Identification and Characterization of Genetic Factors Involved in Candida-Bacterial Interactions

Sean J. Fox
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Identification and Characterization of Genetic Factors Involved in *Candida*-Bacterial Interactions

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A dissertation
presented to
the faculty of the Department of Biomedical Sciences
East Tennessee State University
In partial fulfillment
of the requirements for the degree
Doctor of Philosophy of Biomedical Sciences

______________________________________________
by
Sean J. Fox
December 2013

______________________________________________
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ABSTRACT

Identification and Characterization of Genetic Factors Involved in *Candida*-Bacterial Interactions

by

Sean J. Fox

Throughout existence, fungi and bacteria have long shared ecological niches and thus engage in numerous interactions to mutually enhance survival or antagonistically gain competitive advantages. Of importance to human health are those interactions that involve bacteria with the opportunistic fungi, *Candida albicans*. An important virulence factor of *C. albicans* is the ability to control morphology, which allows the transition between yeast, pseudohyphal, and hyphal phenotypes. Morphological control in *C. albicans* is governed by quorum sensing and the secreted autoregulatory molecule farnesol. Quorum sensing allows individual cells to sense the environment and respond as a group. Bacteria also use quorum sensing to communicate and control virulence. Despite their abundance in nature, very little is known about the interactions of *C. albicans* with bacteria on a genetic and molecular level. The objective of our research is to identify the genetic elements involved in *C. albicans*-bacterial interactions and characterize the genes that may participate in these relationships. To accomplish this, we screened a *C. albicans* mutant library for the ability to filament in the presence of *Escherichia coli, Pseudomonas aeruginosa*, and *Staphylococcus aureus*, where
typically, these 3 bacterial species inhibit C. albicans filamentation. We identified 836 C. albicans mutants that displayed a filamentous phenotype in the presence of bacteria. Collectively, 295 of these mutants filamented in the presence of all 3 bacterial species. Candidates were subsequently sequenced to identify the location of the mutation and the affected genetic element. CDR4, a putative ABC transporter, and ALS6, a putative adhesion, were further characterized for their specific involvement in Candida-bacterial interactions. Using a filamentation assay, cdr4 and als6 deletion strains demonstrated a decreased response to the inhibitory effects of farnesol as well as bacterial molecules known to inhibit the production of hyphal-filaments. Additionally, the ability of cdr4 and als6 deletion strains to attach and form biofilms was significantly enhanced even in the presence of farnesol and bacterial inhibitors. The results of this study contribute to the body of knowledge involving polymicrobial interactions, and these findings may lead to new antifungal targets for therapeutic interventions.
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ABBREVIATIONS

Agr  Acessory Gene Regulator
AI-2  Autoinducer 2
AIP  Autoinducing Peptide
ALS  Agglutin Like Sequence
BDSF  *Burkholderia* Diffusible Signal Factor
BEC  Buccal Epithelial Cells
BSI  Blood Stream Infection
cAMP-PKA  Cyclic Adenosine Monophosphate Protein Kinase A
CDR  *Candida* Drug Resistance
CF  Cystic Fibrosis
CGD  *Candida* Genome Database
CSP  Competence Stimulating Peptide
DSF  Diffusible Signal Factor
HHQ  2-heptyl-4-quinolone
HSL  Homoserine Lactone
HWP  Hyphal Wall Protein
LD  Lethal Dose
LPS  Lipopolysaccharide
LB  Luria Broth
M199  Medium 199
MAPK  Mitogen Activated Protein Kinase
MRS  Major Repeat Sequence
ORF  Open Reading Frame
PPG  Peptidoglycan
PQS  *Pseudomonas* Quinolone Signal
QS  Quorum Sensing
QSM  Quorum Sensing Molecule
RT-PCR  Reverse Transcription Polymerase Chain Reaction
SAP  Secreted Aspartyl Proteinases
SDSF  *Streptococcus* Diffusible Signal Factor
VAP  Ventilator Associated Pneumonia
YPD  Yeast Peptone Dextrose
YWP  Yeast Wall Protein
CHAPTER 1

INTRODUCTION

Historically, microorganisms have been viewed as pathogens with a majority of the medical and scientific community’s knowledge of microbes derived from pure cultures. However, rarely in the environment and the human body does one find such a pure culture and distinct nature of single isolated microorganisms. In fact, the human body alone is inhabited by trillions of microbes representing thousands of species that comprise the normal flora. These microbial communities exhibit dynamic interactions, express a spectrum of relationships, and employ several forms of communication. Research into polymicrobial interactions is quickly gaining interest due to the implications of the effects on both human health and disease. However, many hurdles remain due to the complex nature of multispecies communities. Understanding the nature of microbial interactions, biofilm formation, alterations of drug efficacy, phenotypic alterations, and medical implications of polymicrobial communities are several areas of interest. Principal among these microbial relationships are fungal-bacterial interactions involving the opportunistic polymorphic fungi, Candida albicans, the most common fungal pathogen.

Candida albicans

C. albicans resides as part of the normal flora in 70% of the population and can be isolated from the skin and gastrointestinal and genito-urinary tracts (Mavor et al. 2005; Kim and Sudbery 2011). While individuals with healthy immune systems can be affected, such as women with recurring vaginitis, those individuals with compromised or
suppressed immune systems face a greater risk of *C. albicans* transitioning from a commensal to deadly pathogen. These at-risk individuals include those undergoing cancer treatment, HIV patients, extreme age (both the very young and very old), indwelling devices, and long-term antibiotic treatment (Giri and Kindo 2012). *C. albicans* causes candidiasis of the tissue and candidemia of the bloodstream (Giri and Kindo 2012). Interestingly, Nucci et al. (2001) examined the origins of candidemia and provided a strong case for the involvement of the human gut’s own *Candida* population as the source. Prevalence of *C. albicans*, especially in the healthcare setting, is staggering as *C. albicans* is the fourth most common organism involved in nosocomial bloodstream infections, the third most commonly isolated organism of catheter related infections, and the second most common opportunistic microorganism of HIV infected patients (Crump and Collignon 2000; Wisplinghoff et al. 2004; Ramage et al. 2006; Hamza et al. 2008; Kim and Sudbery 2011;). Mortality of *C. albicans* infections, particularly when involving the bloodstream, can reach as high as 35-40%, especially for patients in an intensive care unit setting (Gudlaugsson et al. 2003).

*C. albicans* has numerous virulence factors at its disposal to enhance survival in the host for both localized and systemic infections. These include diverse adhesins, the ability to form biofilms (both monospecies and polymicrobial), degradative enzymes, numerous drug resistance mechanisms, and lastly, which has garnered much research of late, the ability to change morphology (Cowen et al. 2002; Sundstrom 2002; Ramage et al. 2005; Schaller et al. 2005). A number of *C. albicans* virulence factors can be linked to specific morphological states. For example, the secreted aspartyl proteinases (SAP) are a group of enzymes responsible for degradation of human proteins. While
Sap1p, Sap2p, and Sap3p are expressed by yeast cells, Sap4p and Sap6p are associated with the hyphal form (Naglik et al. 2003). Likewise, adhesions are also associated with different morphologies. Agglutin-like sequence protein (Als3p) and hyphal wall protein (Hwp1p) are hyphal specific, yeast wall protein (Ywp1p) is yeast specific, and enhanced adhesion to polystyrene protein (Eap1p) is expressed by both morphological forms (Nobile and Mitchell 2006; Li et al. 2007; Liu and Filler 2011). Moreover, mature biofilm formation and some instances of drug resistance depend upon the ability of *C. albicans* to transition from yeast to hyphae (Baillie and Douglas 1999). Thus, the virulence attribute of polymorphism appears to be an ideal target for *C. albicans* research.

**Polymorphic Nature of *C. albicans***

*C. albicans* infections and overall virulence comprises the reversible transition between unicellular yeast, pseudohyphal, and hyphal forms. A variety of environmental factors shape morphological transition and can be induced in the laboratory via numerous controllable conditions. Some of the more potent inducers of hyphal formation are serum, pH (neutral), temperature (37°C), and the type of media used, such as M199, a synthetic tissue culture media (Han et al. 2011). Each morphological state is associated with specific roles in *C. albicans* pathogenesis and to be effectively virulent, it is essential for *C. albicans* to interconvert between morphologies (Sudbery et al. 2011). The yeast morphology is necessary for initial attachment, colonization, and dissemination of the organism at sites of infection (Saville et al. 2003). The hyphal form has the crucial responsibilities of host cell and tissue invasion as well as immune evasion (Shareck and Belhumeur 2011; Brand 2012). Infection of tissue and access to
the bloodstream requires the hyphal form to breach epithelial cells and their associated barriers to enter critical areas of the human body (Grubb et al. 2008, Wachtler et al. 2012). Hyphae are also important in escape from host immune cells, specifically macrophages (Lorenz et al. 2004). When *C. albicans* is locked into one morphological form, virulence is attenuated. For example, using a mouse model, treatment of systemic candidiasis was successful by inhibiting filamentation using a *Candida* strain engineered to alter its morphology based on the presence of doxycycline (Saville et al. 2006). Finally, *C. albicans* is part of the normal flora of most immunologically competent individuals and the host immune system has adapted to recognize cues from *C. albicans* when it shifts from a commensal to a pathogen. Moyes et al. (2010) observed that the yeast form adheres to host cells and is tolerated by the immune system, while a switch to hyphal morphology alerts and elicits a response from immune cells. This is clear evidence that morphogenesis is important for *C. albicans* to colonize and infect the host.

**Signaling Pathways of Morphogenesis**

*C. albicans* regulates its morphology in response to numerous cues perceived from the varied habitats in which it resides. These cues include temperature, nitrogen levels, serum, amino acid availability, pH, nutrient limitations, serum, quorum sensing, and solid matrixes (Sudbery 2011). Each environmental cue has an associated membrane detector or directly interacts with a genetic factor of the respective signaling system. For instance, temperature (37°C) is sensed by the heat shock protein Hsp90p, amino acids are detected by the G-protein coupled receptor (Gpr1p), nitrogen levels are detected via the ammonium permease (Mep2p), and CO$_2$ levels are detected through
the carbonic anhydrase (Nce103p) (Biswas and Morschhauser 2005; Maiden et al. 2005; Shapiro et al. 2009; Klengel et al. 2013). In contrast, embedded agar matrix, serum, and QS molecules act directly upon a component of their respective signaling cascade (Sudbery 2011). Consequently, with the varied cues that C. albicans responds to, there are a number of signaling pathways that have been identified to control morphology. Five of the most studied pathways include the cyclic AMP-protein kinase A (cAMP-PKA) pathway, the mitogen-activated protein kinase (MAPK) pathway, the pH pathway, the embedded matrix pathway, and a negative regulation of hyphae pathway. Each of the pathways also involve a variety of positive transcriptional regulators (Efg1p, Cph1p, Czf1p) and negative transcriptional regulators (Tup1p, Nrg1p, Rfg1p) of hyphal development (Sudbery 2011).

