These negative results should be repeated at progressively lower doxycycline concentrations.

Discussion

From this study, we found that excess biotin or lipoic acid was unable to rescue the growth of any strains in the yeasts collection when the indicated gene was turned off by 10 µg/ml doxycycline. This is possibly due to the essentiality of these genes. The rationale for using excess biotin or lipoic acid is to undertand whether biotin uptake participate in the function of these essential genes. When we supply high biotin, we would assume the regulation of biotin uptake will take place depending on other environmental factors. In yeasts, *VHT1* (Vitamin H Transporter 1) will be responsible for

both of the import and export of biotin. It is regulated as in excess biotin, there will be lower amount of *VHT1* expressed [66]. As such, the *S. cerevisiae* cells might not have the high internal biotin concentrations, which in turn will not affect any post-modification of internal components of the cells [20]. In conclusion, another approach will be beneficial to elucidate the roles of excess biotin in yeasts, especially in *C. albicans*.

Future Directions

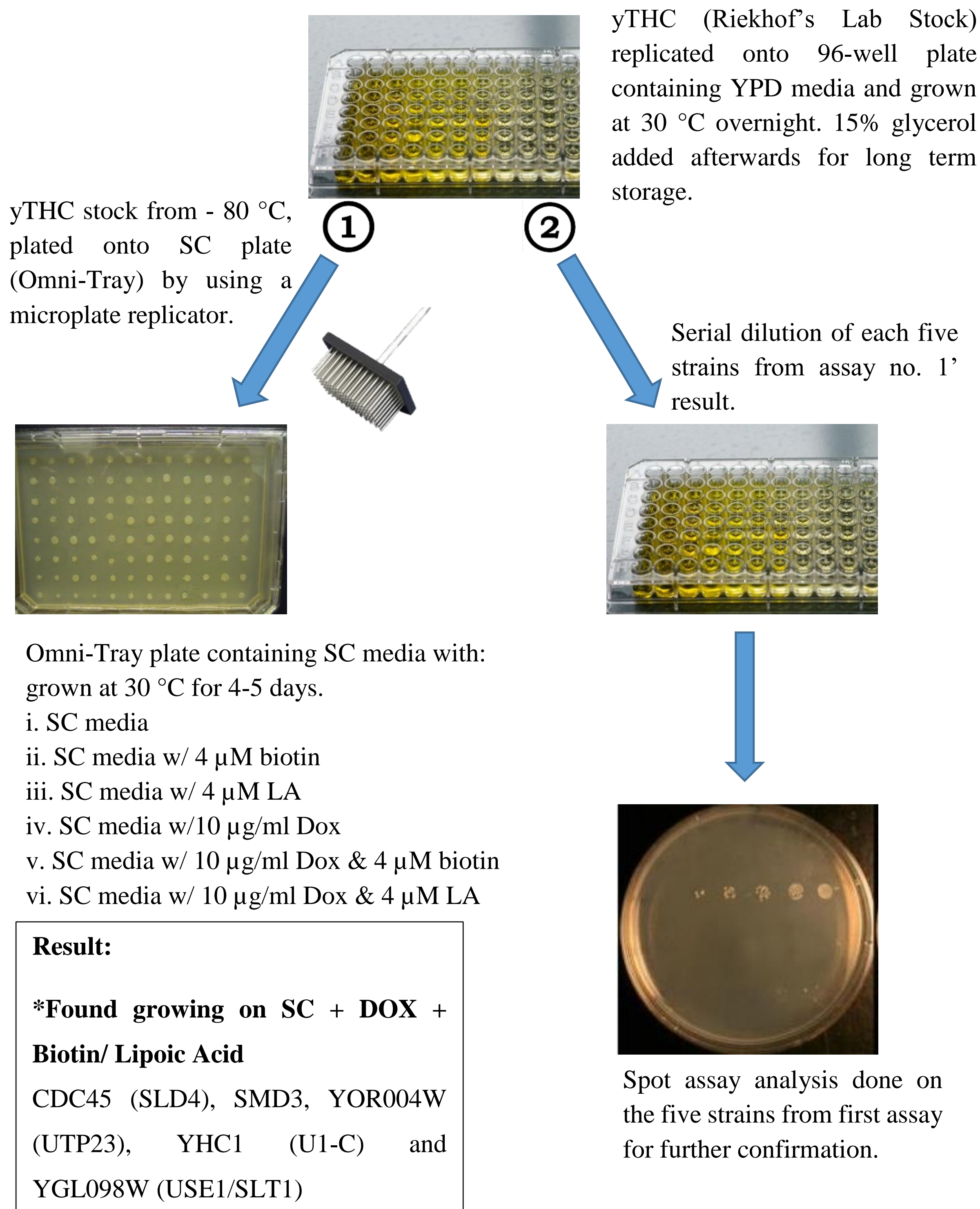
1. We have obtained the Homann collection [67] of transcription factor knockouts from the Fungal Genetics Stock Center. This collection contains 317 strains representing 155 transcription regulators (TRs). We will identify which TRs are necessary for high biotin GTF.
2. We have preliminary evidence from western blot analysis (Fig 3.3c) that biotinylated proteins were evidently higher in cells that had been growing with excess (4 µM) biotin than cells that were biotin-starved. Ocassionally, we observed resting cells to have less biotinylated proteins, which could indicate that essential biotin-dependent enzymes such as pyruvate carboxylase (PC) and acetyl-CoA carboxylase (ACC) are degraded when growth is concluded and then resynthesized when growth resumes. Alternatively, it could mean that biotinylation is reversible and growth can be turned on or off merely by controlling the presence or absence of bioin on these enzymes. To distinguish these possibilities, we need antibodies directed towards epitopes on PC and ACC other than the biotin attachment site. An antibody to *S. cerevisiae* PC was not reactive towards *C. albicans* PC (data not shown). Accordingly, we have ordered two antibodies (YenZym, San Francisco), which recognize 20 aa portions of *C. albicans* PC and ACC as predicted by their known sequences. These antibodies should be available in January 2017.
3. A plausible model for why such a high concentration of biotin is required is that we need to build up the cyctoplasmic biotin to a sufficintly high level that we force the biotin into another compartment. A likely candidate for such a compartment is the

