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Sensing Surfaces: A Non-filamentous C. albicans Response

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A Non-filamentous *C. albicans* Response

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ABSTRACT:

Resulting infections of mucosal tissues by *Candida albicans* present difficulty in treatment due to the formation of biofilms and invasion of tissue directed by thigmo-tropic responses of hyphal cells. Initiation of biofilm formation, however, is largely dependent on yeast-form cells. Their ability to sense surfaces, however, has yet to be examined. In the present study, an initial genetic profiling of the surface sensing response by non-filamentous *Candida albicans* was generated. In order to assess the ability for yeast-form *C. albicans* cells to recognize surfaces, four differentially solidified YPD plate types were created using agar, noble agar, Gelrite, and carrageenan. Genetic expression was assessed after 30 minutes growth on solid plates via qRT-PCR and compared against liquid YPD conditions. Of the 15 genes tested, 4 genes were significantly differentially expressed across all plate types including Rhd3, Nce103, Hwp1, and Ece1. This analysis indicates a potential surface sensing response by yeast-form *C. albicans* cells characterized by inhibition of biofilm forming genes and the discernment of growth under atmospheric conditions on noninducing surfaces.

Keywords: fungal surface recognition, *Candida albicans*, non-filamentous, qRT-PCR, solid surfaces, biofilm

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INTRODUCTION

The human microbiome is largely characterized by bacterial populations making up the overwhelming majority. Indeed, 99.9% of all microbial cells belonging to only a few species (Huffnagle & Noverr, 2013). As a result a large portion of research has focused on the bacterial component of the microbiome. The remaining 0.1% of the microbiome, however, possesses an equal, if not greater impact on human health despite its lower percent of sheer cell numbers (Huffnagle & Noverr, 2013). Being commonly referred to as the 'rare biosphere' this small group of microbes is involved in many of the diseases associated with the microbiome throughout the body (Huffnagle & Noverr, 2013). Important contributors to this group include fungi that, like other rare biosphere microbes, only represent a small percent of commensal microbes. Despite this, fungi are still found in every human microbiome with their combined presence known as the mycobiome (Huffnagle & Noverr, 2013). Chief among commensal fungi are *Candida* species of which *Candida albicans* has the largest role in disease across mucosal tissues in addition to being the most abundant *Candida* species found in up to 75% of the US population's oral cavities (Mayer et al., 2013). *Candida* infections as a result are common with over ¾ of all women experiencing vulvovaginal (yeast) infections due to *Candida spp.* at least once in their lives (Mayer et al., 2013).

Importantly, *Candida* infections can be split up into two main categories: mild, superficial infections of the skin and mucosal tissues, like the previously mentioned yeast infections, or severe systemic infections that have fatality rates of over 50% in immunocompromised and elderly populations (Kollu & LaJeunesse, 2021). Notably, both types of infections occur as the result of dysregulation of the immune system and or surrounding microbiome, like after antibiotic usage (Brand, 2012). This is because *Candida albicans*' populations are largely held in

check by surrounding microbes and through more nuanced immune regulation (Brand, 2012). The precise interactions of *Candida albicans* and the immune system, however, are still not fully understood (Richardson & Moyes, 2015). Nevertheless, due to the regulatory role of the surrounding microbiome and its significant bacterial make up, broad spectrum antibiotic usage creates advantageous environments for fungal expansion allowing *Candida spp.* to overtake mucosal tissues leading to infection.

The ability for *Candida albicans* to cause infection, however, is dependent on its ability to transition between yeast and hyphae morphologies through a process known as filamentation (Almeida & Brand, 2017). Filamentation then is characterized by the reversible, unbroken, parallel extension of its cell wall to form hyphae and is what makes *Candida albicans* an opportunistic pathogen as its virulence is dependent on its ability to filament (Brand, 2012). Filamentation is induced based on environmental cues including elevated temperature (> 37 ºC), elevated pH (> 7), exposure to serum, and the absence of quorum sensing factors, like farnesol, indicating low cell densities ($< 10^7$ cells per ml) (Mayer et al., 2013). Hyphae formation then represents a specific response that is highly site dependent being linked to a subset of genes associated with virulence factors and hypha specific proteins (Brand, 2012). Commonly identified proteins within this subset include Hwp1, Als1, Sap genes, and Ece1. Hwp1 and Als1 proteins function in cellular specific adhesion through encoded glycosylphosphatidylinositol (GPI) linked cell surface proteins (Mayer et al., 2013). The multiple expressed Sap genes encode secreted hydrolases similar to Ece1 which encodes the precursor for candidalysin, a peptide toxin that causes molecular damage aiding in scavenging of host cell nutrients (Richardson & Moyes, 2015). Together these genes allow for filaments to adhere to and invade tissues or other surfaces

having a large role in virulence giving its morphological change in hyphal formation its ability cause infections.

Hyphae formation is also essential for proper biofilm formation with filamentous fungal biofilms characterized by their pseudomembranous structure (Brand, 2012). In *Candida albicans*, biofilm formation follows a sequential process initiated by the adherence of yeast cells to a surface followed by the proliferation and further adherence of these cells in addition to hyphae transition of the upper biofilm and buildup of extracellular matrix (ECM) (Mayer et al., 2013). Interestingly, this process can occur on both biotic and abiotic surfaces . Commonly adhered surfaces include not only mucosal and other bodily tissues but also medical implants like dentures, stents, and prosthetics. Biofilms however are not simply stable, passive features but instead are highly dynamic posing an even greater health concern than filamentous cells on their own due to difficultly in their clearance (Brand, 2012). Much of the difficulty in treatment lies in increased expression of drug efflux pumps removing antifungal compounds and the incorporation of other oral microbes such as *Streptococcus mutans* and *Streptococcus gordonii* (Brand, 2012). These bacteria add to the complexity of the structure and further block diffusion of drug to the biofilm's center along with regulating fungal cell proliferation via secreted quorum sensing factors (Brand, 2012). Biofilms also release virulent cells, allowing for greater dissemination of the pathogen (Mayer et al., 2013). Beyond treatment evasion biofilms also show strong tendencies to invade underlying substrates (Brand, 2012). This includes mucosal tissue surfaces along with plastic and other abiotic surfaces even when little nutrient value is present (Fan et al., 2013). Such invasion is likely the result of the incorporation of filamentous cells within the biofilm as normal functioning of both biofilm and hyphae require proper surface recognition.