Of the known pathways, the most studied and most important is the cAMP-PKA pathway. This pathway controls, directly or indirectly, a number of the signals generated from environmental cues such as temperature, amino acids, CO2, serum, and nitrogen (Sudbery 2011). Activation of cAMP-PKA occurs via the adenyl cyclase, Cyr1p, which funnels the signals from multiple environment cues (Rocha et al. 2001). Cyr1p can be activated via a Ras1p dependent or independent manner. Ras1p is a membrane associated GTPase that upon physical association with Cyr1p causes an increase in cAMP levels (Biswas et al. 2007). Ras1p dependent activation of Cyr1p is through serum, nitrogen, and temperature cues. In contrast, independent activation of Cyr1p can occur through serum associated bacterial peptidoglycan, amino acid, and CO2 cues by directly associating with Cyr1p (Biswas et al. 2007). The ultimate target of
the cAMP-PKA pathway is the transcriptional factor Efg1p, which is the foremost positive regulator of hyphal development (Sohn et al. 2003).

The second pathway, MAPK, is also involved in signal response to temperature, nitrogen, and serum (Sudbery 2011). MAPK signaling is via Ras1p and the transcriptional target is the positive regulator Cph1p (Monge et al. 2006). While the cAMP-PKA and MAPK pathways have a number of shared environmental triggers, as well as genetic signaling components, the pH pathway only shares the target transcriptional regulator Efg1p (Sudbery 2011). In this pathway Rim21p, a membrane associated sensor, detects the pH of the environment and causes the proteolytic cleavage of the transcriptional regulator Rim101p that targets genes associated with hyphal development (Biswas et al. 2007). Rim101p, in turn, bypasses the signaling of the cAMP-PKA pathway and directly regulates Efg1p (Biswas et al. 2007). The fourth pathway, embedded matrix, involves the hyphal induction pathways associated with *C. albicans* when cells become embedded into agar. This pathway is also independent of the cAMP-PKA and MAPK pathways and the transcriptional target is CSF1 (Sudbery 2011). The last pathway, negative regulation, is associated with 3 different transcriptional repressors of hyphal development. Tup1p is the dominant repressor and has been implicated in the regulation of morphogenesis involving quorum sensing (Braun 1997; Kebaara et al. 2008). The other 2 transcriptional factors, Nrg1p and Rfg1p, seem to work in tandem with Tup1p and even recruit Tup1p when repressing filamentation (Kadosh and Johnson 2001; Murad et al. 2001). Thus, the events controlling *C. albicans* morphogenesis encompass a variety of environmental triggers that appear to regulate morphogenesis through multiple routes.
Quorum Sensing

Microorganisms are highly adaptable and, through close contact in shared environments, have developed means of communicating with each other. Cell to cell communication in the microbial world is a complex network that spans intraspecies, interspecies, and inter-kingdom interactions involving prokaryotic and eukaryotic microbes. The term quorum sensing (QS) has been used to describe this communication process involving small secreted molecules. QS is based on 2 main components, quorum sensing molecules (QSMs) and the increase in cell density of a population. Microorganisms secrete QSMs into the environment and, as the cell density of the population increases, so do the concentration of QSMs. Eventually, the population reaches a tipping point where the QSMs reach a threshold concentration. Once this threshold is met, the population can operate as a ‘quorum’ and activate signal transduction pathways to activate or repress genes responsible for a variety of biological functions, including motility, bioluminescence, morphology, and virulence (Antunes et al. 2010). Certain criteria should be applied to differentiate QSMs from other molecules a microorganism emits. These criteria include extracellular secretion/accrual in response to a certain change (such as cell growth), a dedicated receptor that recognizes the QSM, and a response by the microorganism that the QSM induces (Atkinson and Williams 2009). While QS has been studied extensively in single species systems, relatively little is known about the cross-kingdom communication of QS and QSMs in the polymicrobial setting of prokaryotic-eukaryotic communities.

Quorum Sensing in C. albicans. The discovery of QS in C. albicans and the QSM farnesol was the first documented example found in eukaryotes (Hornby et al.
The notion that morphology may be controlled by QSMs was observed when *C. albicans* cells were added to spent culture media, which induced this morphological change (Hornby et al. 2001). The *C. albicans* QSM farnesol acts in a density dependent manner. At concentrations below $1 \times 10^6$ cells/ml, *C. albicans* typically display the hyphal morphology (Kruppa 2009). However, as the cell density increases above this concentration, there is an accumulation of farnesol molecules in the environment and the cells revert to the yeast morphology (Hornby et al. 2001). Farnesol, at concentrations ranging from 10-250 µM, has the ability to inhibit the yeast to hyphal transition, even when *C. albicans* is grown under hyphal inducing conditions (Kruppa et al. 2004). Farnesol at this range inhibits biofilm formation but does not affect *C. albicans* growth rate or block elongation of pre-existing hyphae (Hornby et al. 2001; Ramage et al. 2002). *C. albicans* cells exposed to farnesol have decreased expression of hyphal formation associated genes, while also exhibiting an increase in drug and oxidative stress resistance (Cao et al. 2005; Enjalbert and Whiteway 2005; Westwater et al. 2005). A number of other molecules that *C. albicans* secretes have been suggested to be additional QSMs. However, most of these molecules either are not relevant at density dependent levels or produce only a small fraction of the change that farnesol exerts. For example, farnesoic acid was originally reported to be a QSM and inhibitor of *C. albicans* morphogenesis but was determined to be found in only one particular strain (Oh et al. 2001; Hornby and Nickerson 2004). Two other molecules, tryptophol and phenylethyl alcohol, were observed to have “autoantibiotic” properties by reducing filamentation in *C. albicans* (Lingappa et al. 1969). However, the concentrations of the 2 molecules proved to be extremely high to mediate morphological
control over *C. albicans* (Lingappa et al. 1969). Conversely, another QSM, tyrosol, has the opposite effect of farnesol. Tyrosol can induce hyphal production in *C. albicans*, which shortens the amount of time required to induce germ tube formation of yeast cells (Chen et al. 2004). This induction of hyphae can also affect the early stages of biofilm formation by increasing biomass (Alem et al. 2006). However, when added exogenously at the same time as farnesol, farnesol mutes the pro-hyphae effects of tyrosol (Alem et al. 2006). Clearly, *C. albicans* secretes a number of factors that control its morphology, but farnesol is the primary regulator of the yeast to hyphal transition.

Our understanding of QS signaling pathways in *C. albicans* is relatively limited and the receptor involved in farnesol response is unknown. Five elements have been implicated as playing roles in *C. albicans* signaling and regulation of QS response. These include Ras1p, Tup1p, Cph1p, Hst7p, and Chk1p (Kruppa 2009). Ras1p is a major regulator of farnesol induced response by signaling through either the cAMP-PKA or MAPK pathways that govern hyphal production (Leberer et al. 2001). Tup1p, a transcriptional regulator, negatively regulates hyphae production in *C. albicans* (Braun 1997). Upon exposure to farnesol, *TUP1* mRNA and protein levels are induced (Kebaara et al. 2008). Cph1p and Hst7p are integral parts of the MAPK signaling cascade and mRNA of both genes are decreased in *C. albicans* cells that are exposed to farnesol (Sato et al. 2004). MAPK signaling is heavily involved in the regulation of filamentation, especially in response to numerous environmental stimuli (Shapiro et al. 2011). Lastly, Chk1p is a histidine kinase involved in cell wall biosynthesis via a 2-component signaling system (Kruppa et al. 2004). *CHK1* mutants exposed to farnesol are not responsive to the inhibitory effects and grow mainly as hyphae (Kruppa et al. 2004).
It is not entirely clear at this point how farnesol response signals through these multiple genetic elements.

**Quorum Sensing in Bacteria.** Bacterial QS and QSMs were first described in the bioluminescence of *Vibrio fischeri* and its mutualistic relationship with the bobtail squid (Kempner and Hanson 1968). Since this initial discovery, the process of QS and the components that constitute the machinery of QS have been described in numerous bacterial species. QS in bacteria can be divided into 3 different classes. The first class belongs to Gram-positive bacteria, such as *S. aureus*, which involves small autoinducing peptides (AIPs) as QSMs, coupled with an accessory gene regulator (*agr*) 2-component regulatory mechanism (Antunes et al. 2010). The *agr* locus encodes *agrA*, *agrB*, *agrC*, and *agrD* (Antunes et al. 2010). *AgrD* encodes the immature AIP, which is cleaved and secreted by the membrane bound AgrBp (Qiu et al. 2005). When extracellular levels of AIP reach a sufficient level, the membrane associated AgrCp binds AIP and activates AgrAp through phosphorylation of ArgCp (Lina et al. 1998). The *agrA* protein then binds to target genes and induces expression of over 70 different genes of which 23 are associated with virulence factors (Antunes et al. 2010).

The second class belongs to Gram-negative bacteria, such as *P. aeruginosa*, and involves homoserine lactone molecules (HSL) coupled with a LasI/LasR system (Antunes et al. 2010). The LasI component makes HSL that is able to diffuse freely across the cell membrane (Antunes et al. 2010). HSL molecules engage the LasR component, which binds to the promoter of the appropriate gene and initiates transcription (Antunes et al. 2010). In actuality, *P. aeruginosa* has 3 different QS systems as well as an “orphan receptor” for QS. In addition to the LasI/LasR QS
system there is a Rhl/C4HSL QS system that works much like the 3OC12HSL/Las system (Antunes et al. 2010). Finally, in the *Pseudomonas* quinolone signal system (PQS) the *pqxABCD* operon is involved in the genesis of 2-heptyl-4-quinolone (HHQ), the precursor of PQS (Diggle et al. 2006). The PQS system links the *Las* system to the *Rhl* system that is under the control of the *Las* to regulate virulence (Diggle et al. 2006; Dekimpe and Deziel 2009).

Finally, the third class belongs to both Gram-positive and Gram-negative bacteria and is considered an interspecies QS communication pathway. This class involves Autoinducer-2 (AI2) as the signaling molecule coupled with a *LuxS/LuxR* system (Antunes et al. 2010). AI2 is synthesized by *LuxS* and this system has been identified in more than 55 bacterial species (De Keersmaecker et al. 2006). Even bacterial species that do not secrete AI2 still have the ability to respond to AI2, which lends credence to the notion that this is a universal molecule of bacterial communication (Dufour and Rao 2011).

