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Potential Role of RTA3 and GNP3 Transport Genes in the Quorum Sensing Response of Candida Albicans

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Potential Role of RTA3 and GNP3 Transport Genes in the Quorum Sensing Response of *Candida albicans*

By

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Abstract

Quorum sensing (QS) is a mechanism of communication between cells to coordinate biological activities. In the opportunistic fungal pathogen *Candida albicans,* QS is important for regulating the organism's ability to change morphology from a yeast form to a hyphal form. Previously, our laboratory identified several genes involved in polymicrobial communication between *C. albicans* and three bacterial species. We hypothesize that interspecies communication and QS utilize the same genetic pathways to control *C. albicans* morphological behavior. In this study, two transport genes, *RTA3* and *GNP3,* and their involvement in QS were examined. To test whether these two genes participate in QS, a filamentation assay was performed using *C. albicans* wild type strain SC3514, along with CHK21, and the haploinsufficient strains *hRTA3* and *hGNP3*. The cells were washed, then incubated in the presence or absence of *tran,trans*-farnesol, cyclic di-GMP, or pyocyanin at 37°C for 2.5 hr in M199, pH 7.5 to induce filamentation. When exposed to farnesol, the *hGNP3* mutant behaved more like the non-responder (CHK21), while the *hRTA3* mutant showed a filamentation rate similar to that of the wild type (SC5314). When exposed to cyclic di-GMP, all four samples reacted almost identically, showing a mild inhibition of filamentation. Pyocyanin was not found to impact filamentation of *C. albicans.* In addition to QS-regulated filamentation, the mutants were evaluated for their ability to form biofilms in the presence or absence of farnesol. The impact of farnesol on biofilm formation for both haploinsufficient mutants (*hRTA3* and *hGNP3*) fell between the ranges for the positive and negative controls. Biofilm assays utilizing cyclic di-GMP showed an increase in biofilm formation for all four samples, indicating an increase in overall biofilm mass despite the decreased filamentation seen in filamentation experiments.

Introduction

Candidiasis

Candidiasis is a broad term referring to a disease state resulting from microbial infection by a member of the genus *Candida.* These infections can manifest themselves in numerous ways, causing a very diverse range of pathologies. *Candida* typically infect mucosal membranes, resulting in disease states such as thrush, onychomycosis, or urinary tract infections (Kabir, 2012). An estimated 75% of females will experience a case of vulvovaginal candidiasis at least once in their lifetime, with 40-50% of those women experiencing at least one recurrence (Sobel, 2007). Additionally, potentially life-threatening systemic infections have been observed, with mortality rates as high as 38% (Kibbler, 2003). Once the fungus enters the bloodstream, it is capable of colonizing many of the internal organs, as many of its virulence traits involve very strong adherence to many tissue types.

Candidiasis has been widely considered a nosocomial infection, and most cases are observed in patients with intravenous catheters or on respiratory ventilators. In some cases, the medical equipment introduces the pathogen to the bloodstream, but in others the infection begins when a previously commensal cell becomes virulent. In these cases, *Candida albicans* is either persorbed through epithelial cells or germinates through intestinal microvilli, allowing a disseminated infection to begin (Chauhan, 2006).

As an opportunistic infection, the majority of cases occur in patients who are somehow immunocompromised, such as the very young, very old, patients taking immunosuppressive drugs or undergoing chemotherapy, and individuals with AIDS. To cause disease, *Candida* strains produce a number of virulence factors that enable them to adhere to body surfaces, destroy

tissues, and evade the host immune system (Raska, 2007). Over the years, the incidence of candidiasis caused by each member of the *Candida* genus has varied, but the overall incidence and outcomes has remained the same for the better part of two decades (Pfaller, 2007).

Candida albicans

Candida albicans is an opportunistic fungal pathogen that can be found in the gut and urinary tracts of around 70% of healthy adults (Shulze, 2009). Though this organism exists in the gut without causing disease under typical immune conditions, immunosuppression can cause this typically commensal microbe to act as a dangerous pathogen. *C. albicans* can be isolated from approximately 60% of all infections with *Candida* species, making it a very important subject of study for researchers attempting to develop new treatment options (Pfaller, 2007).

Crucial to this organism's ability to cause disease is its ability to spontaneously and reversibly change phenotype (Soll, 2014). Depending on a number of external factors, *C. albicans* can shift from a yeast-like form, which is normally associated with a commensal relationship, to a hyphal form, a filamentous shape more commonly associated with invasive infections. These factors include temperature, pH, available nutrition, and cell density (Figure 1).