In hyphae, some evidence points towards surface recognition as a result of general cell wall perturbations across cell wall adhesion zones activating stretch sensors within the cell, that when coupled with chemical signaling, help to identify the specific surface (Almeida & Brand, 2017). However, more evidence points to asymmetrical growth of hyphae along surfaces driving *Candida albicans*' thigmo-tropic response (Thomson et al., 2015).This is because asymmetrical growth serves to orient polarity protein complexes, known to direct hyphal positioning, as close to the substrate as possible allowing for precise navigation and reorientation of the hyphal tip around obstacles across the surface (Thomson et al., 2015). The corresponding response is highly specific with the ability to recognize ridges less than half the height of the hyphae (Thomson et al., 2015). Asymmetrical growth then allows for hyphal tips to follow contours and gaps on surfaces and importantly direct hyphae tips towards softer regions through sensing surface stiffness, directing direct invasion (Thomson et al., 2015). Additionally, previous work has shown that thigmotropic responses are contingent upon adhesion to the surface as poorly adhered cells have reduced surface recognition (Thomson et al., 2015). Greater adherence correspondingly also shows greater reorientation of hyphal tips (Thomson et al., 2015). The critical role of hyphal cells to biofilm formation and subsequent invasion of substrate then can be explained as seen when hyphal formation is inhibited, biofilm integrity is greatly reduced (Tsuchimori et al., 2000). However, what is not known is if yeast cells possess a similar ability to recognize surfaces. Yeast cells, like hyphae, are able to adhere to surfaces and play an essential role in initiating biofilm formation adhering first and acting as a base across both biotic and abiotic surfaces. As these surfaces require the presence of differentially expressed adhesion molecules, yeast cells likely possess some ability to differentiate between surfaces. Additionally, their adhesion alone, while often cited as a trigger for filamentation is not exclusive to inducing

filamentation meaning some response by yeast cells to surfaces is occurring that is able to be regulated. Further, previous work in our lab has shown differential expression across liquid and solid rich media conditions (YPD) at 30 minutes, pointing towards a potential specific response to solidified surfaces. This response, however, has yet to be examined across surfaces beyond the traditional agar plate. Our goal for this work was to generate an initial genetic profile of non-filamentous *Candida albicans* yeast form cells across solid surfaces using qRT-PCR analysis of 15 previously defined genes that differentiated liquid and solid YPD media growth at 30 minutes (Figure 1). *Candida albicans* yeast-form cells were tested across 4 solid conditions to determine if a universal response to solid surfaces is present and, if so, further characterize it. Our results showed 4 of the 15 genes to be significantly differently regulated across all surfaces. Our results demonstrate an expanded role of yeast cells in biofilm initiation and holds implication for other microbial recognition of surfaces that may have been previously overlooked.

Sugar Metabolism	Stress Induced	Cell Wall	Replication
HGT2 ↑	STF2 ↑	BCR1 ↓	IME2 ↓
TYE7 ↑	PIL1 ↑	HWP1 ↓	
UCF1 ↑	NCE103 ↓	ECE1 ↓	
C1_09320C_A ↓	GSY1 ↑	PGK1 ↑	
ROB1 ↑		RHD3 ↑	

Figure 1. Selected significantly differentially expressed genes from RNA-seq analysis of solid vs liquid conditions at 30 minutes. Genes fall into four main categories with blue arrows indicating upregulation and red arrows indicating downregulation in solid YPD agar conditions compared to liquid YPD at 30 minutes.

MATERIALS AND METHODS

Strains and media

All analyses were performed using *Candida albicans* strain SC5314 grown on yeast-extract-peptone-dextrose (YPD) media following standard protocol as outlined previously by

Sherman (1991). To generate a range of solid surfaces YPD media was solidified using agar and known agar substitutes including noble agar (RPI), carrageenan (Sigma), Gelrite (RPI), guar gum (Sigma), and 300 bloom porcine gelatin (Sigma). Based on literature weight per volume percent formulations were formulated and tested across a range of weight per volume percentage (Jain et al., 2005; Mateen et al., 2012; Watson and Apirion, 1976). Final formulations included 1.6% w/v agar, 1.8% w/v noble agar, 3% w/v carrageenan, 0.08% w/v Gelrite, 10% w/v gelatin, and 7% w/v gaur gum. Plates were allowed to solidify for at least 48 hours to ensure fully hardened surfaces across each plate type.

Pre-testing analysis

Cells were grown overnight in 3 ml of YPD media at 30º with shaking. In 1 ml aliquots, overnight samples were washed twice in equal volume phosphate buffered saline (PBS) at pH 7.2. Cells were then resuspended in an equal volume of PBS. Plates were preheated at 30º for at least 4 hours to ensure consistent temperature throughout the plate before being inoculated with 200 µl of washed cells and 140 µl of sterile water. Cells were spread using glass beads and incubated at 30º for 3 hours. In triplicate, solid plates were imaged on an Evos FL inverted microscope at 40x magnification at time points 30 minutes and 3 hours. Cell morphologies were compared to the agar solidified plates to detect filamentous growth as a result of the properties of the solidification agents at either time point. Plates without visible filaments were selected to continue with further analysis on.

