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Analyzing the Role of a Protein Downregulated After Induction of Filamentous Growth In *Candida albicans*

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Abstract

Candida albicans is a commensal fungus, normally living with its human host, however, it has the ability to cause invasive infection. *Candida albicans* is the fourth most frequent nosocomial infection affecting a vulnerable immunocompromised population. *Candida albicans* exhibits different morphologies including yeast, pseudohyphae, and hyphae. The varying morphological potential of this organism is a virulence trait. Because of this, research has focused on what drives activation of hyphal formation as well as what impedes it. During a filamentation assay, a novel observation pertaining to a subgroup of proteins being downregulated early after germination, was made. In this study, we constructed an over-expression strain of one of these proteins and have used known hypha inducing media to determine if it will have an impact on filamentation. With the conditions tested thus far, no significant impacts on morphology have been observed.

Candida albicans is a commensal fungus, normally colonizing the gastrointestinal tract and other mucosal membranes of its human host. Yet, *Candida* has virulence potential associated with different environmental cues affecting signal transduction pathways, making *Candida* an opportunistic pathogen as well. Changes in host immunity and complex environmental factors play a large role in *C. albicans* transformation from commensal to pathogen. *Candida* is able to change its phenotypic state between a single celled yeast to filamentous, hyphal or pseudohyphal, morphologies under varying circumstances. There are superficial types of *Candida* infections. Such infections are prevalent in AIDS patients whom frequently suffer from oropharyngeal infections known as oral thrush (1–3). Or, it is also known that 75% of women will suffer some form of *Candida* related vulvogenital infection at least once in their lifetime, impacting their quality of life (4). *Candida* albicans is a top cause of nosocomial infections worldwide (5). In fact, there are about 400,000 bloodstream infections worldwide with a 46-75% mortality rate and 10,000 deaths in the United States alone, all attributed to a more severe form of *Candida* bloodstream infection (Reviewed in (6)). The high mortality rate of this organism makes understanding the associated virulence mechanisms paramount.

Aside from the high mortality rate, the associated prevalence of *Candida* infections is important. A reason for rise in fungal infections can be associated with the growing immunocompromised populations in a hospital setting, a side effect of the medical advancements granting an increased volume of patients with hospital stays. Those advancements include organ transplantation, stem cell transplantation, cancer therapies such as chemotherapy, premature infant deliveries and care, and implanted medical devices (7). These medical treatments compromise the immunity of patients by some degree enabling *Candida* bloodstream infections. Treatment difficulties have arisen as drug resistance has occurred in virulent strains of *candida* (8). Treatment difficulties are also exacerbated

by the lack of efficacy in detecting techniques from cultured based diagnostic tests typically used in detecting fungal infections; it is reported that about 50% of invasive candidiasis cases were missed by this way of detection (9).

C. albicans has developed strategies in order to survive in different niches of a human host and cause disease. A highlight here would be the ability of this organism to undergo morphological switching under varying circumstances (10). Although the importance of morphological switching as a virulence trait was accepted, there wasn't a manner of testing whether or not this theory was 'true' in experimental studies until a group constructed a strain that could be regulated between yeast and filamentous growth form (11). This study was the first time ever where the theory of morphological switching being necessary for virulence, was proven evident. Morphology becomes important during biofilm formation, another virulence trait of *C. albicans*. Biofilms are formed when yeast cells adhere to a surface, commonly medical implanted devices like heart valves or pacemakers, followed by hyphal morphogenesis, and eventually the film forms in a population of filamentous and yeast cells which produce polysaccharides, proteins and nucleic acids (12–14). When biofilms form, they have the potential of impairing the function of the devices they form on, having the correct surface to adhere to, as well as maintaining the ability to sustain infection. (15).

Environmental factors responsible for activation of *Candida albicans* hyphal growth include: presence of serum, neutral pH, 5% CO_2, and low nitrogen (10, 14, 16, 17). These environmental cues activate important pathways such as the mitogen activated protein kinase (MAPK), cyclic adenosine monophosphate (cAMP), calcineurin, and HOG pathways (10, 18). However, activation of hyphal growth must be accompanied by downregulation of filament repressors, such as Nrg1 (14, 19).

Previous work in the lab identified a group of proteins that were seen to be degraded early during filamentation when induced in 37°C, a known activation factor of hyphal formation. This finding

presented the question if the presence of these proteins was necessary for morphogenesis from yeast form to filamentous growth, as well as what their true function is. The focus of this study is one of these proteins. However, this protein remains uncharacterized and is called C1_05590_C. The primary way of analyzing this protein was by increasing its expression levels followed by testing over-expression strains under the influence of known filamentation activating cues. This allowed for the determination of whether or not this protein influences the transition from yeast to filamentous growth. Furthermore, the importance of deciphering events of morphological switching involves the better understanding of this phenomenon to drive clinical applications directed in reducing the high mortality rates associated with *Candida* infections.

RESULTS AND DISCUSSION

Wild-type strain SC5314 was used as a control and was grown along *sa*-C1_05590_C mutants from the same cell line. There were no significant differences between the two mutant strains and the wild-type strain when grown in different liquid media. When grown in yeast conditions (28 °C) in liquid YPD, all cells remained in yeast form. When grown in filamentous inducing conditions corresponding to YPD, GLcNAc, YNB, RPMI, and Spider media, filamentation was clearly observed amongst all streaked colonies examined. This is reflective of the growth conditions of the mutants not being affecting differently in any significant pathways involved in the morphological switching from yeast to filamentous growth form than the wild type strain.