**Drug Treatment and Resistance**

*C. albicans* has relatively few therapeutic treatments compared to the number of antibiotics available for bacterial infections. There are 4 main classes of drug targets/treatments for *C. albicans*: the azoles, polyenes, fluorocytosines, and echinocandins. Each drug target/treatment has benefits and drawbacks. The azoles, which include fluconazole, target the membrane by inhibiting an enzyme essential in ergosterol biosynthesis (Tobudic et al. 2010). These drugs are fungistatic as they inhibit growth and do not actually kill *C. albicans*. In cases of resistance to azoles, there are
several mechanisms, which include overexpression of lanosterol, an intermediate in the ergosterol pathway, as well as mutations that affect the binding of azoles to their targets (Cannon et al. 2007). Unfortunately, because azoles are fungistatic, this can lead to selective survival that increase the chances of resistance (Cannon et al. 2007). The polyenes, which include nystatin, target and bind to ergosterol, allowing pore formation in the membrane and reduces the integrity of the cell (Cannon et al. 2007). Polyenes are fungicidal and resistance is rare making them very useful in the treatment of systemic Candida infections (Cannon et al. 2007). When resistance occurs, it is due to a reduction of ergosterol in the plasma membrane (Cannon et al. 2007). The disadvantage of polyenes is host toxicity, especially during prolonged use, due to the similarity of ergosterol to human cholesterol (Shapiro et al. 2011). The echinocandins, including caspofungin, are the newest of the drug classes and inhibit cell wall biosynthesis, thus causing a loss in cell wall integrity (Shapiro et al. 2011). Less is known about the long-term effects of echinocandins and there have been documented instances of drug resistance (Shapiro et al. 2011). In all 3 drug classes, drug resistance is associated with modulation of the drug target, increase in drug exporters, acquired responses to stress, and biofilm formation (Cannon et al. 2007). In respect to morphology, drug resistance and mature biofilm growth that exhibit increased drug resistance require the ability to form both yeast and hyphal morphologies (Chandra et al. 2001). The harmful side effects of current drugs, the increase in resistance to current treatments, and the lack of new antifungal targets demonstrate a need for novel applications of known virulence inhibitors for C. albicans. Thus, the polymorphic nature of C. albicans seems an attractive candidate. Targeting and controlling virulence factors
rather than eradicating and selecting for drug resistance may be an ideal method of combating *C. albicans*, especially because it is part of the normal flora.

**Candida-Bacterial Interactions**

The interaction of *Candida* with bacteria is quickly becoming an important area of medical research for a number of reasons. *C. albicans* is normally a commensal organism until conditions change that benefits its growth and pathogenicity. There is mounting evidence that *Candida* infections spawn from a person’s own normal flora (Wargo and Hogan 2006). Research has implicated that the normal flora bacterial members, in addition to many other beneficial attributes, keep the commensal *Candida* in check (Mason et al. 2012). When imbalances occur, as in antibiotic treatment, the imbalance tips the scale in favor of *Candida* overgrowth to transition from normal flora to pathogenic (Mason et al. 2012). Secondly, *Candida*-bacterial interactions can be harmful or beneficial to the host and understanding these interactions could aid in human health. Beneficial interactions include chemical and physical interactions that indicate bacteria can inhibit *Candida* virulence factors and suppress overgrowth (Boris and Barbés 2000). Harmful interactions include increased mortality from polymicrobial infections and *Candida*-bacterial biofilms that intensify persistence and severity of infection as well as increased drug resistance (Carlson 1982; Harriott and Noverr 2009; Peleg et al. 2010). Lastly, the understanding of polymicrobial interactions is, at best, in its infancy. There exists a great potential for the development of new antifungals and antimicrobials derived from the uncharacterized world of *Candida*-bacterial interactions.
Sites of Candida-Bacterial Interactions

**Systemic Infections.** Perhaps the most dangerous form of polymicrobial interaction to human health involves bloodstream infections (BSI). Once microorganisms gain access to the bloodstream, dissemination and infection can manifest in practically any part of the human body. *C. albicans* is the fourth most common causative agent of nosocomial BSIs and 27% to 56% of *C. albicans* BSIs are polymicrobial (Wisplinghoff et al. 2004; Klotz et al. 2007). In a study of *Candida* BSI patients, the most commonly isolated bacterial species with *Candida* was *S. aureus* (20%), while *P. aeruginosa* (8%), and *E. coli* (4%) were also coisolated (Klotz et al. 2007). Patients with bacteremia and candidemia have reduced survival rates compared to those with candidemia alone (Dyess et al. 1985).

**Burn Wounds.** A breach in the skin barrier is an example of trauma that can leave an individual at risk for microbial infections. The principal bacterial species found in burns include *Klebsiella, Pseudomonas,* and *Staphylococcus* (Gupta et al. 2005). As the number of bacterial species present in the burn wound increases, the presence of *Candida* decreases, suggesting an antagonistic relationship in this setting (Gupta et al. 2005). *Pseudomonas* and *C. albicans* are almost never co-isolated in burn wounds (Gupta et al. 2005). This particular observation adds to the continuing theme of antagonism between *P. aeruginosa* and *C. albicans.*

**Lung/Pulmonary Tract.** *Pseudomonas,* *Staphylococcus,* and *Candida* species are 3 of the most common agents associated with ventilator-associated pneumonia (VAP) (Adair et al.1999). Patients with mechanical ventilation who have colonization of
*Candida* in the respiratory tract have an increased risk of *Pseudomonas* VAP (Azoulay et al. 2006). Ader et al. highlighted this important association as treatment of *Candida* colonization of the respiratory tract with antifungals reduces the risk of *Pseudomonas* associated VAP (Ader et al. 2008). *C. albicans* co-infections may also impair the host immune system’s ability to deal with *Pseudomonas* VAP (Roux et al. 2009). These observations suggest that *Candida-Pseudomonas* interactions in burn wounds and VAP are fundamentally different.

While *Candida* can be found in the lungs of healthy individuals, patients with cystic fibrosis (CF) provide microorganisms an ideal habitat where *Candida*-bacterial interactions appear abundant. A study of the microbial populations of the sputa of patients with CF found that *C. albicans* was present in 78% of the patients (Valenza et al. 2008). Bacterial colonizers included *S. aureus* (63%), *P. aeruginosa* (50%), and *E. coli* (5%) (Valenza et al. 2008). The population of microbes in the CF lung changes over time, with *S. aureus* associated with young children and *P. aeruginosa* in 80% of CF lungs of people in their 20s (Hauser et al. 2011). This association with *P. aeruginosa* also resulted in a decline in health (Hauser et al. 2011). *C. albicans* colonization in CF patients is best predicted by the cocolonization by *P. aeruginosa*, and this cocolonization between the 2 leads to increased exacerbation of symptoms (Chotirmall et al. 2010).

**The Oral Cavity.** Oral colonization and infections are highly polymicrobial in nature. In a study of patients with atrophic denture stomatitis, 78% of patients had coinfections with both *S. aureus* and *C. albicans* (Monroy et al. 2005). In periodontal disease there is a strong association of *Staphylococcus* with *Candida* (Cuesta et al. 2009).
2010). Of the patients who have periodontal disease, *Staphylococcus* species were found in 69.5% of the patients, while *Candida* species were identified in 42.7%, with the most common species being *S. aureus* and *C. albicans* (Cuesta et al. 2010). In dental prosthesis wearers, *C. albicans* can be isolated from 86% and *S. aureus* was found in 84% of patients with atrophic denture stomatitis (Monroy et al. 2005).

**Vaginal Infections.** Normal flora bacteria play a pivotal role in preventing *C. albicans* vaginal infections. An estimated 75% of women will experience at least one vaginal infection, with about 5-10% of women having recurrent vaginal candidiasis (Sobel 1988). *Lactobacillus* species, which constitute a majority of the vaginal microbiome, have been shown to have inhibitory effects on *Candida* and can aid in preventing *Candida* infections. Antibiotic use disrupts this protection by destroying the bacterial biome allowing *Candida* overgrowth (Noverr and Huffnagle 2004; Strus et al. 2005). Fluconazole treatment in conjunction with probiotic use of *Lactobacillus* was found to aid in the treatment of vaginal candidiasis (Martinez, Franceschini et al. 2009). In addition, *Lactobacillus* has been shown to reduce the ability of *C. albicans* to adhere to and infect tissue (Martinez, Seney, et al. 2009).

*Candida* Biofilms

*C. albicans* biofilm formation can be divided into stages based on time and development. The early stage (0-2 hours) encompasses attachment and primarily involves the yeast morphological form of *C. albicans* (Chandra et al. 2001). The intermediate stage (12-30 hours) involves yeast, pseudohyphal, and immature true hyphae, and the final phase, maturation (38-72 hours), involves yeast, pseudohyphal,
and mature hyphae (Chandra et al. 2001). The fully mature biofilm stage has 3D architecture, channels for fluid movement, and is encased in an extracellular matrix (Ramage, Martínez, et al. 2006). Finally, the dissemination stage results in the release of yeast from terminal hyphae of the mature biofilm into the environment (Uppuluri et al. 2010). Morphological transition is important for the development of fully mature *C. albicans* biofilms. Wild type strains of *C. albicans* form a normal robust biofilm with a thin yeast foundation layer and thick upper hyphal layers (Baillie and Douglas 1999). Conversely, a mutant locked in the yeast morphology could only form a yeast base layer, while a mutant locked into the hyphal morphology formed only an outer hyphal layer (Baillie and Douglas 1999). Additionally, the locked yeast or hyphal forms show decreased biofilm integrity and increased antifungal drug sensitivity (Baillie and Douglas 1999).

*Candida* biofilms play a significant role in nosocomial infections associated with implanted devices. Kojic et al. (2004) identified nosocomial agents associated with commonly implanted devices. *Candida*, as the causative agent, correlated with vascular catheters (10%), prosthetic valves (10%), ventricular assist devices (28-66%), ventriculoperitoneal shunts (6-15%), and urinary catheters (10-30%) (Kojic and Darouiche 2004). A second benefit of biofilms is an increase in antifungal drug resistance. Fluconazole resistance in biofilms can be up to 400 times that of planktonic cells, with the MIC of fluconazole 128 times higher in biofilms than in planktonic cells (Seneviratne et al. 2008). This increased resistance can also be found with other azoles and amphotericin B used to treat *Candida* infections (Hawser and Douglas 1995). Lastly, *Candida*-bacterial mixed biofilms have numerous benefits to the
microorganisms involved including increased attachment opportunities, nutritional needs, stability, drug resistance, and protection from host immune defense (Adam et al. 2002; Bamford et al. 2009; Harriott and Noverr 2009; Shirtliff et al. 2009). For example, S. aureus forms poor biofilms, but when mixed with C. albicans can associate with hyphae and form robust biofilms (Harriott and Noverr 2009). Additionally, P. aeruginosa use C. albicans biofilms to establish infection by attaching to the hyphae form and use them as a scaffold to form biofilms. Whether as monospecies or polyspecies, biofilms have an enormous impact on human health with 65% of human infections attributed to biofilms (Li and Tian 2012).

**Candida-Bacterial Physical Interactions**

*Candida and E. coli.* The physical interaction and type of relationship *E. coli* and *C. albicans* share vary depending on the aspect being examined. In general, *E. coli* seems to have a suppressive effect on *C. albicans* growth. *E. coli* inhibits *C. albicans* germ tube formation, but delineation of this effect, whether it be mediated by physical interactions, chemical interactions, or a combination of the two, has not been substantially examined thus far (Nair et al. 2001). Studies involving the physical interactions, particularly attachment, have produced conflicting results. For example, when using human cells, preincubation of *E. coli* significantly increased the number of *C. albicans* cells that were able to attach to human cells and it was hypothesized that *E. coli* fimbriae participated in this process (Makrides, 1983). The same *E. coli* strain was also used in a separate study and showed an increase in *C. albicans* attachment to abiotic surfaces (Nair and Samaranayake 1996a). Under microscopic observation, *E. coli* cells were reported to act as attachment sites for the yeast form of *C. albicans* (Nair
and Samaranayake 1996a; Nair and Samaranayake 1996b). However, there was a decrease in overall *C. albicans* attachment when epithelial cells were preincubated with *E. coli* (Nair and Samaranayake 1996b). Conversely, preincubation of abiotic surfaces with nonpathogenic *E. coli* inhibited attachment of *C. albicans*, but it is unknown whether this is due to *E. coli* out competing *C. albicans* for space or due to a secreted factor (Trautner et al. 2010). In a biofilm model, *E. coli* growth is increased when co-cultured with *C. albicans*, compared to the biofilm mass of *E. coli* alone (Thein et al. 2007). There is a negative effect with a decrease in viable cell mass of *C. albicans* as the concentration of *E. coli* increased (Thein et al. 2007). This study also found that after 48 hours, *C. albicans* blastospores and a majority of hyphae were nonviable, but *E. coli* remained viable in dual species biofilms (Thein et al. 2007). These results indicate that the interactions between *E. coli* and *C. albicans* vary depending upon a series of factors such as which microorganism is in greatest concentration, which attaches first, and how the relationship evolves over time.