mitochondrion. We have not found any information regarding how biotin gets in and out of mitochondria in yeasts, leading us to hypothesize how one would identify such a mitochondrial biotin transporter. This approach led us to the tet-off collection in *S. cerevisiae;* if we can identify a mitochondrial biotin transporter in *S. cerevisiae* then we could study its orthology in *C. albicans.*

Table 4.1 Five strains were identified to growth when supplemented with high concentration of biotin/ lipoic acid

Mutated Genes	Info obtained *Saccharomyces* Genome Database (SGD)[68]
CDC45 (SLD4)	It is a DNA replication initiation factor, elongation machinery recruiter, and it binds tightly to ssDNA.
SMD3	It is known as the Core Sm Protein Sm D3, and a part of the spliceosomal U1, U2, U4, and U5 snRNPs.
YOR004W (UTP23)	It is a component of the small subunit processome; involved in 40S ribosomal subunit biogenesis; interacts with snR30 and is required for dissociation of snR30 from large pre-ribosomal particles.
YHC1 (U1-C)	It is a component of the U1 snRNP complex required for pre-mRNA splicing.
YGL098W (USE1/SLT1)	Essential SNARE protein localized to the ER; involved in retrograde traffic from the Golgi to the ER and Sey1p-independent homotypic ER fusion; required for efficient nuclear fusion during mating; forms a complex with the SNAREs Sec22p, Sec20p and Ufe1p.