RNA extraction and cDNA generation

Overnight samples of strain SC5314 were grown at 30º with shaking. Cells were subsequently washed twice and resuspended in equal volumes of PBS. Preheated plates (4 hours)

at 30ºC were inoculated with 200 µl of washed cells and 140 µl of sterile water spread evenly using glass beads. After incubating at 30ºC for 30 minutes cells were collected from plates using a cell scraper and 2 ml of sterile water. Cells were immediately spun down to remove supernatant before proceeding with RNA extraction using an Rneasy mini kit with on-column DNase treatment (Qiagen). Upon combination in 70% ethanol RNA was held at -4ºC overnight before proceeding with the RNA isolation. Fully isolated RNA quality was assessed on a Nanodrop machine before being used in cDNA synthesis. Following standard procedures, cDNA was generated using the Thermo Scientific Maxima First Strand cDNA Synthesis Kit with remaining RNA stored at -80ºC for long term storage. Genomic contamination assays were performed on all samples using a -RT control with ideal CT differences greater than 10 cycles. RNA was also extracted from the liquid YPD condition at 30 minutes via introduction of 110 µl of PBS washed cells into 50 µl of prewarmed YPD media at 30ºC. Cells were incubated for 30 minutes at 30ºC before being collected through vacuum filtration using 0.2 micron paper filter. Filter paper was immediately frozen in a sterile 50 mL conical tube overnight before subsequent removal of cells using 2 ml of cold sterile water alternating vertexing at max speed and scraping of the paper filter surface for 1 minute. Collected cells were spun down, supernatant removed, and RNA extraction performed as described previously.

qRT-PCR analysis

Prior to qPCR analysis, generated gene primer pair efficiencies was performed using a 1:10 genomic DNA dilution series. qPCR analysis was performed using 20 µl reactions consisted of 9 µl of primer master mix and 11 µl of cDNA master mix. Primer master mixes were generated using a 2:5 microliter ratio of each primer pair to nuclease free H_2O per reaction. cDNA master mixes were generated using a 1:10 microliter ratio of +RT cDNA to 2x SYBER green per

reaction (Thermo Scientific). The previously identified housekeeping gene HSP90, a stable expressed heat shock factor, was used to control for RNA concentration variations between replicates within plate conditions. Reactions were run in a quantitative PCR machine.

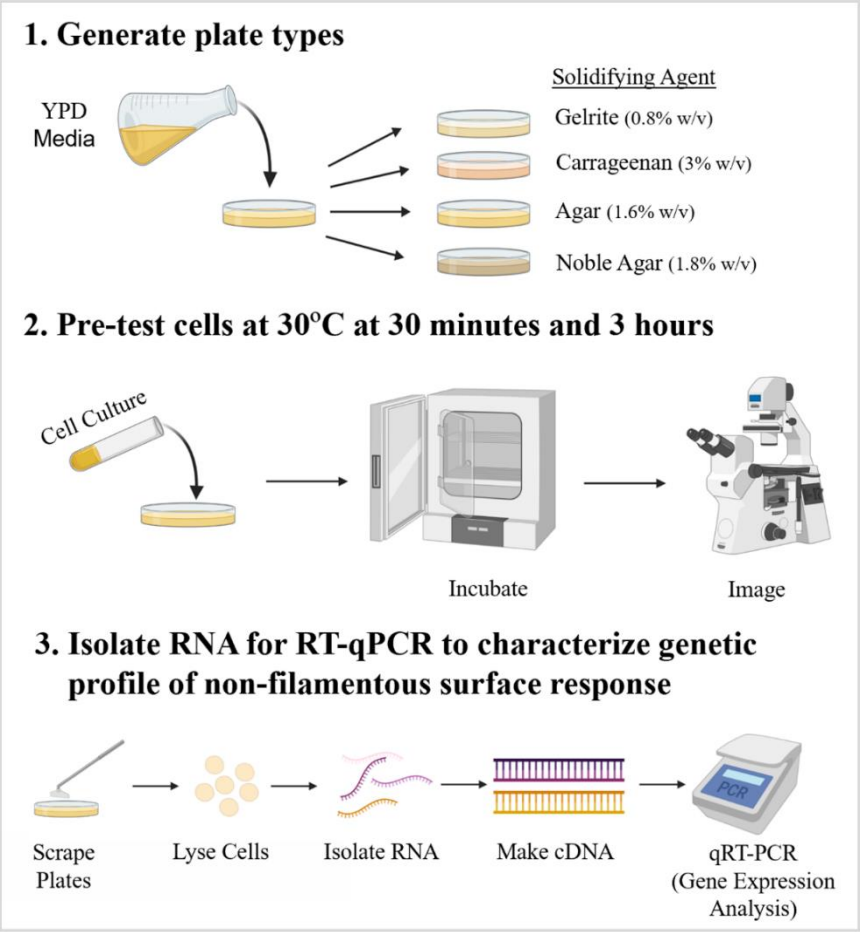

Figure 2. Expression analysis scheme for solid surface recognition. Plate types were generated with a YPD base and variable solidification agent. Plates were then assessed for non-filamentous cell profiles at 30 minutes and 3 hours. Non-filamentous cells were then collected after 30 minutes growth, RNA extracted, and qRT-PCR performed to characterize the genetic response.