*Candida* and *P. aeruginosa*. Arguably, the majority of research into *Candida*-bacterial relationships has centered on the interactions of *C. albicans* with *P. aeruginosa*, which seem to have an antagonistic relationship. Hogan et al. found that when coincubated *P. aeruginosa* was able to attach to, form biofilms on, and ultimately kill *C. albicans* hyphae (Hogan and Kolter 2002). In contrast, *P. aeruginosa* was unable to attach to yeast cells, which retain their viability (Hogan and Kolter 2002). Subsequently, a number of factors were identified that were required for *P. aeruginosa* interactions with *C. albicans* hyphae (see Table 1.1). Type IV pili of *P. aeruginosa* appear necessary for physical interactions with *C. albicans*, as type IV pili mutants are
attenuated in their attachment to hyphae and have delayed killing time (Hogan and Kolter 2002). Additionally, the presence of bacteria was required for the killing of C. albicans hyphae, whereas bacterial media alone did not have this effect (Hogan and Kolter 2002). However, another study showed that cell free bacterial media alone can kill hyphae but not as effectively as live bacteria (Brand et al. 2008).

Another factor involved in the physical interaction and killing by P. aeruginosa is the glycosylation status of the C. albicans cell wall (Brand et al. 2008). C. albicans strains that have defects in O-linked glycosylation were more susceptible to P. aeruginosa contact mediated cell death, suggesting that cell wall glycans play a role in attachment as well as cell viability (Brand et al. 2008). Cell death may also be linked to attachment. Brand et al. found that C. albicans cell lysis occurs at points of P. aeruginosa microcolony attachment (Brand et al. 2008). This suggests that contact dependent factors play a role in the antagonistic relationship of P. aeruginosa and C. albicans. In mixed Candida-Pseudomonas biofilms, competitive attachment is prevalent. In a study of attachment and biofilm growth in mixed biofilms, the ability to produce glycocalyx affected Candida-Pseudomonas interactions (El-Azizi et al. 2004). Glycocalyx deficient P. aeruginosa biofilms reduced the attachment and biofilm growth of C. albicans, while glycocalyx positive P. aeruginosa biofilms increased C. albicans attachment (El-Azizi et al. 2004). Conversely, C. albicans preformed biofilms enhanced the adhesion and biofilm growth of nonglycocalyx producing P. aeruginosa (El-Azizi et al. 2004). When bacteria and C. albicans were added simultaneously, there was a decrease in C. albicans attachment despite the glycocalyx status of the P. aeruginosa (El-Azizi et al. 2004). The antagonistic relationship between C. albicans and P.
*P. aeruginosa* can also be seen over the course of biofilm growth. *P. aeruginosa* exudes a suppressive effect on *C. albicans* during the attachment phase (57% reduction), maturation phase (67% reduction), and fully mature biofilms (99% reduction) (Bandara et al. 2010). *Candida-Pseudomonas* interactions continue to be the most studied polymicrobial interactions due to their opposing effects upon each other and serve as a template for eukaryotic-prokaryotic relationships.

**Candida and S. aureus.** *C. albicans* and *S. aureus* appear to have a mutualistic relationship, with *S. aureus* being the third most common microorganism coisolated in polymicrobial *C. albicans* bloodstream infections (Klotz et al. 2007). This synergism and accompanying physical interactions are best observed in mixed *Candida-S. aureus* biofilms. *S. aureus* preferentially associates and forms colonies on hyphae without harming the viability of hyphae as with *P. aeruginosa* coculture (Harriott and Noverr 2009; Harriott and Noverr 2010; Peters et al. 2010). *C. albicans* also confers the ability of *S. aureus* to form biofilms in serum, where typically it cannot, while also protecting *S. aureus* from antibiotics (Harriott and Noverr 2009). It is hypothesized that *C. albicans* encases *S. aureus* in an extracellular matrix, thereby obstructing the effects of antibiotics on the bacteria (Harriott and Noverr 2009). *S. aureus* attachment is strongest at the tip and middle of *C. albicans* hyphae with weaker attachment to *S. aureus* to yeast cells (Ovchinnikova et al. 2012). Using mutants deficient in hyphal production, it was shown that morphogenesis is instrumental in the ability of *S. aureus* to form mixed biofilms with *C. albicans* (Harriott and Noverr 2010). Only mutants that have the ability to attach to abiotic surfaces can increase antibiotic resistance of *S. aureus* (Harriott and Noverr 2010). Using atomic force microscopy in conjunction with
C. albicans mutants, Peters et al. found that Als3p proved to be a significant player in the attachment of C. albicans to S. aureus (see Table 1.1) (Peters et al. 2012). The expression of Als3p in S. cerevisiae allowed the binding of S. aureus, to which it normally cannot attach (Peters et al. 2012). The most interesting finding involving physical interactions of C. albicans and S. aureus is the synergistic tissue invasion of coinfections. S. aureus, which normally cannot invade tissue, appears to use C. albicans hyphae to gain tissue access. In murine tongues it was shown that S. aureus could only penetrate the tissue when attached to the hyphal form of C. albicans and abolishing C. albicans Als3p negated this effect (Peters et al. 2012). Attachment most likely involves other factors yet to be identified. While live C. albicans cells are not necessary for S. aureus to form biofilms, heat inactivated or formalin exposed C. albicans cannot support S. aureus attachment/biofilm growth, indicating additional Candida surface components are involved (Harriott and Noverr 2010). Morphology also plays a role in the relationship between C. albicans and S. aureus. In C. albicans hyphal biofilms there is an upregulation of S. aureus proteins associated with metabolism and protein synthesis (Peters et al. 2010). However, in yeast biofilms, S. aureus induces protein expression related to cell stress response in C. albicans (Peters et al. 2010). The factors of C. albicans morphogenesis, hyphal association, biofilm formation, and antibiotic resistance all form new perspectives on the importance of understanding Candida-S. aureus coinfections and the importance of understanding the physical interactions of the 2 microorganisms.

Other Important Candida – Bacterial Interactions. Acinetobacter baumannii inhibits C. albicans filamentation by attaching to the hyphal form, leading to the death of
filaments, but leaves yeast cells viable (Peleg et al. 2008). An outer membrane protein, ompA, of *A. baumannii* is involved in attachment to *C. albicans* and necessary for the killing of hyphae (see **Table 1.1**) (Gaddy et al. 2009). *Salmonella enterica* serovar *typhimurium* adheres to *C. albicans* filaments causing filament death (Kim and Mylonakis 2011). In contrast to *A. baumannii* and *S. typhimurium*, *Streptococcus gordonii* appears to share a mutualistic relationship with *C. albicans* by aiding in the colonization of the oral cavity by promoting hyphal formation of *C. albicans* (Bamford et al. 2009). There are a number of factors involved in attachment of *S. gordonii* to *C. albicans*, including cell surface polypeptides and salivary adhesions (Holmes et al. 1996). *C. albicans* adhesin proteins have been shown to be required for *S. gordonii* attachment, namely Als3p, Eap1p, and Hwp1p (Nobbs et al. 2010). *S. gordonii* SspB, of the antigen family peptides, attaches to *C. albicans* Als3p (Bamford et al. 2009; Silverman et al. 2010). *S. gordonii* takes up salivary proteins and displays them on their surface that *C. albicans* can use for attachment (O’Sullivan et al. 2000). Another *Streptococcus* species, *S. mutans*, uses glucosyltransferases to attach to *C. albicans* (Gregoire et al. 2011). *S. salivarius* attaches to and covers *C. albicans* hyphae (Ishijima et al. 2012).

**Table 1.1** Attachment factors associated with *Candida*-bacterial interactions.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Proposed Attachment Factors</th>
<th>Attachment Yeast</th>
<th>Attachment Hyphae</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Acinetobacter baumannii</em></td>
<td><em>A. baumannii</em>: ompA</td>
<td>Limited</td>
<td>Yes</td>
<td>(Gaddy et al. 2009)</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td><em>E. coli</em>: Unknown (fimbriae?)</td>
<td>Yes</td>
<td>?</td>
<td>(Makrides, 1983, Microbios)</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td><em>P. aeruginosa</em>: Unknown (pili?)</td>
<td>No</td>
<td>Yes</td>
<td>(Hogan and Kolter 2002; Brand et al. 2008)</td>
</tr>
<tr>
<td><em>Salmonella</em></td>
<td><em>S. enterica</em>: sopB</td>
<td>Limited</td>
<td>Yes</td>
<td>(Kim and Mylonakis)</td>
</tr>
</tbody>
</table>
Candida-Bacterial Chemical Interactions

*Candida and E. coli*. Chemical interactions between *E. coli* and *C. albicans* remain ill defined. *E. coli* is known to use the QSM AI2, but when Bandara et al. examined the effect of AI2 on a panel of *Candida* species in regards to biofilm growth, there was to be no effect on *C. albicans* (Bandara 2011). A relatively new QSM, AI3, and associated QS system has been described in *E. coli*; however, the signaling pathways and the resulting effects on virulence are still being investigated (Antunes et al. 2010). It is quite possible *E. coli*, given its overwhelming abundance in the human body, exudes a number of other QSMs or chemical metabolites that are unknown at this time.