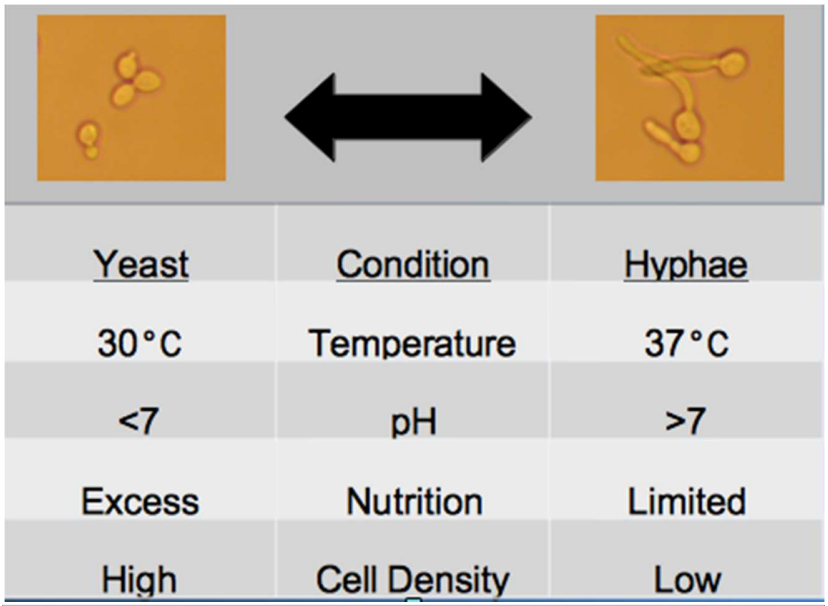


Figure 1: Factors impacting *Candida albicans* morphology

The ability to shift between a yeast and hyphal form has been associated with many biological processes in *C. albicans*. Gene expression, adhesion, hydrophobicity, frequency of recombination, presentation of surface antigens, and pathogenicity have all been associated with this organism's ability to change phenotypes (Soll, 2014). An intermediate phase between these two clearly defined forms, known as a pseudohyphal form, is commonly observed in laboratory settings. The yeast form, which is typically not associated with disease, reproduces via budding or binary fission to produce other yeast cells. When grown under filamentation conditions, hyphal cells grow out of yeast through production of germ tubes. After germ tube formation, a septum forms to separate the hyphal and yeast cell, creating a true hyphal cell (Gow, 1997). Pseudohyphal cells reproduce via a budding mechanism, and many of the cells remain attached to one another. Examples of these types of morphologies can be seen in Figure 2.