Supplementary Figure 4.1



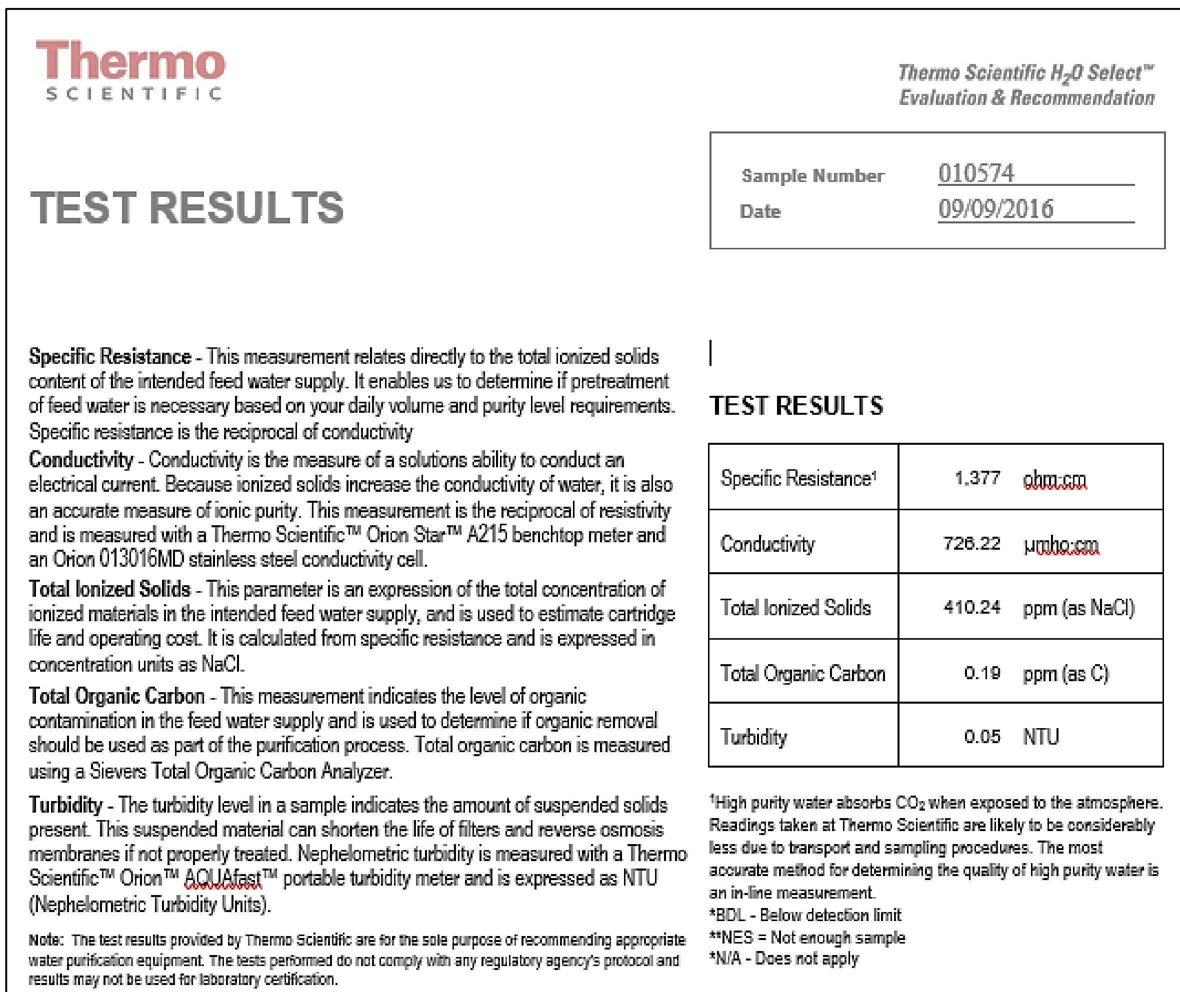
APPENDIX

A. Western blot analyses on cells that had been supplemented with biotin (4 µM) or biotin-starved in either growth [64] or filamentation inducing media (bottom). The positive control used is pyruvate carboxylase (125kDa). Consistent trend seen was smaller biotinylated proteins were observed more in cells grown with biotin.

B. Water analysis was run to check the endotoxin levels from water sources used in conducting germ-tubes differentiation assays.

Label	Water Sources	Endotoxin (EU/mL)	% GTF 4 hr
A	Super Saver (OLD)	10.4	60
B	Super Saver (Albumin-D. tube)	760	90
C	Super Saver (Filtered)	<0.097	85
D	Milli-Q (Dr. Atkin) Plastic Bottle	4.53	80
E	Milli-Q (Dr. Atkin) Glass Bottle	0.124	70
F	Milli-Q (Dr. Atkin) Filtered	<0.097	70
G	Milli-Q (East Campus)	16.9	70
H	Milli-Q (Othmer Hall)	<0.097	80
I	Always Save	<0.097	90
J	Protein Core	1.34	80
K	Distilled Water (Beadle)	0.781	70
L	Tap Water (Beadle)	1.84	65

C. Water analysis on the building distilled water (Beadle Center E150) was ran by Thermo Scientific, CA. This water source was used for conducting germ-tubes differentiation assays.



Thermo SCIENTIFIC

Thermo Scientific H_2O Select™ Evaluation & Recommendation

TEST RESULTS

Sample Number 010574
Date 09/09/2016

Specific Resistance - This measurement relates directly to the total ionized solids content of the intended feed water supply. It enables us to determine if pretreatment of feed water is necessary based on your daily volume and purity level requirements. Specific resistance is the reciprocal of conductivity

Conductivity - Conductivity is the measure of a solutions ability to conduct an electrical current. Because ionized solids increase the conductivity of water, it is also an accurate measure of ionic purity. This measurement is the reciprocal of resistivity and is measured with a Thermo Scientific™ Orion Star™ A215 benchtop meter and an Orion 013016MD stainless steel conductivity cell.

Total Ionized Solids - This parameter is an expression of the total concentration of ionized materials in the intended feed water supply, and is used to estimate cartridge life and operating cost. It is calculated from specific resistance and is expressed in concentration units as NaCl.