Bacterial lipopolysaccharide (LPS) may serve as a communication messenger between bacteria and *Candida*. In a study of *E. coli* LPS on biofilm formation, there was no effect on *C. albicans* biofilms, but it did have effects on other *Candida* species (Bandara et al. 2009). *In vivo*, *E. coli* LPS injected into mice along with *C. albicans* have a number of effects including increased virulence and increased number of cells in tissue (Akagawa et al. 1995; Henry-Stanley et al. 2003).
Candida and P. aeruginosa. P. aeruginosa has an arsenal of secreted molecules and metabolites at its disposal, of which many have been shown to influence C. albicans (see Table 1.2). Hogan et al. demonstrated that P. aeruginosa mutants with genetic defects in secreted factor genes, such as PlcS and PlcR (phopholipase C), PhnAB (phenazines), GacA (HSL and pyocyanin), LasR (QS molecules), RhlR (QS molecules), and RpoN (QS response) are attenuated in the killing of C. albicans hyphae (Hogan and Kolter 2002). Hogan et al. (2004) also identified P. aeruginosa genes essential in inhibition of C. albicans morphological changes, including genes (lasL, lasR, rhlL, rhlR) involved in the secretion of HSL molecules. This led to the identification of 3-oxo-C12 homoserine lactone (3OC12HSL), a QSM of P. aeruginosa, as having a significant effect on C. albicans morphology (Hogan et al. 2004). Purified 3OC12HSL inhibits C. albicans filamentation, reverts preformed filaments back to the yeast form, induces expression of yeast genes, and decreases expression of hyphal genes (Hogan et al. 2004). Testing a battery of similar molecules, it was found that this effect on C. albicans filamentation was linked to a similar 12 carbon chain structure that is shared between 3OC12HSL and the C. albicans QSM farnesol (Hornby et al. 2001; Hogan et al. 2004). HSL production is strain specific among P. aeruginosa and media from HSL producing strains is able to inhibit C. albicans morphological transition, while inhibition is not found with reduced HSL producing strains (McAlester et al. 2008). In contrast, supernatants from P. aeruginosa HSL producers and nonproducers inhibited C. albicans biofilm formation at the maturation phase but not at other biofilm stages (Holcombe et al. 2010). Gene expression of C. albicans biofilms exposed to P. aeruginosa supernatant from both HSL producing and non-HSL producing strains affect
different genes but also regulate expression of an overlapping 238 genes (Holcombe et al. 2010; Reen et al. 2011). HHQ, a precursor to PQS, inhibits C. albicans biofilm formation without affecting adhesion or morphology (Reen et al. 2011). Phenazines also reduce C. albicans viability, ability to produce hyphae, attachment, alter metabolism, and influence biofilm formation (Morales et al. 2013). P. aeruginosa cis-2-decenoic acid induces C. albicans biofilm dispersion, as well as that of many other bacterial biofilms (Davies and Marques 2009). Pyocyanin and 1-hydroxyphenazine inhibit C. albicans growth and pyocyanin treated C. albicans cells lack hyphal development (Kerr et al. 1999). Taken together, the numerous molecules involved in Candida-Pseudomonas interactions highlight the steps each microorganism will take to gain an advantage.

Candida and S. aureus. Although S. aureus and C. albicans have a number of intricate physical associations, particularly with the hyphal form, and are found together in numerous areas of the human body, relatively little is known about their chemical interactions. While S. aureus employs a peptide system of QS, to date these AIPs have not been tested for influence on C. albicans.

Other Notable Candida-Bacterial Chemical Interactions. Acinetobacter baumannii spent bacterial media from the stationary phase growth inhibits C. albicans filamentation and biofilm growth but not to the same extent of live cells (Peleg et al. 2008). A. baumannii implements a LuxI autoinducer system, but mutations in LuxI do not inhibit filamentation (Peleg et al. 2008). However, a mutant of the A. baumannii gacS sensor kinase has reduced filament inhibition and cell death of C. albicans, indicating secreted molecules may play a role in this process (Peleg et al. 2008).
*Xanthomonas campestris* produces a diffusible signal factor (DSF), cis-11-methyl-2-dodecenoic acid, that acts as a signaling molecule to control virulence (Wang et al. 2003). DSF is similar structurally to farnesol and can inhibit the yeast to hyphal shift in *C. albicans* albeit with less potency (Wang et al. 2003).

*Burkholderia cenocepacia* produces a signal molecule very similar to *X. campestris* DSF and structurally similar to the *C. albicans* QSM (Zhang et al. 2011). This signal molecule, cis-2-dodecenoic acid (BDSF), is a potent inhibitor of *C. albicans* germ tube formation with stronger inhibitory effects than that of farnesol, HSL, and DSF (Boon et al. 2008). Zhang et al. expanded on these findings and found that BDSF is an effective inhibitor of *C. albicans* biofilm formation but has limited effects on preformed biofilms (Zhang et al. 2011). Also, BDSF greatly reduces the expression of hyphal specific genes *HWP1* and *ALS3* (Zhang et al. 2011).

*Enterococcus faecalis* supernatant inhibits *C. albicans* filamentation in a *Caenorhabditis elegans* model, while also inhibiting biofilm formation, but does not seem to kill *C. albicans* (Cruz et al. 2013). *E. faecalis* uses small peptides as QSMs, GBAP, and the Fsr QS system. GBAP does not seem to be involved, but the Fsr system does play a role in the inhibitory effects on *C. albicans* (Cruz et al. 2013).

*Streptococcus mutans* secretes a number of molecules that have effects on *C. albicans*. The first molecule shown to influence *C. albicans* was trans-2 decenoic acid, which shares similar structure to farnesol and *X. campestris* DSF, thus was given the name *Streptococcus* diffusible factor (SDSF) (see Table 1.2) (Vilchez et al. 2010). SDSF from stationary cultures inhibits *C. albicans* hyphal formation, but, this effect is
only observed at 2 and 4 hours of incubation and dissipates after 6 hours (Vilchez et al. 2010). SDSF was also able to reduce the expression of hyphal specific genes \textit{HWP1} and \textit{SAP5} (Vilchez et al. 2010). Researchers found that SDSF production was not limited to \textit{S. mutans} but also found in \textit{S. sanguinis}, \textit{S. oralis}, and \textit{S. mitis} (Vílchez et al. 2010). Another molecule produced by \textit{S. mutans}, mutanobactin A, has been shown to inhibit the yeast to hyphal morphological shift (Joyner et al. 2010). \textit{S. mutans} can inhibit germ tube formation even when physically separated from \textit{C. albicans}. Spent media from 4 hours inhibits germ tube formation, but 6, 8, and 24 hour does not (Jarosz et al. 2009). Competence stimulating peptide (CSP), a 22 amino acid peptide, acts as a QSM of \textit{S. mutans} and is produced in the beginning of exponential growth phase (Jarosz et al 2009). Mutants of \textit{comC} that encodes CSP could not reproduce this inhibition (Jarosz et al. 2009). CSP does not inhibit growth nor evoke stress response or significantly inhibit biofilm formation (Jarosz et al. 2009). Other \textit{Streptococcus} species tested had varying effects similar to \textit{S. mutans} (Jarosz et al. 2009).

Indole is a molecule shared among both Gram-positive and Gram-negative bacteria that is hypothesized to be a QSM of \textit{E. coli} used in the communication of biofilm populations (Lee and Lee 2010). Raut et al. observed that physiologically relevant levels of indole inhibited \textit{C. albicans} growth, germ tube formation, adhesion, and blocked biofilm formation (Raut et al. 2012). Oh et al. (2012) found that indole was able to reduce the attachment of \textit{C. albicans} to human epithelial cells at concentrations that did not affect the viability of human cells. Additionally, in a \textit{C. elegans} model, indole was able to inhibit filamentation, increase survival of the worm, and led to an overall lower \textit{C. albicans} burden (Oh et al. 2012). When examining the effects of indole
on *C. albicans* gene expression it was unexpectedly found that genes that are normally affected by bacterial influences on *C. albicans* virulence, most notably *ALS3* and *HWP1*, were unaffected; however, *NRG1*, a transcriptional repressor of filamentation, was greatly increased (Oh et al. 2012).

Peptidoglycan (PPG) constitutes the cell wall of both Gram-positive (in large amounts) and Gram-negative bacteria (in smaller amounts). Xu et al. (2008) found that PPG from *E. coli* and *S. aureus* did not alter *C. albicans* morphogenesis, but when PPG is hydrolyzed it can promote hyphal morphology. The subunit of PPG that was shown to be the active inducer of hyphae was muramyl dipeptide (Xu et al. 2008). Purified and synthetic muramyl dipeptides interact with Cyr1p and Ras1p for the signaling of hyphal development (Xu et al. 2008). Serum, depending on the source, is a potent inducer of hyphae and PPG can be readily found in serum (Xu et al. 2008). PPG is also found in the intestines where *C. albicans* may use it to induce filamentation for invasion (Xu et al. 2008).

Lipopolysaccharides (LPS), large lipid polysaccharides, are major constituents of Gram-negative bacterial membranes. As mentioned previously, *E. coli* LPS has no effect on *C. albicans* biofilms but has varying effects on other *Candida* species (Bandara et al. 2009). Bandara et al. (2010) also examined different bacterial LPS effects on biofilm growth using a number of different *Candida* species, again finding that the effect is *Candida* species specific. *Serratia marcescens*, and *Salmonella typhimurium* LPS had no significant changes in *C. albicans* biofilms at any stage (Bandara et al. 2010). *P. aeruginosa* LPS significantly increased *C. albicans* metabolic activity during biofilm attachment (0-90 minutes) phase, with increases in yeast cell
numbers as well as the presence of numerous dead *C. albicans* cells (Bandara et al. 2010). *Klebsiella pneumonia* LPS also produced significant increases in metabolic activity at both the attachment and maturation (90 min-24 hours) of *C. albicans* biofilms (Bandara et al. 2010). The overall message was that LPS from different bacterial species affect different *Candida* species in different ways in a time, concentration, and species dependent manner (Bandara et al. 2010). Generally, higher concentrations of LPS from both bacteria inhibited *C. albicans* metabolic activity, while lower concentrations enhanced metabolic activity (Bandara et al. 2013). *P. aeruginosa* LPS resulted in increased yeast cell morphology and decreased hyphal cell morphology in biofilms with “patchy” architecture, but the cells were still viable (Bandara et al. 2013). LPS from both *P. aeruginosa* and *K. pneumonia* increased expression of hyphal specific genes and *P. aeruginosa* LPS inhibited protein synthesis in *C. albicans* (Bandara et al. 2013). One factor that could explain the varying effects of LPS on *C. albicans* could be the small differences in LPS structure between strains as well as differences between species.

*Lactobacillus rhamnosus* bacteria inhibit *C. albicans* germ tube formation, while heat killed bacteria are unable to (Nover and Huffnagle 2004). Bacterial supernatant from 2 hour (30-55%) and 24 hour (92-98%) growth of *L. casei, L. paracasei*, and *L. rhamnosus* all inhibited germ tube formation (Nover and Huffnagle 2004). Noverr et al. tested a number of short chain fatty acids readily produced by *Lactobacillus* and butyric acid, inhibited in a concentration dependent manner (Nover and Huffnagle 2004). When gene expression is examined in *C. albicans*-Lactobacillus coculture there are
changes drug exporters, stress responses, transport, signal transduction, and targets of fluconazole gene expression (Köhler et al. 2012).

*Streptococcus gordonii* stationary phase media can induce hyphal formation in *C. albicans*, but a *S. gordonii luxS* mutant, with a deletion for Al2 QS, cannot produce the induction of hyphae (Bamford et al. 2009). Precursors of Al2 used to mimic Al2 could not rescue the lux2 mutant’s ability to induce hyphae in *C. albicans* (Bamford et al. 2009). Thus, either the precursors of Al2 used in this experiment are a poor substitute or there are other secreted factors causing hyphal induction (Bamford et al. 2009).