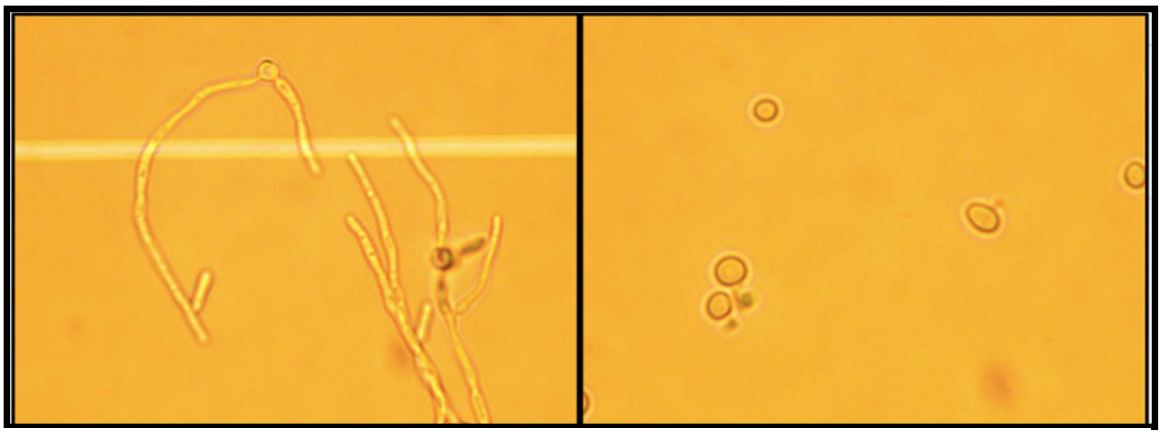


Figure 2: Example hyphal (left) and yeast (right) morphologies

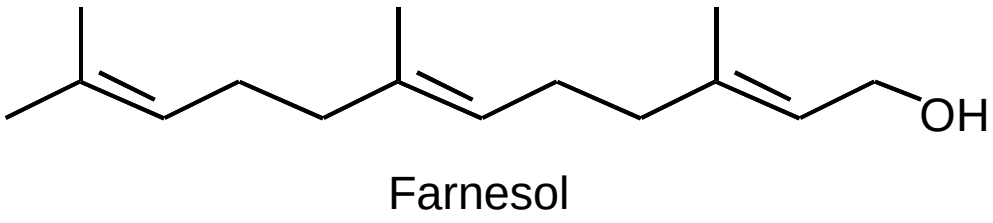
Quorum Sensing

In *C. albicans,* the morphological shift between yeast and hyphal forms is controlled via quorum sensing (QS). In QS, each cell produces QS molecules, and, upon reaching a certain concentration, certain effects are achieved. These molecules are small, diffusible, and their effects are varied depending on the specific organism with which they are interacting. The effects of QS molecules vary, from processes like biofilm formation to bioluminescence, but each action is similar in that it can only be achieved when enough of the participating cells work together. One of the more dramatic and commonly cited quorum sensing phenomena is the bioluminescence of marine bacteria *Vibrio harveyi* and *Allivibrio fischeri*, which does not take place until enough cells can "light up" simultaneously and be seen (Bassler, 2002).

Initially, the molecules phenylethyl alcohol and tryptophol were isolated from growths of *C. albicans,* and though they were found to inhibit growth at certain concentrations, further research proved they did not impact germ tube formation (Hogan 2006). Years later, farnesol was revealed to be a major quorum sensing molecule for *C. albicans.* When this molecule is present at sufficient concentration, germ tube formation is inhibited, resulting a phenotypic

shift toward yeast cells (Nickerson, 2006). A growth density of greater than 10^6 cells per milliliter is typically sufficient to ensure formation of yeast cells, with lower concentrations resulting in primarily hyphal growth. Though farnesol can block formation of new germ tubes, the molecule does not prevent elongation of pre-existing germ tubes (Mosel, 2005). Additionally, the commitment to either growth morphology due to the action of farnesol is very temporary and cells can be induced to shift towards either morphology within 2 hours – typically during stationary phase (Nickerson, 2006).

A second molecule, tyrosol, has also been shown to act as a quorum sensing molecule to induce the formation of hyphal cells (Chen, 2004). When transferred to a minimal growth medium, *C. albicans* experiences a significant "lag phase" before beginning its normal growth cycle, but addition of tyrosol eliminates this delay. The opposing actions of tyrosol and farnesol point to an intricate system of positive and negative control that exists between the two. The structure of farnesol can be seen in Figure 3.



Farnesol

Figure 3: Structure of Farnesol

The exact mechanisms for the export and uptake of farnesol are, as of yet, unknown,

though the ability to exploit these pathways represents an exciting possibility for the production of future treatment options. Exploitation of quorum-sensing pathways to attenuate virulence is an elegant solution that has been previously studied in other opportunistic pathogens for which conventional treatments are often ineffective. For example, farnesol has been previously shown to inhibit formation of PQS, a quorum-sensing molecule in *P. aeruginosa,* which indirectly impacts multiple virulence traits (LaSarre, 2013).

Basis of Study

Transport genes *RTA3* and *GNP3* have been previously established to play a role in the import or export of other molecules in *C. albicans.* These transport genes were further explored using haploinsufficient mutants of each gene to determine their potential role in quorum sensing responses. Additionally, the molecules cyclic di-GMP and pyocyanin were tested due to *C. albicans'* response to other microbes when growing in a biofilm. *C. albicans* strain CHK21 was included as a negative control, as it has been previously established to ignore the presence of farnesol.

Materials and Methods

Media

Yeast Peptone Dextrose (YPD) medium – 20g dextrose, 20g peptone, 10g yeast extract in 1 liter distilled water. Autoclaved to sterilize. Plates were made by adding 20g bacto agar prior to autoclaving.

Medium 199 – 9.5g M199, 18.7g Tris Buffer in 1L distilled water, then pH adjusted to 7.5. Medium was filter sterilized.

BHI (Brain Heart Infusion) agar medium – 37g Brain Heart Infusion, 18g agar, and 50mg kanamycin in distilled water. Autoclaved to sterilize.

Yeast strains

Procedures for this study utilized *C. albicans* wild type SC5314, null mutant CHK21 (*chk21,chk21)*, and haploinsufficient mutants *hRTA3* (*rta3,RTA3)* and *hGNP3 (gnp3,GNP3)*.

Molecule preparation

Farnesol - 3mM *trans,trans*-farnesol was prepared in ethanol. This solution was made fresh weekly and stored at 4°C to prevent degradation of the farnesol. 1µL of this solution was used per 1 mL of media, making a working solution of 300µM.