Total Organic Carbon - This measurement indicates the level of organic contamination in the feed water supply and is used to determine if organic removal should be used as part of the purification process. Total organic carbon is measured using a Sievers Total Organic Carbon Analyzer.

Turbidity - The turbidity level in a sample indicates the amount of suspended solids present. This suspended material can shorten the life of filters and reverse osmosis membranes if not properly treated. Nephelometric turbidity is measured with a Thermo Scientific™ Orion™ AQUAfast™ portable turbidity meter and is expressed as NTU (Nephelometric Turbidity Units).

Note: The test results provided by Thermo Scientific are for the sole purpose of recommending appropriate water purification equipment. The tests performed do not comply with any regulatory agency's protocol and results may not be used for laboratory certification.

TEST RESULTS

Specific Resistance[1]	1,377	ohm:cm
Conductivity	726.22	µmho:cm
Total Ionized Solids	410.24	ppm (as NaCl)
Total Organic Carbon	0.19	ppm (as C)
Turbidity	0.05	NTU

[1]High purity water absorbs CO_2 when exposed to the atmosphere. Readings taken at Thermo Scientific are likely to be considerably less due to transport and sampling procedures. The most accurate method for determining the quality of high purity water is an in-line measurement.
*BDL - Below detection limit
**NES = Not enough sample
*N/A - Does not apply

D. Multiple sequence alignment (ClustalW) analysis was done on *VHT1* (Vitamin H Transporter1) for *S. cerevisiae, C. albicans* and *S. pombe.* This comparison was done in order to identify similarities in domains between the three species.