RESULTS

Plate formulations

Agar is almost universally used as the solidifying agent of choice when making solid media for microorganisms. Our initial studies, suggesting that yeast cells may be sensing surface, were based on the difference in gene expression observed between liquid and solid versions of rich

media, with the agar solidifying agent as the only difference in the media recipes. It is possible that the differing response to media may have been driven by agar itself, and not the solid nature of the plates compared to liquid growth. Our goal for this work was to determine if yeast cells grown in rich media using other solidifying agents would show a similar gene expression pattern. In order to generate differently solidified solid plates, five known agar substitutes were used. Of the five, only three substitutes resulted in a hardened surface comparable to agar. This included noble agar, carrageenan, and Gelrite, but guar gum and gelatin failed to form fully solidified plates (Figure 3). Gelatin solidified plates formed a hardened solid surface at room temperature but melted at elevated temperatures at and above 30ºC. Guar gum failed to form a hardened surface instead forming a highly viscous liquid at room temperature transitioning from its fully liquid state after leaving the autoclave to a highly viscous semi solid state within 60 seconds regardless of concentration. Both plates were unusable in subsequent expression analyses.

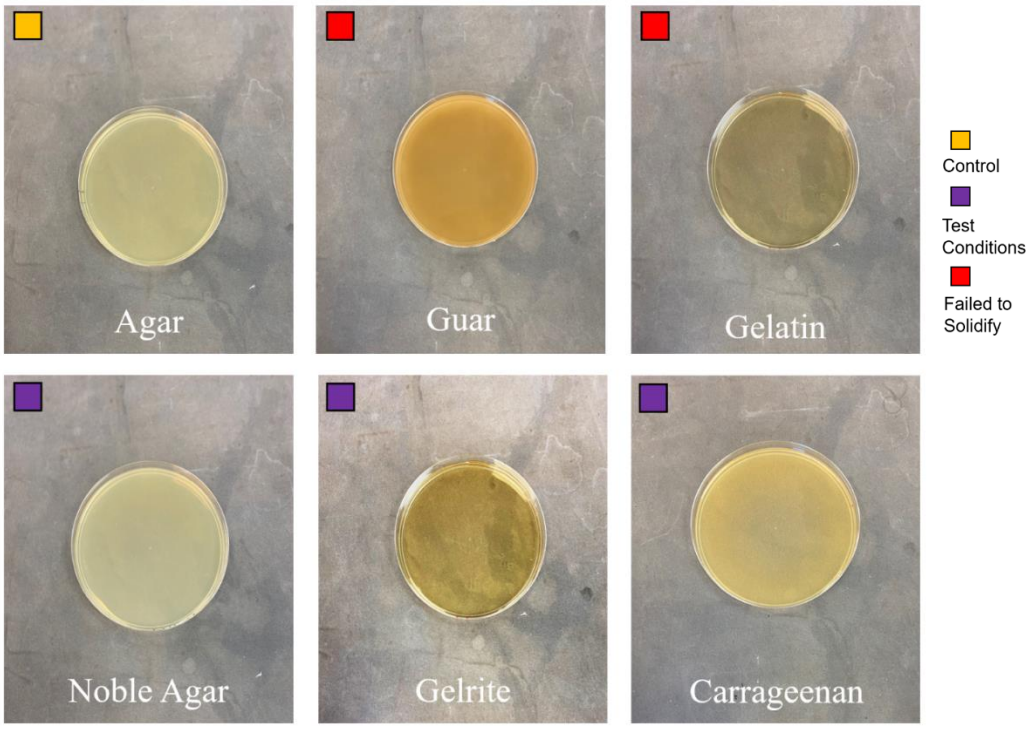

Figure 3. Solid plate formulations. Plates were made with a YPD base using differing solidification agents as indicated on each image. Orange squares represent the solid control condition, purple squares represent the tested conditions, and red squares represent plates that failed to solidify fully and were not used in the analysis.

Non-filamentous growth pretesting

To ensure only yeast cells were assessed in genetic testing, cell morphologies were analyzed after prolonged incubation at 30ºC at 30 minute and 3 hour time points on solid conditions. All 3 agar substitute solidified plates exclusively showed yeast cell morphologies with no difference from the control agar plate type at both 30 minutes and 3 hours post incubation indicating solidifying agents were inducing compounds (Figure 4). Similar concentrations of cells were seen across plates with any present clustering of cells attributed to the evaporation of the water used to help spread the cells initially, clumping cells together.