Cyclic diguanylate (c-di-GMP) – stock 2 mg per ml solution was prepared. Experiments utilized 50 µl of stock c-di-GMP solution per ml of M199, yielding a final concentration of 100 µg per ml.

Pyocyanin – 50 mM stock solution was diluted to a 50 µM working solution.

Concentration was increased ten-fold twice during experimentation.

Standard growth conditions

Each strain of *C. albicans* was maintained at 30°C on Petri plates containing YPD agar, and stored at 4°C. Samples were inoculated into 5 mL liquid YPD medium overnight at 30°C with rotary shaking. Cells were harvested by centrifugation (5000 rpm), washed 3 times with sterile distilled water, then diluted to concentrations necessary for the type of experiment being performed.

Filamentation assay procedure

To induce hyphal development, 5 x 10^5 cells were added to 1mL Medium 199, pH 7.5, and incubated for 2.5 hours at 37°C. To determine if the strains responded to 300µM farnesol, 100 µg/mL cyclic di-GMP, or various concentrations of pyocyanin, each of the previously mentioned compounds was utilized per mL of medium. A total of 8 microcentrifuge tubes were prepared per molecule, 2 tubes per strain, with 1 tube acting as a control and the other tube containing the molecule of interest in order to observe the response. The molecules were added at time 0, just before cells were introduced to hyphal developing conditions. Samples were centrifuged after incubation to concentrate the cells for counting and morphological determination using a Neubauer chamber.

Biofilm assay procedure – Farnesol

Serial dilution used to create $10^3 - 10^6$ cells/mL dilutions. For each dilution, a 100µL

inoculum was placed in a 96-well plate pre-coated with 5% fetal calf serum. Cells were allowed to adhere for 2 hours to the well bottom at 37°C. The medium was removed and unattached cells removed by washing with 100µL dH2O, and then 100µL fresh M199 was added to each well. For half of the samples, the fresh M199 contained the molecules of interest (300µM farnesol, 100 µg/mL cyclic di-GMP). The plate was then incubated at 37°C for 48 hours to allow for biofilm development, after which the excess media was removed and biofilms were washed with 100µL dH2O to remove unattached cells, then treated with 100µL of methanol for 10 min. For staining of the biofilms, we followed the protocol of Peeters et al. Briefly, methanol was then removed and plates were dried for 20 minutes, then stained with 100µL of .00757% Crystal Violet. After staining for 20 minutes, excess Crystal Violet was removed, and 150µL of 33% acetic acid was added to each well to lyse the cells and release the dye. Optical density of the resulting liquids was measured at 590nm. Biofilm studies using pyocyanin were not performed due to the molecule's lack of impact on filamentation.

Results

Effects of farnesol on filamentation of mutants *hRTA3* and *hGNP3:*

Tests were conducted to determine the role of transport genes *RTA3* and *GNP3*. Their roles were explored via exposure to three types of molecules: farnesol, cyclic di-GMP, and pyocyanin. Haploinsufficient strains *hRTA3* and h*GNP3*, along with a positive and negative control, were placed in conditions that normally induce filamentation. A control tube for each strain, and another tube containing farnesol, was observed to note the molecule's effect, most importantly the response of the haploinsufficient mutants. Farnesol greatly inhibited filamentation in the SC5314 strain, but the effect was much less dramatic for CHK21, which largely ignored the presence of farnesol. Haploinsufficient mutant *hRTA3* showed a percent filamentation similar to the wild type, but the *hGNP3* mutant filamented more strongly. Both haploinsufficient showed a difference in percent filamentation between the positive and negative controls (Figure 4).