S. cerevisiae
S. pombe
C. albicans

```
CLUSTAL multiple sequence alignment by MUSCLE (3.8)

sp|P53241|VHT1_YEAST                MTISNKSWRSYFPHLRKLPEDDQYLYSDDTNSSIIAEEELHHSVDKSSKTDVTAETTAVE
sp|O13880|VHT1_SCHPO                ---MASEW----------PETSRASSVEENPKLNIPEIVESVSDSKPSLKNQFSTTVIDS
tr|Q59UG7|Q59UG7_CANAL              -----------------------------------MSNSVSEAESRGHKSLEIEEYQIEE
                                                                        .     : ..   .         .

sp|P53241|VHT1_YEAST                PHPHNLRHDLPYEVRDEAGRKWWKYFDEFEYRVNKEYKKSRKWYEFLYPNHTTQTKAERR
sp|O13880|VHT1_SCHPO                SDLNVFNDGAETTVKEQ------EFTSSELRRLQKLRL-----------------KMDLR
tr|Q59UG7|Q59UG7_CANAL              YQIDNHTNDESSEILDP------------HQLNKVYR------------------KLDLR
                                            .    : :               .::*                    * : *

sp|P53241|VHT1_YEAST                LLYKLDIIIALYFFMLCWSKSVDLN-NYTNAYVSNMKEDLNMKGNDYVYTS-TIANVGAI
sp|O13880|VHT1_SCHPO                I---IPCLWILYFLSCCLRFTVSLSFTMNTAQGHSLIQTLSGYSAHYLALGLALFYVGYI
tr|Q59UG7|Q59UG7_CANAL              I---IPALWCLYFLTSFGSNCYGLTLTMNRAEGHSLAQQLHLTSKDTSTAS-ALYYVGYI
                                    :   :  :  ***:        .*. . . *   .: : *   .      . ::  ** *

sp|P53241|VHT1_YEAST                VFQLPFMYLLPRFPSHIILP--VMDLGWTWFTFACYRA-----NSLAELRAYRFILSAFG
sp|O13880|VHT1_SCHPO                IFEVPSNLMMAFIEPRIWVSRIQLTIG---VVGACHAVLGTKHGNAQSYVALRFFLGVAE
tr|Q59UG7|Q59UG7_CANAL              IFDVPMNLIMTKVSPQTWLARIVITVG---LVYTCYHVL----HNAGGVIAVRFISGVVG
                                    :*::*   ::. . ..  :.   : :*   .. :*: .      .     * **: ..

sp|P53241|VHT1_YEAST                AAYYPVSQYILGCWYAPDEINSRVCLFFCGQQLGSVTSGLLQSRIFKSLNGVHGLAGWRW
sp|O13880|VHT1_SCHPO                SGLWPGLAYYMSRWYRGKHLGKRIGWYYTAAQIAAAAVSLVSAG-FQKMDGARGLYGYQW
tr|Q59UG7|Q59UG7_CANAL              AGTWPGLSYYVSLWYPNDRSTRRIGYYFTAAQISAAVAGLVSAG-FQKMDGVHGYTGWQW
                                    :. :*   * :. **  .    *:  :: . *:.:...*:.:   *:.::*..*  *:.*

sp|P53241|VHT1_YEAST                MFLIDAIAISLPTAIIGFFVIPGVPSKC-------------YSLFLTDEEIRIARARNKR
sp|O13880|VHT1_SCHPO                MFLIWGV-VAIAQALSIPWWLPAVASKEHRKSLSSFIPLPKWMKTLSPQRIGFLTPADKS
tr|Q59UG7|Q59UG7_CANAL              MYLVYGV-ITMTVGIALLWWLPDRPFIGVKSESNRLVEF--YRKFTTPPHP--LNEQEKE
                                    *:*: .: :::. .:   : :*  .                :    :          :*

sp|P53241|VHT1_YEAST                NQIKDGVDKSKLAPLWSRKLWKKVFCTPAFWVLVV--FDTCSWNNMTAYSGSYTLWLKSN
sp|O13880|VHT1_SCHPO                LHSRYIAEMNVGKR-WQWSDLLKSCLDLRVWPFILMYFGIVGVGNGIFNYCT-LIIEEIN
tr|Q59UG7|Q59UG7_CANAL              LHRKDLEHRYKLLK-WTWKDVVNIITDLRIWPLIIMYFGVVGTGFGLAVFGS-TIIATNN
                                     : .           * ..   :      .* :::  *.  . .       :  :    *

sp|P53241|VHT1_YEAST                TKYSIAQVNNLSV---IPACLGFAYVIFCAFGADLFRCKWIFMVFAAIMNTVSCALLIKW
sp|O13880|VHT1_SCHPO                PSFSGIDISLLNAPIWLADALGI--VTVMPLYDRFHK-KFSFFTGSCLI-IIAGLAVANY
tr|Q59UG7|Q59UG7_CANAL              PNLTSIQVSLLYAPIWLFDLGGI--LIITPFADRFKKFRALIFSGACVI-IIVGMIVTTF
                                    .. :  ::. * .   :    *:  : . .:   : . .  ::  :.::  :    : .:

sp|P53241|VHT1_YEAST                DIPSKAKWYAFFTTYFSVAASP--CL-WSFINDFLRFDPQVKAITWIAIYSFSQ-----S
sp|O13880|VHT1_SCHPO                APRAWSRYGGLLMIGFGLGPTVPICMAWCSASMAKTYGDVGVASSLALVTGLGNLGSVVT
tr|Q59UG7|Q59UG7_CANAL              AHGSWNKYGGLLIAGFGLGPTVPICMSWSAEIFQKRYGDVGNAVSAALVSGLGNLGSVTS
                                       :. .: .::   *.:..:   *: *.       :.    * :   : .:.:     :

sp|P53241|VHT1_YEAST                TYAWIPTLAWPTVESPRFKTGYTVSLIFGAIYGLWTFVVLFFYKRNEKKHALGNGIILYD
sp|O13880|VHT1_SCHPO                TYALYSG--WPGD--PTFRKSNDVCIALIGVSIIACGIEFLLDKTGFGQFNASFNNHDHE
tr|Q59UG7|Q59UG7_CANAL              TYALYSG--WPEDKARLYRNSNMMLVLMLGVSIIASGVCQLI------------------
                                    ***  .   **      :...  : : : .:  :   :  ::

sp|P53241|VHT1_YEAST                SNKGEELPEFVKKNMEERDGYYYLKRSS
sp|O13880|VHT1_SCHPO                VEDEQEMTDIKPALPSSQQADA------
tr|Q59UG7|Q59UG7_CANAL              -KDKVRQPK-------------------
                                     :.    ..
```

Residue	Colour	Property
AVFPMILW	RED	Small (small+ hydrophobic (incl.aromatic -Y))
DE	BLUE	Acidic
RK	MAGENTA	Basic - H
STYHCNGQ	GREEN	Hydroxyl + sulfhydryl + amine + G
Others	Grey	Unusual amino/imino acids etc

- An * (asterisk) indicates positions which have a single, fully conserved residue.
- A : (colon) indicates conservation between groups of strongly similar properties scoring > 0.5 in the Gonnet PAM 250 matrix.
- A . (period) indicates conservation between groups of weakly similar properties scoring =< 0.5 in the Gonnet PAM 250 matrix.

```
Percent Identity  Matrix - created by Clustal2.1


  1: sp|P53241|VHT1_YEAST     100.00   16.51   21.56
  2: sp|O13880|VHT1_SCHPO      16.51  100.00   43.78
  3: tr|Q59UG7|Q59UG7_CANAL    21.56   43.78  100.00
```

REFERENCES

1. Tati, S., *Physiological Studies on Candida albicans*, in *Biological Sciences*. 2010, University of Nebraska-Lincoln.