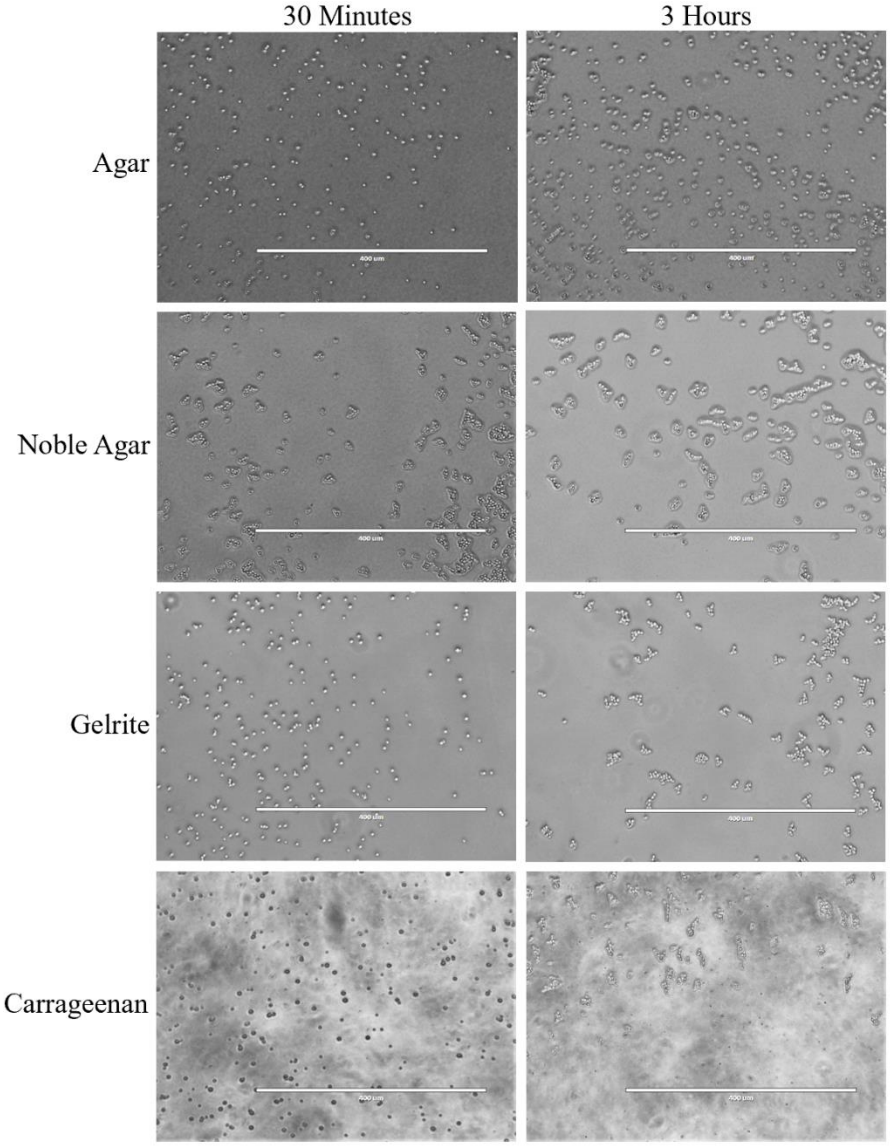

Figure 4. Pre-testing of solid plate formulations to ensure non-filamentous cell growth. Each plate type was imaged at both 30 minutes and 3 hours in triplicate to ensure filamentous growth was not induced by the solidification agent. Cells were imaged using an EVOS FL inverted microscope at 40x magnification.

RNA isolation and cDNA synthesis

To determine the purity of isolated RNA, 1 µl RNA aliquot readings from each sample were taken using a Nanodrop machine. RNA samples showed highly pure RNA samples with ratios above 2.1 centered around 2.19 for each solid plate and liquid YPD replicate except noble agar replicate C that had a 260/280 ratio below 2 at 1.94. This still is considered acceptable as a 260/280 ratio of around 2 is generally accepted as pure for RNA with above 1.8 denoting a pure DNA sample. RNA 260/230 readings also indicated a pure sample with values above 2 (Table 1). This is expected as 260/230 values are typically higher than their respective 280/260 values. Of note, agar replicate B had a lowered 260/230 ratio of 1.95. This lowered 260/230 ratio indicates the potential presence of organic contaminants like phenol, TRIzol, or peptide bonds that absorb at 230 nm pointing to a potential problem within the sample extraction process. This reading, however, is also considered acceptable as 260/230 values are more variable than 280/260 values and this value centers around 2 which is considered pure. Extracted RNA concentrations were between 100 ng/µl and 350 ng/µl (Table 1). These values were on the lower end of the desired range but resulted in cDNA concentrations centered around 550 ng/µl or above (data not shown) sufficient for qRT-PCR analysis.

cDNA replicates were assessed for genomic contamination prior to gene expression analysis using the negative RT samples using *C. albicans* housekeeping gene TDH3.

Table 1
Extracted RNA sample nanodrop purities across solid and liquid conditions.

Sample	Concentration (ng/µl)	A_{260}/A_{280} Ratio	A_{260}/A_{230} Ratio
Agar			
A	247.6	2.18	2.43
B	164.1	2.19	1.95
C	256.4	2.19	2.52
Noble Agar			
A	222.5	2.20	2.37
B	156.3	2.19	2.50
C	348.8	1.94	2.38
Carrageenan			
A	108.6	2.19	2.36
B	153.9	2.18	2.10
C	173.9	2.11	2.05
Gelrite			
A	133.4	2.19	2.46
B	147.9	2.19	2.44
C	102.7	2.19	2.44
Liquid YPD			
A	132.0	2.19	2.19
B	166.3	2.18	2.55
C	135.0	2.17	2.46

RNA was extracted after 30 minutes growth for each condition.

Primer efficiencies

To verify selected primers could properly replicate the desired gene sequence of interest, primer pair efficiencies were measured using genomic DNA dilution series. Generated primer pairs showed efficiencies between 90 and 105 percent across all primer pairs indicating primer specificity and lack of dimer formation (Figure 5).