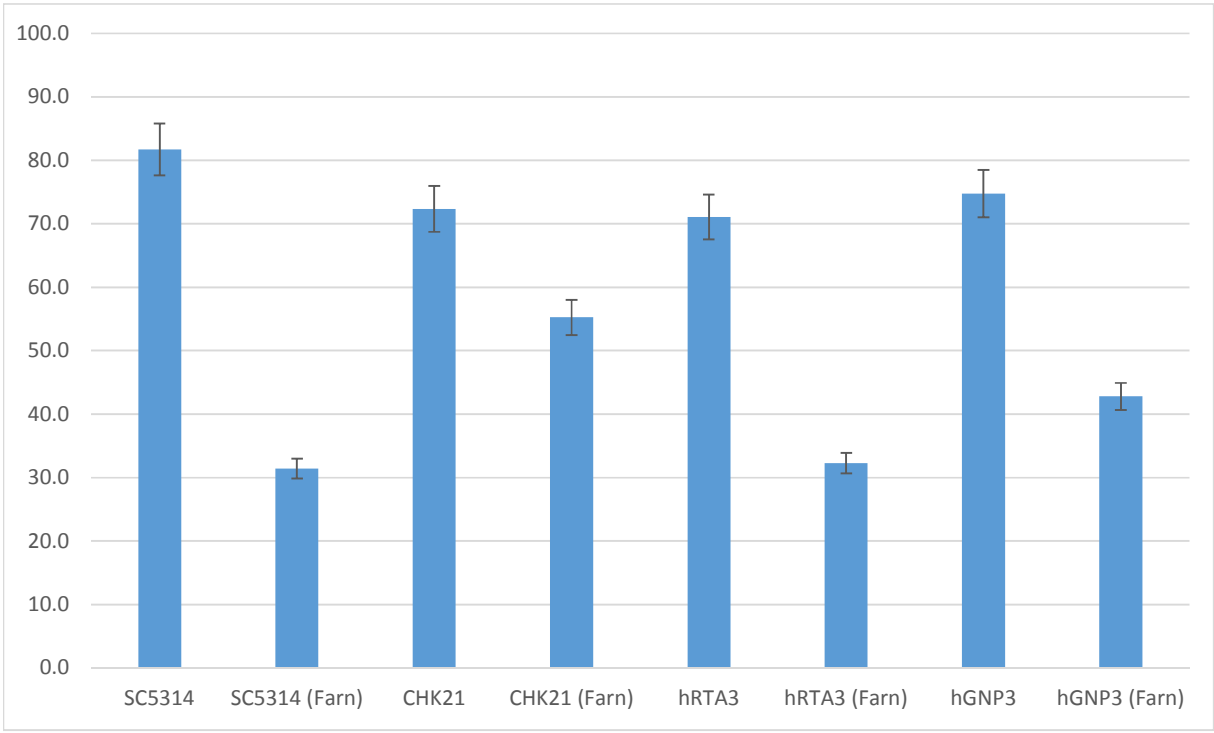


Figure 4: Percent Filamentation In The Presence of Farnesol

Effects of cyclic di-GMP on filamentation of mutants *hRTA3* and *hGNP3*:

Haploinsufficient strains *hRTA3* and h*GNP3*, along with a positive and negative control, were placed in conditions that normally induce filamentation. A control tube for each strain, and another tube containing cyclic di-GMP, was observed to note the molecule's effect. Cyclic-di-GMP effected both the SC5314 and CHK21 strains almost equally, with very similar results for both haploinsufficient strains. Overall, this molecule was not found to greatly inhibit the filamentation of *C. albicans;* however, it did effect each sample almost equally, suggesting that the mechanism for uptake of cyclic-di-GMP may be different than that of farnesol. (Figure 5)