2. CDC. *Fungal Diseases*. October 6, 2016 [cited 2016 October 30].

3. Gow, N.A., *A developmental program for Candida commensalism.* Nat Genet, 2013. **45**(9): p. 967-8.

4. Kron, S.J., C.A. Styles, and G.R. Fink, *Symmetric cell division in pseudohyphae of the yeast Saccharomyces cerevisiae.* Mol Biol Cell, 1994. **5**(9): p. 1003-22.

5. Berman, J. and P.E. Sudbery, *Candida Albicans: a molecular revolution built on lessons from budding yeast.* Nat Rev Genet, 2002. **3**(12): p. 918-30.

6. Alvarez, F.J. and J.B. Konopka, *Identification of an N-acetylglucosamine transporter that mediates hyphal induction in Candida albicans.* Molecular biology of the cell, 2007. **18**(3): p. 965-975.

7. Lee, K.L., H.R. Buckley, and C.C. Campbell, *An amino acid liquid synthetic medium for the development of mycelial and yeast forms of Candida Albicans.* Sabouraudia, 1975. **13**(2): p. 148-53.

8. Rocha, C.R., et al., *Signaling through adenylyl cyclase is essential for hyphal growth and virulence in the pathogenic fungus Candida albicans.* Mol Biol Cell, 2001. **12**(11): p. 3631-43.

9. Sudbery, P.E., *Growth of Candida albicans hyphae.* Nature Reviews Microbiology, 2011. **9**(10): p. 737-748.

10. Bayer, E.A. and M. Wilchek, *The biotin transport system in yeast.* Methods Enzymol, 1979. **62**: p. 371-8.

11. Hall, C. and F.S. Dietrich, *The reacquisition of biotin prototrophy in Saccharomyces cerevisiae involved horizontal gene transfer, gene duplication and gene clustering.* Genetics, 2007. **177**(4): p. 2293-307.

12. McVeigh, I. and E. Bell, *The amino acid and vitamin requirements of Candida albicans Y-475 and Mycoderma vini Y-939.* Bulletin of the Torrey Botanical Club, 1951: p. 134-144.

13. Pendini, N.R., et al., *Biotin protein ligase from Candida albicans: expression, purification and development of a novel assay.* Arch Biochem Biophys, 2008. **479**(2): p. 163-9.

14. Hoja, U., et al., *Pleiotropic phenotype of acetyl-CoA-carboxylase-defective yeast cells--viability of a BPL1-amber mutation depending on its readthrough by normal tRNA(Gln)(CAG).* Eur J Biochem, 1998. **254**(3): p. 520-6.

15. Hasslacher, M., et al., *Acetyl-CoA carboxylase from yeast is an essential enzyme and is regulated by factors that control phospholipid metabolism.* J Biol Chem, 1993. **268**(15): p. 10946-52.

16. Hoja, U., et al., *HFA1 encoding an organelle-specific acetyl-CoA carboxylase controls mitochondrial fatty acid synthesis in Saccharomyces cerevisiae.* J Biol Chem, 2004. **279**(21): p. 21779-86.

17. Brewster, N.K., et al., *Regulation of pyruvate carboxylase isozyme (PYC1, PYC2) gene expression in Saccharomyces cerevisiae during fermentative and nonfermentative growth.* Arch Biochem Biophys, 1994. **311**(1): p. 62-71.

18. Navarathna, D.H., et al., *Dur3 is the major urea transporter in Candida albicans and is co-regulated with the urea amidolyase Dur1,2.* Microbiology, 2011. **157**(Pt 1): p. 270-9.

19. Kim, H.S., et al., *Identification of the tRNA-binding protein Arc1p as a novel target of in vivo biotinylation in Saccharomyces cerevisiae.* J Biol Chem, 2004. **279**(41): p. 42445-52.

20. Hasim, S., et al., *Histone biotinylation in Candida albicans.* FEMS Yeast Res, 2013. **13**(6): p. 529-39.

21. Fitzpatrick, D.A., et al., *Analysis of gene evolution and metabolic pathways using the Candida Gene Order Browser.* BMC Genomics, 2010. **11**: p. 290.

22. Rogers, T.O. and H.C. Lichstein, *Regulation of biotin transport in Saccharomyces cerevisiae.* J Bacteriol, 1969. **100**(2): p. 565-72.