*Salmonella typhimurium* media from stationary growth inhibits *C. albicans* filamentation, whereas media from exponential growth does not (Tampakakis et al. 2009). There is no known QSM associated with *S. typhimurium*; however, it does have a QS sensor sdiA that can respond to other bacterial QSMs (Tampakakis et al. 2009). Supernatant form *S. typhimurium* stationary growth suppresses *C. albicans* TEC1, HWP1, ALS3, and CDC42 gene expression (Tampakakis et al. 2009).

**Table 1.2** Bacterial molecules associated with *Candida*-bacterial interactions.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Proposed Molecule(s)</th>
<th>Effect on <em>C. albicans</em></th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Acinetobacter baumannii</em></td>
<td>Unknown</td>
<td>Inhibitory</td>
<td>(Peleg et al. 2008)</td>
</tr>
<tr>
<td><em>Burkholderia cenocepacia</em></td>
<td>Dodecenoic acid</td>
<td>Inhibitory</td>
<td>(Zhang et al. 2011)</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>Unknown</td>
<td>Inhibitory</td>
<td>(Nair et al. 2001)</td>
</tr>
<tr>
<td><em>Lactobacillus rhamnosus</em></td>
<td>Butyric acid</td>
<td>Inhibitory</td>
<td>(Noverr and Huffnagle 2004)</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>HSL, HHQ, Decenoic acid, Pyocyanin, Phenazine</td>
<td>Inhibitory</td>
<td>(Kerr et al. 1999; Hogan et al. 2004; Davies and Marques 2009)</td>
</tr>
<tr>
<td><em>Salmonella typhimurium</em></td>
<td>Unknown</td>
<td>Inhibitory</td>
<td>(Tampakakis et al. 2009)</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>Unknown</td>
<td>Inhibitory</td>
<td></td>
</tr>
</tbody>
</table>
**Table 1.2** (continued)

<table>
<thead>
<tr>
<th><strong>Streptococcus gordonii</strong></th>
<th>AI-2</th>
<th>Enhancing</th>
<th>(Bamford et al. 2009)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Streptococcus mutans</strong></td>
<td>Decenoic acid, CSP Mutanobactin A</td>
<td>Inhibitory</td>
<td>(Jarosz et al. 2009; Joyner et al. 2010; Vilchez et al. 2010)</td>
</tr>
<tr>
<td><strong>Xanthomonas campestris</strong></td>
<td>Dodecenoic acid</td>
<td>Inhibitory</td>
<td>(Wang et al. 2003)</td>
</tr>
<tr>
<td>Various bacteria</td>
<td>Indole</td>
<td>Inhibitory</td>
<td>(Raut et al. 2012)</td>
</tr>
<tr>
<td>Various bacteria</td>
<td>LPS</td>
<td>Varying</td>
<td>(H.M.H.N. Bandara et al. 2010)</td>
</tr>
<tr>
<td>Various bacteria</td>
<td>PGP subunits</td>
<td>Enhancing</td>
<td>(Xu et al. 2008)</td>
</tr>
</tbody>
</table>

**Farnesol’s Effect on Bacteria**

Farnesol has numerous observed effects on several bacteria (see Table 1.3). For instance, swarming motility of *P. aeruginosa* is inhibited by farnesol (McAlester et al. 2008). Additionally, farnesol inhibits the production of PQS in *P. aeruginosa*, which directly inhibits pyocyanin production (Cugini et al. 2007). This action can be suppressed by higher levels of PQS (Cugini et al. 2007). Cugini et al. (2010) also found that *P. aeruginosa* strains defective in the LasR QS system and PQS production can be restored by the addition of farnesol resulting in increased pyocyanin and phenazine production.

Farnesol is able to compromise *S. aureus* and *S. epidermidis* membrane integrity and sensitize them to antimicrobials (see Table 1.3) (Meiller et al. 2006; Pammi et al. 2012). In *S. aureus* biofilms extremely high concentrations of farnesol (30mM) are able to inhibit formation, reduce the number of viable bacteria, and prevent further growth of preformed biofilms (Unnanuntana et al. 2009).

When *C. albicans* establishes a quorum, after 8 hours of biofilm growth, it is able to inhibit *A. baumannii* growth (Peleg et al. 2008). Synthetic farnesol added to media
also inhibited *A. baumannii* growth and mutants defective in farnesol production are unable to inhibit *A. baumannii* (Peleg et al. 2008).

Finally, *S. mutans* membrane permeability is hindered in the presence of farnesol (Jeon et al. 2011). Additionally, when applied to mixed species biofilms of commonly found oral bacteria, farnesol inhibits the competitiveness of *S. mutans* and reduces its dominance over other species in the polymicrobial biofilms (Jeon et al. 2011).

In polymicrobial communities of bacteria and *Candida* there is a microbial chess match to gain a competitive advantage for limited resources and space. Often, with every movement by one microorganism, especially when involving chemical molecules, there is a counter move made by the competing microorganism. Farnesol appears to serve as both a regulator of *C. albicans* morphogenesis as well as a chemical counter attack to bacterial competitors.

**Table 1.3** Reported effects of *Candida albicans* farnesol on bacteria.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Effect of Farnesol on Bacteria</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Acinetobacter baumannii</em></td>
<td>Inhibits growth</td>
<td>(Peleg et al. 2008)</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>Increases antibiotic sensitivity</td>
<td>(Brehm-stecher and Johnson 2003)</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>Inhibits PQS, pyocyanin, swarming motility</td>
<td>(Cugini et al. 2007; McAlester et al. 2008; Cugini et al. 2010)</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>Compromises cell membrane integrity, Increases antibiotic sensitivity</td>
<td>(Meillereet al. 2006)</td>
</tr>
<tr>
<td><em>Staphylococcus epidermidis</em></td>
<td>Compromises cell membrane integrity, Increases antibiotic effectiveness</td>
<td>(Pammi et al. 2012)</td>
</tr>
<tr>
<td><em>Streptococcus mutans</em></td>
<td>Compromises membrane integrity, Prevents biofilm dominance</td>
<td>(Jeon et al. 2011)</td>
</tr>
</tbody>
</table>
Specific Aims

Recent research has shown that *C. albicans* can respond to bacterial QS molecules (Hogan and Kolter 2002; Boon et al. 2008; Peleg et al. 2008). In addition to QSMs, bacteria secrete metabolites, antibiotics, and other molecules into their environment that could also be types of communication. This would imply that there are signaling or genetic pathways that *C. albicans* uses to detect bacterial QSMs, as well as other metabolites, that regulate *C. albicans* ability to filament. Thus, we hypothesize that *C. albicans* uses more than one pathway to regulate filamentation in the presence of bacteria. In many instances bacteria can inhibit the ability of *C. albicans* to change its morphology, thus inhibiting the yeast to hyphal shift (Hogan and Kolter 2002; Tampakakis et al. 2009). The understanding of this process and the pathways involved has, thus far, not been defined. Only a single gene *CHK1* has been shown to be genetically linked to *C. albicans* QS, which cannot account for all modes Candida-bacterial interactions (Kruppa et al. 2004). To better characterize this process, the implementation of a genetic screen to aid in the identification of genes that are involved in the regulation of *C. albicans* ability to filament in the presence of Gram-positive and Gram-negative bacteria could prove very useful. This would allow for identification of common as well as bacteria-specific linked genes/pathways that modulate filamentation of *C. albicans*.

**AIM 1:** Screen a *C. albicans* transposon insertion library to identify candidates that filament in the presence of bacteria and identify the insertion site of the transposon within the *C. albicans* genome (Chapter 2).
**AIM 2:** Characterize the involvement of *C. albicans CDR4* in the regulation of filamentation in the presence of bacteria (Chapter 3).

**AIM 3:** Characterize the involvement of *C. albicans ALS6* in the regulation of filamentation in the presence of bacteria (Chapter 3).
CHAPTER 2

CHARACTERIZATION OF GENETIC DETERMINANTS THAT MODULATE CANDIDA ALBICANS FILAMENTATION IN THE PRESENCE OF BACTERIA

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Keywords: Candida albicans, polymicrobial interactions, haploinsufficiency, quorum sensing, filamentation
Abstract

In the human body, fungi and bacteria share many niches where the close contact of these organisms maintains a balance among the microbial population. However, when this microbial balance is disrupted, as with antibiotic treatment, other bacteria or fungi can grow uninhibited. *C. albicans* is the most common opportunistic fungal pathogen affecting humans and can uniquely control its morphogenesis between yeast, pseudohyphal, and hyphal forms. Numerous studies have shown that *C. albicans* interactions with bacteria can impact its ability to undergo morphogenesis; however, the genetics that govern this morphological control via these bacterial interactions are still relatively unknown. To aid in the understanding of the cross-kingdom interactions of *C. albicans* with bacteria and the impact on morphology we utilized a haploinsufficiency based *C. albicans* mutant screen to test for the ability of *C. albicans* to produce hyphae in the presence of three bacterial species (*Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*). Of the 18,144 mutant strains tested, 295 mutants produced hyphae in the presence of all three bacterial species. The 295 mutants identified 132 points of insertion, which included identified/predicted genes, major repeat sequences, and a number of non-coding/unannotated transcripts. One gene, *CDR4*, displayed increased expression when co-cultured with *S. aureus*, but not *E. coli* or *P. aeruginosa*. Our data demonstrates the ability to use a large scale library screen to identify genes involved in *Candida*-bacterial interactions and provides the foundation for comprehending the genetic pathways relating to bacterial control of *C. albicans* morphogenesis.
Introduction

Polymicrobial communities of bacteria and fungi play a pivotal role in both human health and disease and can be found on nearly all facets of the human body including the skin, mouth, lungs, gastrointestinal, urinary, and reproductive tracts [1]. As varied as the habitats these microbes reside in are the types of interactions (both chemical and physical) that the opportunistic fungal pathogen *Candida albicans* and bacteria have with one another, which are only recently being fully appreciated by the scientific community [1,2]. *C. albicans* ability to change morphology from a budding yeast to a filamentous hyphae is considered a major virulence factor [3,4] that is influenced through a variety of environmental and host factors, including temperature, serum, pH, nutrient availability, and quorum sensing (QS) [5]. QS in *C. albicans* is regulated by the molecule farnesol [6], which when present at saturating levels, inhibits the ability of *C. albicans* to shift from yeast to hyphal form. This type of chemical communication among a population has also been well documented in bacteria [7–10]. Recent research has shown that bacterially secreted quorum sensing molecules (QSMs) and other metabolites can influence *C. albicans* morphology as well. For example, *Pseudomonas aeruginosa* secretes the QS molecule (QSM) 3-oxo-C12 homoserine lactone (3OC12HSL) that can block *C. albicans* filamentation [11]. Likewise, *Burkholderia cenocepacia*’s diffusible signal molecule, cis-2 dodecenoic acid, has been shown to inhibit *C. albicans* germ tube formation [12]. Similar inhibitory effects can be observed when *C. albicans* interacts with the bacteria *Acinetobacter baumannii* and *Streptococcus mutans* or the yeast *Saccharomyces boulardii* [13–15]. Interestingly, different bacterial species from the same genus, as in the case of *Streptococcus*, can
have opposing effects on *C. albicans* morphology. For instance, the oral bacterium *S. mutans* inhibits hyphal formation of *C. albicans* via the QSMs trans-2-dcenoic acid and competence stimulating peptides [14,16]; however, *S. gordonii* appears to stimulate *C. albicans* hyphae formation in an effort to aid in the colonization of the oral cavity [17]. These results suggest that *C. albicans* has mechanisms in place that can recognize these bacterial molecules in the environment and respond accordingly.