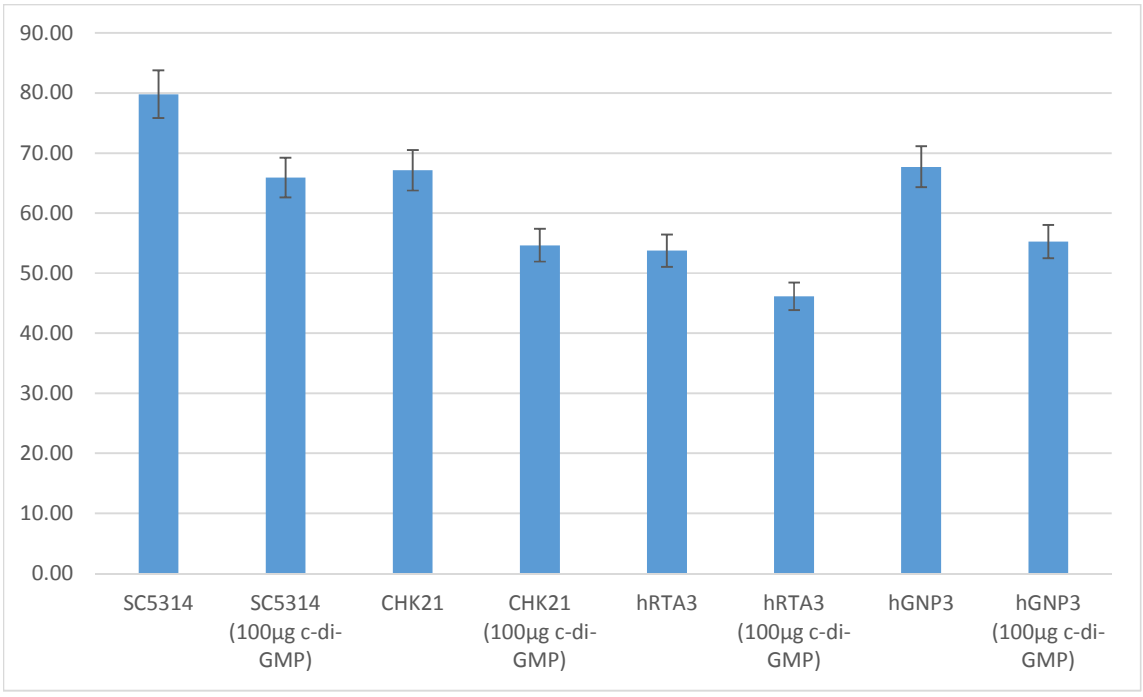


Figure 5 – Percent Filamentation In The Presence of Cyclic-Di-GMP

Effects of pyocyanin on filamentation of mutants *hRTA3* and *hGNP3*:

Despite increasing the concentration 10x twice for a series of 3 tests, pyocyanin was not shown to impact that filamentation of *C. albicans* (data not shown).

Effects of farnesol on biofilm formation of mutants *hRTA3* and *hGNP3:*

To determine the impact of farnesol on biofilm formation in *C. albicans* mutants *hRTA3* and *hGNP3,* serial dilutions were placed in a 96-well plate. After a two-hour period of adherence, biofilms were washed then exposed to 300µM farnesol during a 48-hour incubation.

As expected, farnesol inhibited biofilm formation in all four strains to some degree. Farnesol drastically reduced biofilm formation in the wild type (SC5314), but the impact was much less apparent in the CHK21 knockout. Impact of farnesol for both haploinsufficient mutants (*hRTA3* and *hGNP3*) generally fell between the ranges for the positive and negative controls (Figure 6).

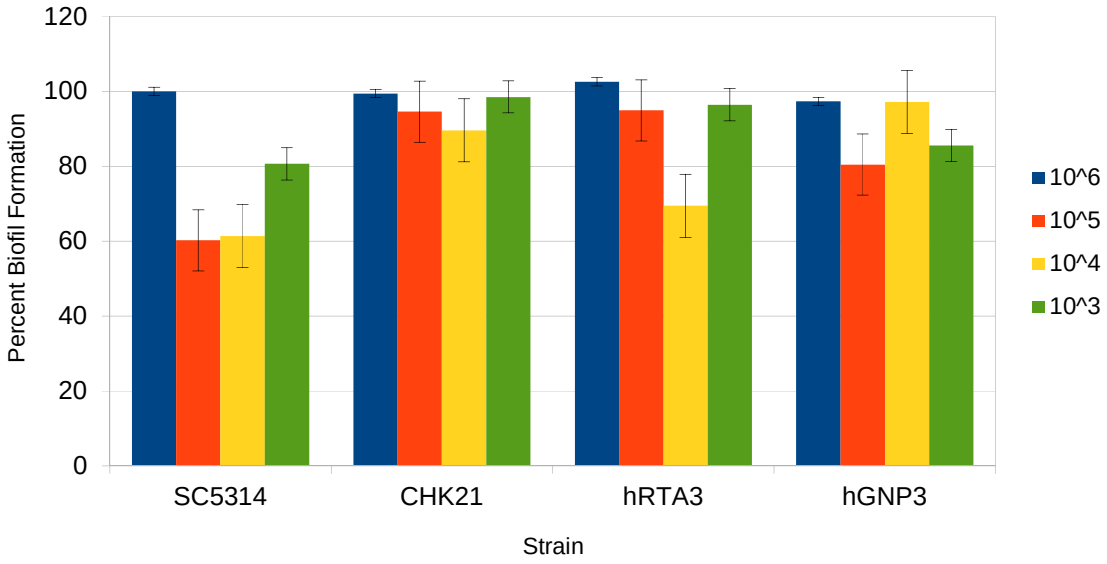


Figure 6: Percent Biofilm Formation In The Presence of Farnesol

Effects of cyclic di-GMP on biofilm formation of mutants *hRTA3* and *hGNP3:*

To determine the impact of cyclic di-GMP on biofilm formation in *C. albicans* mutants *hRTA3* and *hGNP3,* serial dilutions were placed in a 96-well plate. After a two-hour period of adherence, biofilms were washed then exposed to 100 µg cyclic di-GMP during a 48-hour incubation. At all concentrations, cyclic di-GMP increased biofilm formation, with the most

notable increases for the 10^5 dilution (Figure 7).