23. Stolz, J., et al., *Identification of the plasma membrane H+-biotin symporter of Saccharomyces cerevisiae by rescue of a fatty acid-auxotrophic mutant.* J Biol Chem, 1999. **274**(26): p. 18741-6.

24. Izumi, Y., Y. Tani, and K. Ogata, *Microbiological biosynthesis of biotin.* Methods Enzymol, 1979. **62**: p. 326-38.

25. Kögl, F. and B. Tönnis, *Über das Bios-Problem. Darstellung von krystallisiertem Biotin aus Eigelb. 20. Mitteilung über pflanzliche Wachstumsstoffe*, in *Hoppe-Seyler´s Zeitschrift für physiologische Chemie*. 1936. p. 43.

26. Suomalainen, H. and E. Oura, *Yeast nutrition and solute uptake*, in *The yeasts*, A.H. Rose and J.S. Harrison, Editors. 1971. p. 3-74.

27. Wu, H., K. Ito, and H. Shimoi, *Identification and characterization of a novel biotin biosynthesis gene in Saccharomyces cerevisiae.* Appl Environ Microbiol, 2005. **71**(11): p. 6845-55.

28. Ohsugi, M. and Y. Imanishi, *Microbiological activity of biotin-vitamers.* J Nutr Sci Vitaminol (Tokyo), 1985. **31**(6): p. 563-72.

29. Phalip, V., et al., *Characterization of the biotin biosynthesis pathway in Saccharomyces cerevisiae and evidence for a cluster containing BIO5, a novel gene involved in vitamer uptake.* Gene, 1999. **232**(1): p. 43-51.

30. Firestone, B.Y. and S.A. Koser, *Growth promoting effect of some biotin analogues for Candida albicans.* J Bacteriol, 1960. **79**: p. 674-6.

31. Yamaguchi, H., *Effect of biotin insufficiency on composition and structure of cell wall of Candida albicans in relation to its mycelial morphogenesis.* J Gen Appl Microbiol, 1974. **20**: p. 271-228.

32. Kulkarni, R.K. and K.W. Nickerson, *Nutritional control of dimorphism inCeratocystis ulmi.* Experimental Mycology, 1981. **5**(2): p. 148-154.

33. Kulkarni, R.K. and K.W. Nickerson, *Nutritional control of dimorphism in Ceratocystis ulmi.* Experimental Mycology, 1981. **5**(2): p. 148-154.

34. Kucsera, J., K. Yarita, and K. Takeo, *Simple detection method for distinguishing dead and living yeast colonies.* J Microbiol Methods, 2000. **41**(1): p. 19-21.

35. Wood, V., et al., *The genome sequence of Schizosaccharomyces pombe.* Nature, 2002. **415**(6874): p. 871-80.

36. Madsen, C.T., et al., *Biotin starvation causes mitochondrial protein hyperacetylation and partial rescue by the SIRT3-like deacetylase Hst4p.* Nat Commun, 2015. **6**: p. 7726.

37. Kohrer, K. and H. Domdey, *Preparation of high molecular weight RNA.* Methods Enzymol, 1991. **194**: p. 398-405.

38. Pendrak, M.L., et al., *Heme oxygenase in Candida albicans is regulated by hemoglobin and is necessary for metabolism of exogenous heme and hemoglobin to alpha-biliverdin.* J Biol Chem, 2004. **279**(5): p. 3426-33.

39. Fukasawa, Y., et al., *MitoFates: improved prediction of mitochondrial targeting sequences and their cleavage sites.* Mol Cell Proteomics, 2015. **14**(4): p. 1113-26.

40. Savojardo, C., et al., *TPpred2: improving the prediction of mitochondrial targeting peptide cleavage sites by exploiting sequence motifs.* Bioinformatics, 2014. **30**(20): p. 2973-4.

41. Emanuelsson, O., et al., *Predicting subcellular localization of proteins based on their N-terminal amino acid sequence.* J Mol Biol, 2000. **300**(4): p. 1005-16.

42. Samols, D., Thornton, C.G., Murtif, V.L., Kumar, G.K., Haase, F.C. and Wood, H.C. , *Evolutionary conservation among biotin enzymes.* J Bol Chem 1988. **263**: p. 6461-6464.

43. Bahn, Y.S. and F.A. Muhlschlegel, *CO2 sensing in fungi and beyond.* Curr Opin Microbiol, 2006. **9**(6): p. 572-8.

44. Sims, W., *Effect of carbon dioxide on the growth and form of Candida albicans.* J Med Microbiol, 1986. **22**(3): p. 203-8.

45. Odds, F.C., *Candida and candidosis: a review and bibliography* in *Journal of Basic Microbiology*. 1988, W.B. Saunders Company / Bailliere Tindall. p. 382-383.