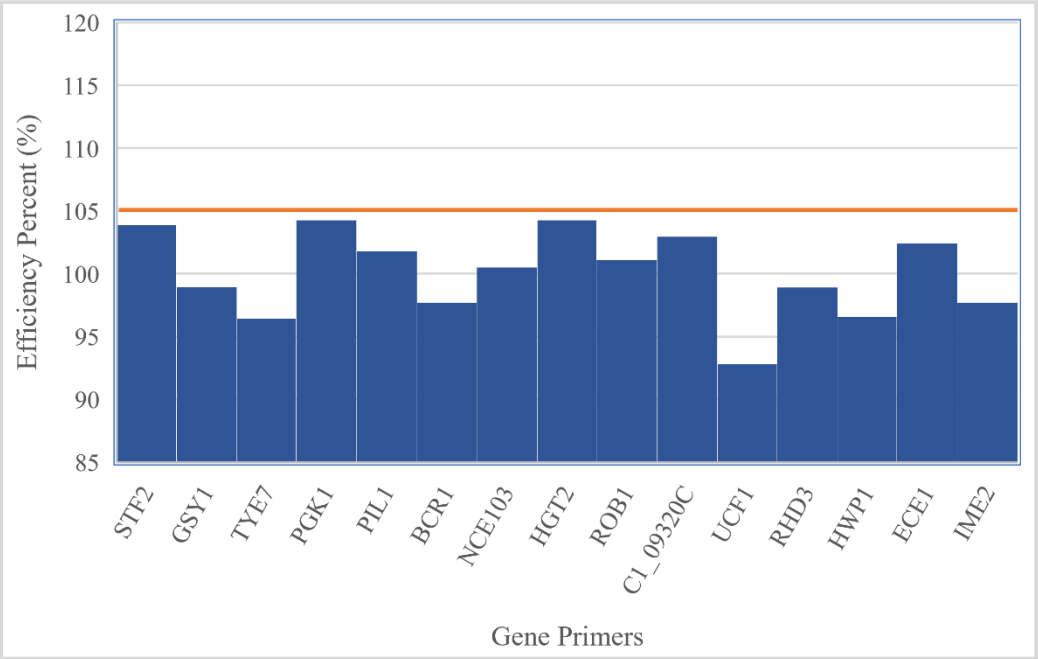

Figure 5. Gene primer efficiencies for qRT-PCR analysis. 5 µM stock primer pairs were assessed using housekeeping gene TDH3 to ensure primer efficiencies were between 90 and 105%. The red line indicates the cut off for overexpression of primer pairs at 105%.

Gene expression analysis

To characterize the genetic response of non-filamentous *Candida albicans* yeast cells to solid surfaces, 9 upregulated and 6 downregulated genes identified previously in an RNA-seq analysis of solid versus liquid YPD conditions at 30 minutes were assessed via qRT-PCR across multiple solid surfaces. Of the 9 RNA-seq assessed upregulated genes, only 1 gene, RHD3, was found to be significantly upregulated in comparison to the liquid condition across all solid conditions (Figure 6). UCF1 showed potential upregulation across carrageenan, noble agar, and agar solidified surfaces. Of these, however, only carrageenan was significantly upregulated with Gelrite plates being significantly downregulated (Figure 6). As a result of its variable expression no clear pattern is able to be drawn meaning it is likely not involved with the recognition of solid surfaces, but instead involved in a carrageenan specific response. Similarly, carrageenan plates generated significant upregulation of PGK1, PIL1, and TYE7. No one other solid plate condition, however, was upregulated indicating a lack of a consistent role in surface recognition

for these genes. Gelrite solidified plates induced significant downregulation of STF2, GSY1, HGT2, and TYE7. This is in direct contrast to previously determined RNA-seq analysis demonstrating significant upregulation of these genes on YPD surfaces meaning the properties of Gelrite are eliciting the opposite response to agar.

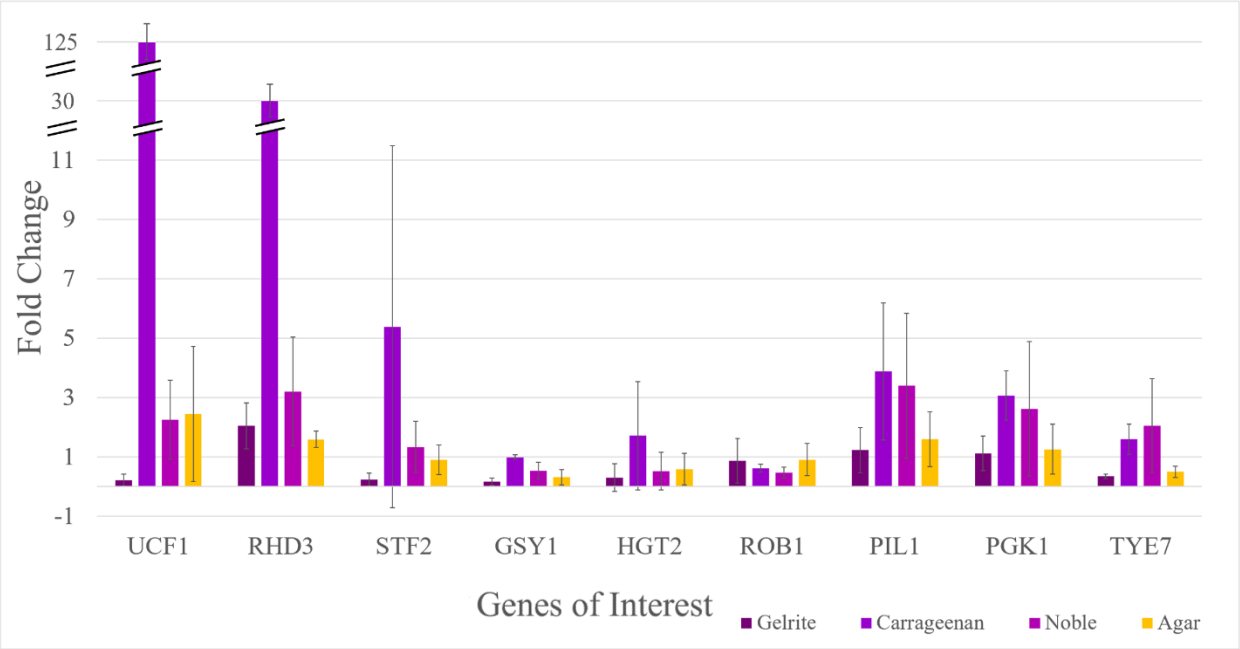

Figure 6. Fold expression difference of RNA-seq upregulated genes across solid conditions. Genetic expression was assessed via qRT-PCR with fold change in expression in comparison to liquid YPD condition at 30 minutes. Dark purple bars indicate Gelrite solidified plates, medium purple bars indicate carrageenan solidified plates, light purple bars indicate noble agar solidified plates, and yellow bars indicate agar solidified plates. Error bars represent standard deviations of biological triplicates (n=3).

Of the six RNA-seq defined downregulated genes, only ECE1 and HWP1 showed significant downregulation across all conditions with NCE103 significantly upregulated across all conditions (Figure 7). Both IME2 and C1_09320 had significant downregulation of the agar solidified condition (Figure 7). Similar downregulation was not seen in the carrageenan or noble agar conditions. Gelrite, however mirrored agar conditions being significantly downregulated across C1_09320 as well. IME2, C1_09320, and BCR1 however all showed variable expression across solid conditions indicating they likely do not play a role in surface recognition (Figure 7).