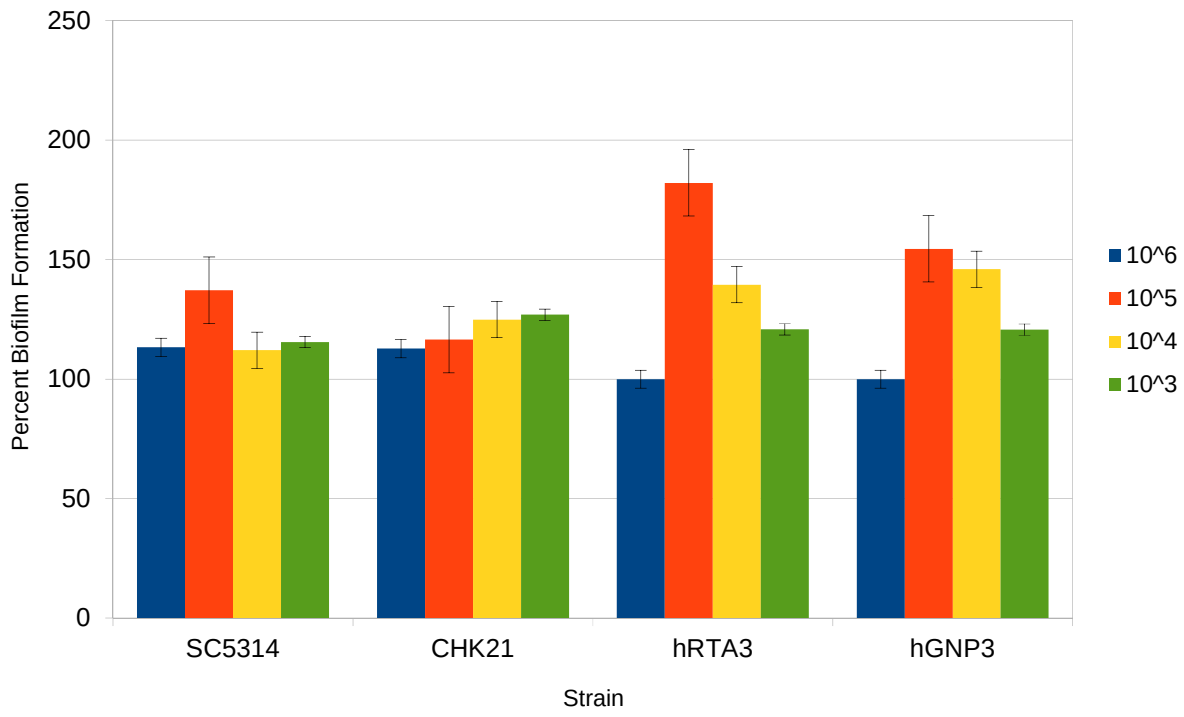


Figure 7 – Percent Biofilm Formation In The Presence of Cyclic-Di-GMP

Discussion

Haploinsufficient mutants *hRTA3* and *hGNP3* were first identified in a laboratory screen conducted by Dr. Sean Fox (Fox, 2013). Both transport gene mutants were shown to filament when co-cultured with three different bacterial species. This screen implicated these transport genes in interspecies communication, and introduced the idea that they may also be involved in intraspecies communication mechanisms such as quorum sensing (QS). All experiments in this study were designed to gain greater insight into the communication mechanisms of *C. albicans* as well as the functions of these two genes.

In the filamentation assays utilizing farnesol, the *hGNP3* mutant behaved more similarly to the known non-responder, *CHK21*, while the *hRTA3* mutant behaved more similarly to the wild type. Thus, the *GNP3* transport gene seems to play a role in the organism's QS response and response to other messenger molecules based on its response in all experiments, while the *RTA3* gene appears to play less of a role in QS, instead being part of the response to other messengers. Both mutants used in all experiments were haploinsufficient, suggesting that a complete knockout of either would have produced even more extreme results. Though these experiments potentially implicate the *GNP3* and *RTA3* transport genes in the organism's QS response and responses to other molecular messengers, additional study is required to figure out their exact mechanism.

C. albicans response to cyclic di-GMP, an established bacterial secondary messenger, was not very dramatic, but filamentation was mildly inhibited. *Pseudomonas aeruginosa* has been previously shown to produce higher levels of cyclic di-GMP when grown alongside *C. albicans* (Chen, 2014). This molecule encourages biofilm formation in *P. aeruginosa*, and the

effect on *C. albicans* filamentation suggests it may also be used to out-compete other organisms in a biofilm. The lack of response to pyocyanin indicates that, while pyocyanin may perform other functions, it is not used to directly compete with *C. albicans*. Other researchers have noted a response produced in *C. albicans* by pyocyanin, but no response was observed during this battery of tests.