46. Ahmad Hussin, N., et al., *Biotin Auxotrophy and Biotin Enhanced Germ Tube Formation in Candida albicans.* Microorganisms, 2016. **4**(3).

47. Arnal, N., et al., *The Arabidopsis Bio2 protein requires mitochondrial targeting for activity.* Plant Mol Biol, 2006. **62**(3): p. 471-9.

48. Zakikhany, K., et al., *In vivo transcript profiling of Candida albicans identifies a gene essential for interepithelial dissemination.* Cell Microbiol, 2007. **9**(12): p. 2938-54.

49. Lan, C.Y., et al., *Regulatory networks affected by iron availability in Candida albicans.* Mol Microbiol, 2004. **53**(5): p. 1451-69.

50. Mattia, E., et al., *Induction of germ tube formation by N-acetyl-D-glucosamine in Candida albicans: uptake of inducer and germinative response.* J Bacteriol, 1982. **152**(2): p. 555-62.

51. Simonetti, N., V. Strippoli, and A. Cassone, *Yeast-mycelial conversion induced by N-acetyl-D-glucosamine in Candida albicans.* Nature, 1974. **250**(464): p. 344-6.

52. Land, G.A., et al., *Factors affecting filamentation in Candida albicans: relationship of the uptake and distribution of proline to morphogenesis.* Infect Immun, 1975. **11**(5): p. 1014-23.

53. Hornby, J.M., et al., *Quorum sensing in the dimorphic fungus Candida albicans is mediated by farnesol.* Appl Environ Microbiol, 2001. **67**(7): p. 2982-92.

54. Dumitru, R., J.M. Hornby, and K.W. Nickerson, *Defined anaerobic growth medium for studying Candida albicans basic biology and resistance to eight antifungal drugs.* Antimicrob Agents Chemother, 2004. **48**(7): p. 2350-4.

55. Lo, H.J., et al., *Nonfilamentous C. albicans mutants are avirulent.* Cell, 1997. **90**(5): p. 939-49.

56. Hornby, J.M., *Quorum Sensing and The Regulation of Morphology in the Dimorphic Fungus Candida albicans*, in *Biological Sciences*. 2003, University of Nebraska-Lincoln: Lincoln, Nebraska. p. 141.

57. Ghosh, S., et al., *Arginine-induced germ tube formation in Candida albicans is essential for escape from murine macrophage line RAW 264.7.* Infection and immunity, 2009. **77**(4): p. 1596-1605.

58. Yamaguchi, H., *Mycelial development and chemical alteration of Candida albicans from biotin insufficiency.* Sabouraudia, 1974. **12**(3): p. 320-8.

59. Yamaguchi, H., *Dimorphism in Candida albicans .I. Morphology-dependent changes in cellular content of macromolecules and respiratory activity.* J Gen Appl Microbiol, 1974. **20**: p. 87-99.

60. Sudbery, P.E., *Growth of Candida albicans hyphae.* Nat Rev Microbiol, 2011. **9**(10): p. 737-48.

61. Doedt, T., et al., *APSES proteins regulate morphogenesis and metabolism in Candida albicans.* Mol Biol Cell, 2004. **15**(7): p. 3167-80.

62. Shapiro, R.S., et al., *Hsp90 orchestrates temperature-dependent Candida albicans morphogenesis via Ras1-PKA signaling.* Curr Biol, 2009. **19**(8): p. 621-9.

63. Tissot, G., et al., *Purification and properties of the chloroplastic form of biotin holocarboxylase synthetase from Arabidopsis thaliana overexpressed in Escherichia coli.* Eur J Biochem, 1998. **258**(2): p. 586-96.

64. Hughes, T.R., et al., *Functional discovery via a compendium of expression profiles.* Cell, 2000. **102**(1): p. 109-126.

65. Baron, U. and H. Bujard, *Tet repressor-based system for regulated gene expression in eukaryotic cells: principles and advances.* Methods Enzymol, 2000. **327**: p. 401-21.

66. Pirner, H.M. and J. Stolz, *Biotin sensing in Saccharomyces cerevisiae is mediated by a conserved DNA element and requires the activity of biotin-protein ligase.* J Biol Chem, 2006. **281**(18): p. 12381-9.

67. Homann, O.R., et al., *A phenotypic profile of the Candida albicans regulatory network.* PLoS Genet, 2009. **5**(12): p. e1000783.

68. Cherry, J.M., et al., *Saccharomyces Genome Database: the genomics resource of budding yeast.* Nucleic Acids Res, 2012. **40**(Database issue): p. D700-5.