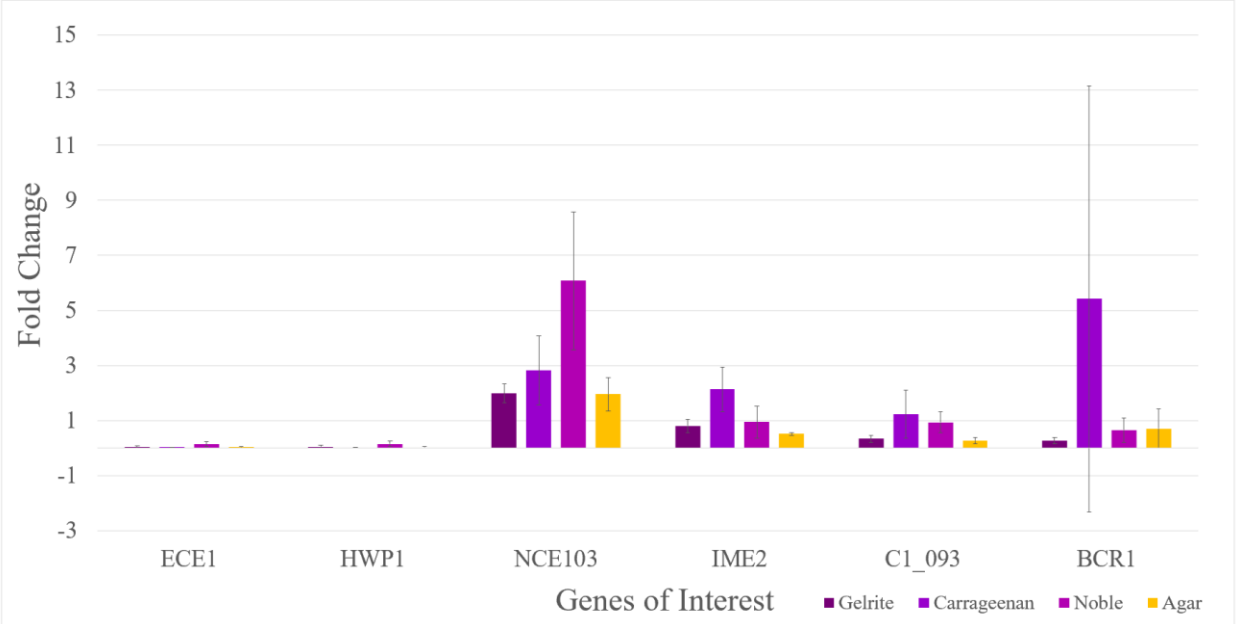

Figure 7. Fold expression difference of RNA-seq downregulated genes across solid conditions. Genetic expression was assessed via qRT-PCR with fold change in expression in comparison to liquid YPD condition at 30 minutes. Dark purple bars indicate Gelrite solidified plates, medium purple bars indicate carrageenan solidified plates, light purple bars indicate noble agar solidified plates, and yellow bars indicate agar solidified plates. Error bars represent standard deviations of biological triplicates (n=3).

DISCUSSION

Candida albicans, despite existing as a commensal fungus in the majority of the US population, is an opportunistic pathogen reliant on its dimorphic nature for virulence (Mayer et al., 2013). In inducing conditions hyphae formation allows for tissue invasion and biofilm formation demonstrating surface recognition responses (Desai & Mitchell, 2015). As a result, yeast form *Candida albicans* cells may also possess some ability to sense surfaces not previously identified.

In order to test this, formulation of differentially solidified plates was generated using multiple agar substitutes in addition to agar. Of the initial five substitutes two, guar gum and gelatin, failed to properly solidify fully for use in experimentation. Initially described weight per volume percentages were subsequently increased in order increase surface hardness. Gelatin

weight per volume percentages ranging from 4% to 10% gelatin were tested. At 4% and higher, plates solidified at room temperature. At 30ºC, however, all formulation percentages melted with no noticeable increase in solidification identified via increase in concentration and was therefore not used in further analyses. Similarly guar gum, a hydrocolloid, failed to form a solid surface between 3% and 6% weight per volume formulations. Lack of sufficient hardening is likely due in part to difficulty in dissolving the guar in solution because of its rapid hydration creating agglomerates that were unable to be further broken down by either heat or increased stirring rate of lab available equipment. In industrial settings this property of guar gum presents notable problems as well but is able to be solved using heavy machinery with sufficient sheer force to break apart agglomerates (Mudgil et al., 2014). In the lab, this was difficult to replicate and resulted in an inability to form consistent solid surfaces. Even when gaur was successfully fully dissolved it resulted in a highly viscous semi solid consistency that was unable to absorb the added 20% glucose or form plates effectively as a result of its viscous nature. This plate type was also not utilized in further analyses as a result.

To generate an initial genetic profile across the differentially solidified surfaces, 15 genes were selected from previously analyzed RNA-seq data between solid and liquid YPD conditions at 30 minutes. Of the RNA-seq identified genes, 15 genes were selected for analysis across all four plate conditions. Genes were selected based on a combination of function, significant fold change between liquid and solid YPD conditions, and base-mean value expression levels 10,000 or higher. This meant that the top differentially expressed genes, both up and down regulated, were not exclusively selected in order to get a more comprehensive perspective across multiple molecular cellular functioning's.

qPCR analysis of the selected 15 differentially expressed RNA-seq genes identified four genes differentially expressed in comparison to liquid conditions across all solid plate conditions. Of these, two genes were significantly upregulated, Rhd3 and Nce103, and two significantly downregulated, Hwp1 and Ece1.