Physical interactions between bacteria and *C. albicans* include both attachment and the development of mixed species biofilms on both biotic and abiotic surfaces [18]. In addition to *P. aeruginosa* secretion of 3OC12HSL, the bacterium preferentially attaches to and kills the *C. albicans* hyphal form, but not the yeast form [19]. Alternatively, *Staphylococcus epidermidis* enjoys the ability of attaching to both the yeast and hyphal forms of *C. albicans*, and when found together as in a mixed biofilm, enhances *C. albicans* resistance to fluconazole [20]. Additionally, *S. aureus* preferentially attaches to *C. albicans* hyphae and these mixed biofilms show increased drug resistance to Vancomycin [21–23]. On the other hand, farnesol has been shown to disrupt *S. aureus* membrane integrity making it more susceptible to antibiotics as well as interfering with biofilm growth [24]. From a virulence standpoint, co-infection of *C. albicans* and other bacterial species, including *S. aureus, E. coli* and *P. aeruginosa*, show synergy with increased mouse mortality when the organisms are infected at sublethal doses [23,25–28]. Furthermore, prior colonization of *E. coli* in the urinary tract enhances *C. albicans* ability to colonize and subsequently cause urinary tract infections where typically it is unable to do so [29]. These trans-kingdom interactions may indicate that microorganisms utilize the nearby molecules to sense and monitor
their shared surrounding, adapt to changes in the local environment, and survive within a mixed species population. Despite the recent influx of research into Candida-bacterial interactions little is known about the genetics behind the mechanisms of communication that govern these interactions, and how they control morphological change in C. albicans.

Multiple species of bacteria are known to inhibit C. albicans filamentation and our goal was to identify mutants that did not respond when cultured in the presence of bacteria. In the present study, we utilized a large scale haploinsufficiency based screen to identify the genetic elements regulating C. albicans filamentation in the presence of bacteria. To our knowledge, this screen is the first to identify genetic determinants involved in polymicrobial interactions of C. albicans with bacteria and how they control morphogenesis of C. albicans. We identified 132 different genetic elements that appear to be involved in the ability of C. albicans to filament in the presence of three different bacteria (E. coli, P. aeruginosa, S. aureus). The results from this screen begin to offer a better understanding of the genetics behind Candida-bacterial interactions as well as factors influencing the morphogenesis of C. albicans.

**Materials and Methods**

**Strains, media, and growth condition.** C. albicans wild type SC5314 [30], was routinely cultured on Yeast Peptone Dextrose (YPD) medium (2% dextrose, 2% peptone, 1% yeast extract, 2% bacto agar) at 30°C. The CDR4 deletion strains [SFLUC6 (∆cdr4/CDR4) and SFLUC4 (∆cdr4/∆cdr4] have been previously described by Morschhäuser et al. [31]. The ALS6 deletion strains [1522 (∆als6/ALS6) and 1420
(Δals6/Δals6)] have been previously described by Hoyer et al. [32]. E. coli (ATCC#33922), P. aeruginosa (ATCC#27853), and S. aureus (ATCC#25923) strains were maintained on Luria Broth (LB) medium (1% tryptone, 1% NaCl, 5% yeast extract, 2% bacto agar) at 37°C.

**Screen for filamentation mutants.** A transposon insertion library of 18,144 C. albicans strains was constructed using C. albicans strain CAI4 [33] and a Tn7 plasmid insertion library created by Uhl et al [34]. To perform the screen for filamentation, the library strains were individually spot replicated, with two centimeter spacing, onto YPD, Medium 199 (M199) (9.5g medium 199 with Earles salts, 18.7g Tris-HCl, 20g bacto agar, pH 7.5), or M199 plates that had been pre-coated with bacterial lawns from fresh 37°C overnight cultures. The YPD plate was incubated at 30°C for 48 hours and served as a positive control for growth and a negative control for filamentation. The M199 control and bacterial plates were incubated at 37°C and were monitored for filamentation for up to seven days. The M199 control plate (without bacteria) served as a positive control for filamentation. Library candidates that filamented in the presence of bacteria were compared to the wild type control (SC5314) and retested twice under the same conditions to confirm the filamentous phenotype.

**Filamentation in liquid media.** C. albicans strains were grown overnight at 30°C, washed three times with dH2O and cells counted with a hemocytometer. 1x10^6 cells/mL were inoculated into pre-warmed medium 199 pH7.5 (37°C, control) or medium 199 pH7.5 containing bacteria that were pre-grown overnight at 37°C. This provided a high concentration of bacteria to simulate crowding conditions for C. albicans. For spent media the bacteria were spun out by centrifugation 5 min at 8,000 x g, then the medium
was filtered to ensure removal of any remaining bacterial cells. The spent media was then inoculated with the *C. albicans* strains. The *Candida strains* were incubated for 2.5 hr at 37°C and morphology was assessed microscopically.

**Mapping of transposon insertion sites.** *C. albicans* library candidates that filamented in the presence of all three bacteria were inoculated into 5mL of YPD and incubated overnight at 30°C with shaking (155rpm). Cells were harvested by centrifugation and chromosomal DNA was obtained using a standard bead extraction [35]. Chromosomal DNA was digested with *BsrGI* (New England), diluted 1:100, and treated with T4 DNA ligase (New England) to allow for the reconstitution of the original insertion plasmid. The ligated DNA was then transformed into *E. coli* TB-1 cells that were made competent with calcium chloride [36]. The transformed cells were plated onto LB agar plates with 50 µg/mL ampicillin and incubated overnight at 37°C. The successfully transformed cells were then grown overnight in 5 mL of LB with 50 µg/mL ampicillin and plasmid DNA was purified using the Promega Wizard® Plus DNA purification system. Purified plasmids were sequenced using primers MKOL544: 5’- GATCTGAGTGAGCATCAACAG-3’ or MKOL525 5’- GCTATGACCATGATTACGCCAGG-3’ that recognize the 5’ and 3’ flanking sequences of the transposon allowing for sequencing on either side of the insertion point. The resulting DNA sequences were used to search the *Candida* Genome Database by BLAST comparison to identify the region of transposon insertion [37].

**Reverse transcription and gene expression.** *C. albicans* SC5314, *E. coli, P. aeruginosa*, and *S. aureus* strains were grown in 50 mLs of YPD at either 30°C or 37°C until they reached mid-log phase. Cells were then harvested by centrifugation, washed
with 1X PBS, and resuspended in 50 mLs of M199. *C. albicans* SC5314 and single species bacteria were then combined in equal amounts and incubated (at 30°C or 37°C) with shaking (155rpm's). Aliquots were taken at 0, 10, 20, 30, 60 minutes post addition, cells were harvested, and samples were frozen. Acid phenol RNA extraction was performed on the samples to extract total RNA [35]. Reverse transcription PCR was performed using a Verso 1-Step RT-PCR kit (Thermo-Fisher). Primers MKOL597 5’-GGCAGATGCC GATACGAGTTCAAATTCG-3’ and MKOL598 5’-CATCAGAAGCCGAACC ATAAGCAGC-3’ were used for *CDR4* RNA detection. The *ENO1* gene served as a loading control using primers MKOL188 5’ – CGACTCCAGGTAACCC- 3’ and MKOL189 5’ – CCCAAGCATCCCCAGTC – 3’. Primers for *ALS6* detection were used as described by Zhao et al [38]. Images were captured using a Syngene G:Box system and analysis was performed using Gene Tools software by SynGene. The experiments were performed three times; the data were quantified and statistically analyzed by a student’s t-test. P-values were calculated and those that were significant (P<0.05) were noted.

**Results**

**Identification of 295 *C. albicans* mutant strains that filament in the presence of three bacterial species.** To identify the genetic elements involved in *C. albicans* polymicrobial interactions, we utilized a *C. albicans* transposon insertion mutant library previously described by Uhl et al [34]. The library was spot replicated, along with a wild-type (SC5314) control onto agar plates of YPD, M199, and M199 containing a lawn of either *E. coli*, *P. aeruginosa* or *S. aureus*. To control for hyperfilamentous mutants and remove them from the screen, strains were grown on YPD at 30°C while mutants that
did not filament were screened out on M199 at 37°C. We chose the test conditions of
growing the strains on freshly spread bacterial lawns as this would result in a
competitive growth condition for the *Candida* strains in the presence of bacteria. Our
choice of the Gram positive, *S. aureus*, and the two Gram negative, *E. coil* and *P.
aeruginosa*, was due to their association with host environments that *C. albicans* is
often known to colonize. We scored the strains to determine if the *C. albicans* mutants
filamented in the presence of one, two or all three bacterial species. Overall we
identified 836 strains (4.60% of the entire mutant population) that filamented in the
presence of one or more of the bacteria. The systematic screening of the transposon
insertion library identified 295 individual strains that produced a filamentous phenotype
in the presence of all three bacterial species. We also identified 271 strains that
filamented in the presence of two of the three bacterial species and 270 that only
filamented in the presence of one species of bacteria. We decided to focus our efforts
on the mutants that filamented in the presence of all three bacterial species as these
mutants would likely be linked to a common mechanism for *C. albicans* to respond to
different species of bacteria. **Figure 2.1** depicts representative phenotypes of both the
wild-type control as well as a *C. albicans* transposon library strains that produced
filaments in the presence of bacteria. On YPD at 30°C, the SC5314 control shows a
colony’s typical yeast, non-filamentous, morphology with characteristic smooth rounded
edges (**Figure 2.1 A, D**) while its growth on M199 without bacteria at 37°C shows
hyphal filaments protruding from the colony (**Figure 2.1 B, E**). SC5314 grown in the
presence of high concentrations of bacteria lack filaments around the colony (**Figure
2.1 C, F) while the mutants identified by our screen exhibit filamentation in the presence of all three bacterial species (Figure 2.1 G-I).

Figure 2.1 Representative colony morphologies. (A) SC5314 wild-type non-filamentous growth on YPD agar (30°C); (B) SC5314 wild-type filamentation on M199 agar (37°C); (C) SC5314 wild-type filamentation inhibition in the presence of *P. aeruginosa* on M199 agar (37°C); (D) 4X magnification of A; (E) 4X magnification of B; (F) 4X magnification of C; (G), (H), (I) Representative examples of library mutant filamentation in the presence of bacteria due to haploinsufficiency on M199 agar (37°C) (4X magnification)

Localization of the Tn7 transposon insertions. To identify the region of genome that the transposon insertion was located, the recovered insertion plasmid constructs were sequenced and the resulting sequences were then mapped by BLAST search of the
Candida Genome Database (CGD) [37]. The 295 library candidates were mapped to 132 points of insertion within genes and other genetic components of the Candida genome, multiple independent candidates were shown to map to the same gene further validating the genetic screen. Fifty percent of the Tn7 insertions mapped within an open reading frame (ORF) while 18% of the insertions mapped to the 5’ upstream region and 13% to the 3’ downstream of an ORF (Supplementary Table 2.1). This accounted for 107 known/predicted genes that fell into an array of categories related to gene function including enzymatic activity, transport, transcription, signaling, and adhesion (Table 2.1). By far, the majority of identified genes and ORFs fell into the category of unknown function. From our screen we noted that the majority of the candidates we identified had not been previously associated with filamentation in general. However the SSU1, FGR10, FGR24, RAS2, MRP2, and CCR4 haploinsufficient mutants we identified in our screen also overlap with the dataset from a haploinsufficient library screen performed by Uhl et al. [34] for filamentous mutants. In their study, Uhl et al [34] screened for hyperfilamentous and less filamentous mutants using different conditions than we utilized in this study. These mutants did not show any hyperfilamentous or less filamentous phenotypes under our control conditions indicating variability between the two experimental approaches.