When strains were grown on solid media under filamentous growth conditions, again, filamentous growth was observed in all strains. For example, a distinct crenulation on spider media is a typical

macroscopic outcome of SC5314 and this was maintained in the mutant strains macroscopic analysis. Invasion into the media was also apparent amongst all strains examined as washing of the cells demonstrated this, indicating again, no impact on filamentous growth form observed in the mutant strains tested. Lee's medium contains a variety of amino acids necessary for induction of filamentous growth from yeast form growth. Similarly, Spider medium is known to induce filamentous growth based on carbon availability. Lastly, GlcNAc is known to stimulate activating pathways of hyphae inducing outcomes, such as activating the cAMP signaling pathway.

METHODS AND MATERIALS

Entailed in this study was the construction of a *Candida albicans* vector in order to insert, clone, and express proteins of interest to analyze their effect under conditions where filamentous growth is known to be activated. These constructs were made up of CIpSAT-SA, the CIp component being a modified version of the CIp10 vector, created specifically for *Candida* integration. The SAT component was introduced to CIp10, encoding a nourseothricin resistance and replacing the URA component of CIp10. Finally, the SA component of CIpSAT-SA is the shorter of the two Actin promoters, which are constitutively on, ensuring the maintenance of elevated levels of C1_05590_C in mutant strains.

However, the protein of interest was first designed into a pMiniT which does not integrate well into *Candida* cells. Xho1 and Mlu1 enzymes allowed for a sub-cloning of C1_05590_C from a pMiniT vector into a CIpSAT-SA construct. Once the final transformation was done, the remainder

of the experiment involved culture assays as the primary way of attempting to characterize the role and function of this novel protein.

Strains and Growth Conditions

C. albicans strain SC5314 and mutant strains *sa*-C1_05590_C were grown on yeast-peptone-dextrose (YPD) agar in 28 °C. The mutant strain was engineered as follows: 5'-Xho1-AUG-His6-C1_05590_C-Myc-TAA-Mlu1-3' in a pMiniT plasmid. The candida plasmid CIp10 was reengineered to replace the URA component with a SAT component encoding nourseothricin resistance for selectivity, as well as incorporating the short act promoter (SA) allowing for constitutive production of the protein of interest. The name of this new construct is CIpSAT-*sa*, maintaining a Xho-1 and Mlu-1 cutsite. The enzymes Xho-1 and Mlu-1 were used to cut out the designed protein from pMiniT and to linearize CIpSAT-*sa*. The insert and linearized CIpSAT-*sa* were ligated, followed by a Stu-1 digestion to prepare the DNA for an electroporation transformation into SC5314 cells (20, 21). To ensure the insert integrated into CIpSAT-*sa*, a double digestion using Xho-1 and Mlu-1 was done and the DNA lengths of the insert-C1_05590_C and CIpSAT-*sa* empty plasmid were compared using gel electrophoresis. After transformation by electroporation was performed, the plated colonies were picked and patched onto another plate. This allowed for colonyPCR followed by gel electrophoresis to confirm that the correct product was growing on the patched plate. Once gel electrophoresis on the double digested DNA and colonyPCR confirmed the correct transformant was present, a YPD plate was streaked with two transformants and a WT strain.

Filamentation Assay

Two approaches were taken for filamentation assays. One approach was by done by streaking YPD grown strains onto plates of YPD, YPD+Serum, Spider, and SLAD. All but SLAD were placed in 37 °C to induce filamentation. SLAD was placed in 28 °C. A second approach involved liquid media. A 1mL aliquot was taken, washed twice with water, and resuspended in water to dilute (1:20) into fresh media including YPD, Spider, Lee's, GLcNAc, RPMI, and YNB with shaking at 37 °C for three hours. Following, microscopy was used to visualize the cells for phenotypic comparison.

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September 6, 2019

David Martin, PhD
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Dear Dr. David Martin,

I will soon have a Bachelor of Science in Biochemistry from Grand Valley State University and I am currently a Ronald E. McNair Scholar at this institution. My regard for the sciences has led me to investigate a component of the organism *Candida albicans* in the Thomas-Cleary Lab, an established laboratory in the biomedical sciences department. Under their direction I have used a microbiological approach in uncovering some research questions regarding an uncharacterized protein in the organism.

In my research I used a microbiological approach to examine the impact of the uncharacterized protein, C1_05590_C, on filamentation. Morphogenesis is an important virulence trait of *C.albicans*. This fungal microorganism lives as a commensal in most of the human population, however, is a known opportunistic pathogen. As a pathogen, *Candida* is a top nosocomial infection, impacting already vulnerable populations. In my recent unpublished article, *Analyzing the Role of a Protein Downregulated After Induction of Filamentous Growth in Candida Albicans,* I explain how I conducted this research. This paper entails two focuses: One questioning what this proteins impact is during morphological switching in *Candida*, and two, my attempt to uncover what this proteins function in the organism is. To do this, I overexpressed the protein by placing it in a vector with a constitutive promoter, allowing for constitutive production of it. This was followed by testing the constitutive mutant under conditions known to induce filamentation.

Infection and Immunity is a journal within the American Society of Microbiology and is fitting for my article because *Candida* is known to cause a deep-seated infection. My article focuses on a novel protein possibly associated with an important virulence trait of *Candida* infections. The impact of this protein remains enigmatic as it has not been observed to evade morphological switching with the media it has been analyzed with. Its function also remains a mystery as the mutant has not been observed to function differently than the wild-type strain. The research I have conducted will bring attention to this uncharacterized protein, for others to continue understanding the underlying mechanisms intrinsic to the virulence trait of phenotypic switching. I have contributed to a starting point, where someone else who is intrigued, can carry on. The only other known information about this novel gene is given by computational predictions.

Thank you,

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