Fungal cell walls function as essential organelles providing shape, strength, and protection for the cell and are arranged in networks of layered polysaccharides and cell wall proteins (Vavala et al., 2013). Rhd3/Pga29 encodes a cell wall protein specific to *Candida albicans* that is not found in other fungi (de Boer et al., 2010). This gene has been shown to be highly expressed exclusively in yeast cell morphologies being subsequently downregulated upon hyphal formation (Heilmann et al., 2011). Interestingly this protein has not been associated with any role in adhesion or cell wall integrity as exposure to different agents that affect cell wall organization and stability, like NaCl, Calcofluor white, and Zymolyase, did not alter expression of Rhd3 (de Boer et al., 2010). Rhd3 deletion mutants also demonstrated overall normal functioning despite lowered virulence (Vavala et al., 2013). This indicates it may have a previously unidentified role associated with yeast cells that may include some functionality in surface recognition. For example, it may be expressed in order to block other protein interactions on cell surfaces to maintain yeast form morphology until strong enough environmental cues override it to signal more specialized actions are needed, like hyphal formation, in which it is subsequently down regulated. This could mean surface recognition only occurs when large enough solids are encountered.

Proper regulation of CO_2 levels is essential for all branches of life with both prokaryotes and eukaryotes including conserved carbonic anhydrases in their genomes (Aguilera et al., 2005). Nce103 plays a critical role in yeast growth on host skin and abiotic surfaces like medical

devices encoding a conserved carbonic anhydrase (Dostál et al., 2020). Like all carbonic anhydrases it serves to hydrate CO_2, however, Nce103 importantly also serves as a CO_2 level sensor for the cell being centralized to the cell wall and plasma membranes of yeast cells (Dostál et al., 2020). Subsequent deletion mutants are unable to grow under normal atmospheric conditions, while still being able to grow in still liquid conditions (Götz et al., 1999). Nce103's expression then is required when encountering atmospheric air containing 0.04% of CO_2 (Aguilera et al., 2005). This indicates that yeast cells may potentially gauge solid surfaces in part based on CO_2 levels. This then only functions for specific surfaces in which are exposed to air, acting as part of a large response that gives it specificity. The upregulation in our samples is likely the result of the evaporation of sterile water used to spread it across the plates leaving cells exposed directly to the air.

Of the down regulated genes, hyphal wall protein 1 or Hwp1, forms important covalent bonds with primary amines found on human epithelial cells and functions to adhere *Candida albicans* cells to mucosal tissues specifically during virulence being highly expressed in biofilm formation. Indeed, the vital role of Hwp1in biofilm formation specifically can be seen in knockout mutants demonstrating normal colonization of the gut and equivalent fatal infection rates and tissue invasion in mice with reduced stability of biofilms. Further, Hwp1 is induced when in contact with either abiotic or biotic surfaces. In light of this, Hwp1 is likely being significantly downregulated in order to ensure adhesion of the yeast cell is not inducing biofilm formation as surrounding environmental cues do not indicate advantageous filamentation presently. This then correspondingly indicates the cell has in some way recognized it is on a surface and is working to ensure non-virulent adhesion of the yeast cell to the solid. Due to the

degree of down regulation the cell appears to be trying to guarantee improper filamentation is not ensuing.

Additionally, commonly expressed together with Hwp1, Ece1 is upregulated in biofilm formation and has been shown to restore partial biofilm maturation in the absence of regulatory transcription factor Bcr1. It, however, unlike Hwp1 is important in tissue damage encoding candidalysin, a peptide toxin. Ece1's downregulation may once again be in response of the cell's recognition of a solid surface in which biofilm formation is likely being downregulated to ensure improper tissue damage is not initiated and yeast cell morphology can be maintained. Its significant downregulation in comparison to liquid conditions indicate the cell knowns it is on a surface as biofilm formation is possible. The mechanism behind this, however, is still unclear.

The remaining 11 tested genes showed variable expression across plate conditions and did not indicate a significant differential expression across liquid and solid conditions (Figures 6 and 7). This may be due to the difference in length of time washed cells were held in PBS before being tested. This is the result of the experimental design in which, in an attempt to keep biological triplicate conditions as similar as possible, plate type triplicates were done together. This meant extended periods of time in PBS separated each plate condition. Due to the variable exposure times to PBS, gene expression then may have been affected most especially seen in later run plate types agar and gelrite and should be considered when assessing the generated results. Some variation between solid plate conditions, however, may be the result of cellular responses to the solidifying agents instead of to the solid surface due to the unique properties of each solidification agent. This, however, likely only plays a minor role as no consistent pattern is seen across similar solidifying agents, like noble agar and agar or carrageenan and agar all being derived from seaweed vegetation (Watson & Apirion, 1976).

RNA is also a transient biological molecule with a very short half life in cells (Chen et al., 2008). This is important for the cell in order to be able to adapt quickly to environment and internal stimuli. It however presents potential difficulties in working with RNA as variation between replicates can be high with the timing of experiments between 30 minutes and a few hours generating largely different expression profiles making precise data collection key.

This study then holds important implications for the yeast cell's role in regulating biofilm formation based upon surface recognition responses that ensure appropriate adhesion is proceeded with via downregulation of Hwp1 and Ece1. Similarly, upregulated geness Nce103 may serve to help indicate if cells are in atmospheric conditions or not along with the still unclear role of Rhd3 potentially including regulating what the cell interacts with. These insights may then may also be valuable in beginning to look at how other microbes function to recognition surfaces within the commensal microbiome and beyond. Future work still remains in further clarification of the role of the four identified differentially expressed genes in surface recognition. How these genes work together, however, is still unclear. Further examination of more differentially expressed genes from the 30 min YPD condition set may help to complete the picture that is non-filamentous surface recognition of *Candida albicans*.

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