Additionally, both transport genes (*RTA3* and *GNP3*) appear to influence biofilm formation. During formation of biofilms, many *C. albicans* cells will filament, so their reduced biofilm formation in the presence of farnesol is not entirely surprising given their reduced filamentation. Again, the degree of impact on the haploinsufficient mutant *hGNP3,* being in between that of the positive and negative controls, implies that a complete knockout would result in an even larger degree of filamentation in the presence of farnesol.

The organism's increased biofilm thickness in the presence of cyclic-di-GMP is surprising given the observed reduction in filamentation in the presence of the same molecule. However, the morphological shift between yeast and hyphal forms is not the only factor that contributes to biofilm formation, so any of these other factors could be responsible for the observed increase in thickness. The overall increase in biofilm mass may be due to an increased production of yeast, though this theory is, as of yet, untested.

References

Bassler B. Small Talk: Cell-to-Cell Communication in Bacteria. *Cell*. 2002;109:421–424. doi: 10.1016/S0092-8674(02)00749-3

Chauhan N, Latge JP, Calderone R. Signaling and oxidant adaptation in *Candida albicans* and *Aspergillus fumigatus*. *Nat Rev Microbiol*. 2006;4: 435–444

Chen AI, Dolben EF, Okegbe C, et al. *Candida albicans* Ethanol Stimulates *Pseudomonas aeruginosa* WspR-Controlled Biofilm Formation as Part of a Cyclic Relationship Involving Phenazines. Kazmierczak BI, ed. *PLoS Pathogens.* 2014;10(10):e1004480. doi:10.1371/journal.ppat.1004480.

Chen H, Fujita M, Feng Q, Clardy J, Fink GR. Tyrosol is a quorum-sensing molecule in *Candida albicans*. *Proceedings of the National Academy of Sciences of the United States of America.* 2004;101(14):5048-5052. doi:10.1073/pnas.0401416101.

Fox SJ, Shelton BT, Kruppa MD. 2013. Characterization of genetic determinants that modulate *Candida albicans* filamentation in the presence of bacteria. PLoS One 8:e71939. 10.1371/journal.pone.0071939

Gow NA. Germ tube growth in *Candida albicans*. *Curr Top Med Mycol*. 1997;8(1–2):43–55.

Hogan DA. Talking to Themselves: Autoregulation and Quorum Sensing in Fungi. *Eukaryotic Cell.* 2006;5(4):613-619. doi:10.1128/EC.5.4.613-619.2006.

Kabir MA, Hussain MA, Ahmad Z. *Candida albicans*: A Model Organism for Studying Fungal Pathogens. *ISRN Microbiology.* 2012;2012:538694. doi:10.5402/2012/538694.

Kibbler CC, Seaton S, Barnes RA, et al. Management and outcome of bloodstream infections due to Candida species in England and Wales. *J Hosp Infect*. 2003;54(1):18–24.

LaSarre B, Federle MJ. Exploiting Quorum Sensing To Confuse Bacterial Pathogens. *Microbiology and Molecular Biology Reviews.* 2013;77(1):73-111. doi:10.1128/MMBR.00046-12.

Mosel DD, Dumitru R, Hornby JM, Atkin AL, Nickerson KW. Farnesol Concentrations Required To Block Germ Tube Formation in *Candida albicans* in the Presence and Absence of Serum. *Applied and Environmental Microbiology.* 2005;71(8):4938-4940. doi:10.1128/AEM.71.8.4938-4940.2005.

Nickerson KW, Atkin AL, Hornby JM. Quorum Sensing in Dimorphic Fungi: Farnesol and Beyond. *Applied and Environmental Microbiology.* 2006;72(6):3805-3813. doi:10.1128/AEM.02765-05.

Peeters E, Nelis HJ, Coenye T. Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. J Microbiol Meth. 2008;72:157–165. doi: 10.1016/j.mimet.2007.11.010.

Pfaller MA, Diekema DJ. Epidemiology of Invasive Candidiasis: a Persistent Public Health Problem. *Clinical Microbiology Reviews.* 2007;20(1):133-163. doi:10.1128/CMR.00029-06.

Raska M, Blakova J, Krupka M, Weigl E. Candidiasis -- do we need to fight or to tolerate the *Candida* fungus? *Folia Microbiol (Praha)*. 2007;52(3):297-312.

Schulze J, Sonnenborn U. Yeast in the Gut: From Commensals to Infectious Agents. Medicine. 2009;106(51–52):837–42.

Sobel JD. Vulvovaginal candidosis. *Lancet.* 2007;369(9577):1961–71.10.1016/S0140-6736(07)60917-9

Soll DR. The role of phenotypic switching in the basic biology and pathogenesis of *Candida albicans. Journal of Oral Microbiology.* 2014;6:10.3402/jom.v6.22993. doi:10.3402/jom.v6.22993.