A number of insertions (5%) were mapped to the RPS and RB2 repeat regions located within the major repeat sequences (MRS) of MRS-1, -2, -4, -6, -7a, -7b, and –R units (Supplementary Table 2.2). There were no Tn7 insertions found within MRS-5, nor the partial RB-2-4a repeat unit. Within the RB2 repeat region, Uhl et al previously identified a family of genes, FGR6, which have no clearly defined function other than
their association with fungal growth [34]. It appears that at least seven of eight FGR6 family members (FGR6-1, FGR6-3, FGR6-4, FGR6-10, orf19.727, orf19.6896, orf19.5775) are involved in regulating filamentous growth in the presence of bacteria. Finally, 14% of the insertions were mapped to non-coding transcripts or unannotated ORFs (Supplementary Table 2.3). Many of these transcripts were characterized previously by RNA-seq analysis [39] and tiling arrays [40], but have not been further characterized regarding their functional roles in the cell. We use the term “transcripts” at this time as the transcribed regions have not been designated officially as protein coding ORFs or regulatory RNA genes in the CGD.

<table>
<thead>
<tr>
<th>Biological Process</th>
<th>Gene/orf identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adhesion</td>
<td>ALS6, EAP1, PGA28, orf19.5813</td>
</tr>
<tr>
<td>Cell wall associated</td>
<td>BMT8, GSC1, GSL1, PGA52</td>
</tr>
<tr>
<td>Cell cycle</td>
<td>IRR1, MAD2, NUF2, TEM1</td>
</tr>
<tr>
<td>Signal transduction</td>
<td>CAS4, RAS2, TOR1</td>
</tr>
<tr>
<td>Transport</td>
<td>CDR4, CRM1, GNP3, MGE1, POM152, RTA3, SSU1, orf19.6592, orf19.6747</td>
</tr>
<tr>
<td>Transcription</td>
<td>BDF1, CCR4, CTA24, CRZ2, HAP31, PHO23, SPT7, SUA72, ZCF11, orf19.536</td>
</tr>
<tr>
<td>Translation</td>
<td>MRP2, TIF5, orf19.4176</td>
</tr>
<tr>
<td>Proteolysis</td>
<td>AXL1, FGR10, orf19.4610</td>
</tr>
<tr>
<td>RNA processing</td>
<td>ILS1, NHP2, POP3, SEN1, orf19.1201, orf19.6736, orf19.6931</td>
</tr>
<tr>
<td>RNA binding</td>
<td>orf19.3124, orf19.4018</td>
</tr>
<tr>
<td>DNA binding</td>
<td>orf19.2579, orf19.7301</td>
</tr>
<tr>
<td>DNA repair</td>
<td>MEC3, SMCS, orf19.4412, orf19.6722, MMS21</td>
</tr>
</tbody>
</table>
Confirmation of phenotypes for Δcdr4/CDR4 and Δals6/ALS6 haploinsufficient mutants. Although we identified independent insertions near or within the same gene multiple times from this screen, to further confirm our observed phenotypes we chose two mutants, Δals6/ALS6 and Δcdr4/CDR4, to be tested using independently constructed heterozygous and homozygous null strains. The ALS6 mutant was chosen as the original Tn7 library insertion point was in the promoter region of this gene, while the CDR4 mutant was chosen as the insertion fell within the open reading frame. The als6 and cdr4 heterozygous and homozygous null strains (obtained from Drs. Hoyer and Morschhäuser) were spot replicated with our haploinsufficient Δals6/ALS6 and Δcdr4/CDR4 library strains onto YPD, M199, and M199/bacterial plates using the same conditions from the original library screen. The phenotypes of the als6 and cdr4 heterozygous and homozygous null strains were similar if not identical to those we saw with our Tn7 mutant isolates (Figure 2.2), thereby confirming our original observations and the validity of using haploinsufficiency for characterizing bacterial-Candida interactions. Furthermore, we examined the morphology of the strains in liquid culture in the presence of bacteria and spent media (Supplementary Figure 2.1). When cells were cultivated under hyphal inducing conditions in M199 we observed all strains
exhibiting a hyphal morphology, whereas the control strain showed clear impairment of filamentation when grown in the presence of all three bacterial species. All the mutant strains filamented in the presence of all three bacterial species with hyphae being the predominant morphology (Supplementray Figure 2.1A). Also when C. albicans was inoculated into spent media that the bacteria had been removed from the mutants were capable of filamenting as hyphae and the wild type strain was impaired as mainly yeast and some pseudohyphae (Supplementary Figure 2.1B). This result also suggests that primary mediator(s) inhibiting C. albicans filamentation are secreted actively by the bacteria.

![Figure 2.2](representative_photos_of_observed_phenotypes_of_SC5314_library_transposon_candidates_and_cdr4_and_als6_heterozygous_and_homozygous.png)

**Figure 2.2** Representative photos of observed phenotypes of SC5314, library transposon candidates, and cdr4 and als6 heterozygous and homozygous
**deletion strains.** YPD growth control (30°C), 40x magnification; M199 filamentation control (37°C), 100x magnification; *E. coli* interactions with *C. albicans* strains, 100x magnification; *P. aeruginosa* interactions with *C. albicans* strains, 100x magnification; *S. aureus* interactions with *C. albicans* strains, 100x magnification.

*C. albicans CDR4 expression increases in the presence of S. aureus.* *C. albicans* and the individual bacterial strains were co-cultured and *CDR4* transcript levels were analyzed over time. The SC5314 cells co-incubated with *S. aureus* at 37°C exhibited over a two-fold increase in *CDR4* expression over the time course of 60 minutes as compared to the SC5314 control without bacteria (**Figure 2.3 and Supplementary Figure 2.2**). There was little to no change in *CDR4* expression in the presence of *E. coli* or *P. aeruginosa*, nor was there any change in *CDR4* expression when tested at 30°C with any of the three bacterial species (data not shown). We also observed that spent bacterial culture media (bacteria removed by filtration) was unable to stimulate the expression of *CDR4* (data not shown). This suggests that a combination of physiologic temperature and (in the case of *S. aureus*) some sort of contact between the organisms is required for *CDR4* induction. Others have shown that changes in morphology and growth may require live bacteria to be present in the media to have a full inhibitory effect on *Candida* filamentation [13]. This would suggest that *C. albicans* interactions with bacteria are multifactorial encompassing both contact dependent mechanisms as well as secreted molecular factors. We also examined the expression of *ALS6*, however no increase in transcript level was observed with any of the growth conditions (data not shown).
Figure 2.3 *C. albicans* CDR4 transcript levels grown in the presence of bacteria. *C. albicans* SC5314, *E. coli*, *P. aeruginosa*, and *S. aureus* were grown to mid log phase in liquid culture, mixed together in equal amounts, co-incubated at 37°C, and aliquots were taken at 0, 10, 20, 30, and 60 minutes post addition. RNA was isolated and expression of CDR4 was measured by reverse transcription. *ENO1* was used as a loading control and reference gene for expression comparisons. Graphical representation of CDR4 expression over time for SC5314 co-incubated with *E. coli*, *P. aeruginosa*, or *S. aureus*. Data is representative of three independent experiments with mean value and standard deviation bars shown. The asterisks indicate a statistically significant difference (P<0.05) in mean intensity of test conditions over the control.

**Discussion**

The microbiome of the human body plays an important role in our health. Currently, studies involving the microbiota have only examined what the microbial population
structures appear to be in both healthy and diseased individuals [41–43]. Because the opportunistic fungus *C. albicans* is a member of normal flora, it is thought that *C. albicans* growth is held in control by other microorganisms as well as the hosts’ immune system. Patients that are immunocompromised or those being treated with broad spectrum antibiotics can create an environment allowing *C. albicans* the opportunity to expand its growth due to the reduction of factors secreted by other organisms that would normally suppress filamentous growth. In order to understand how both Gram positive and Gram negative bacteria impair *C. albicans* ability to filament we performed a genetic screen using haploinsufficiency to identify common targets in *C. albicans* that are used by both types of bacteria to impair *C. albicans* growth. Though it is an imperfect means to screen for all possible genes related to a particular phenotype due to the diploid nature of *C. albicans*, haploinsufficiency has been used previously to identify strains showing reduced or increased filamentation [34] as well as changes in fitness related to drug susceptibility and growth rates [44]. Using this approach we were successful in identifying 107 genes/ORFs that likely play some role in *C. albicans* filamentation response in the presence of various bacterial species.

The genes identified from this screen fell into a variety of biological functions including adhesion, cell cycle, enzymatic activity, signaling, transcription, and transport. For example, the identification of several genes involved in various types of transport was quite surprising. Several of the genes we identified were associated with the plasma membrane (*CDR4, RTA3*, and *GNP3*) while others were associated with nuclear (*CRM1, POM152*) or mitochondrial (*MGE1, SSU1, orf19.7358*) transport. The transporters associated with the plasma membrane likely are used by *C. albicans* for
import/export of bacterial metabolites and QS molecules from the surrounding environment. We speculate that mutations in the *CRM1* or *POM152* genes may be interfering with export/import of signaling proteins or transcriptional regulators required to respond to the presence of bacteria in the environment. It is quite possible that *MGE1, SSU1*, or orf19.7358 transporters may be involved in transporting bacterial molecules that interfere with several other genes associated with mitochondrial function (*DNM1, GCU1, MRP2*, orf19.346, orf19.1201, orf19.4176, orf194018, orf19.4472, and orf19.7152), as mutants of these genes were also shown to be impaired in the filamentation response to all three bacteria. The *cdr4, rta3* and *gnp3* mutants are interesting in that Rta3p is a predicted flipase, which may participate in lipid molecule translocation across the membrane. Likewise the Gnp3p is predicted to be a high affinity glutamine transporter which could serve as a point for small peptide transport. Additionally, Cdr4p has no characterized functional role even though it is a member of a family of ABC transporters, Cdr1-3p [45,46]. Cdr1p and Cdr2p are important in *C. albicans* drug resistance [46,47], while Cdr3p is involved in import of phospholipids [45]. It is possible that Cdr4p plays a role in the import of small peptides or other molecules as ABC transporters have been implicated in peptide transport in *S. aureus* as well as autoinducer-2 transport in *E. coli* [48,49]. We did observe that heterozygous and homozygous mutants of *CDR4* display identical phenotypes to that of our original library isolate, further implicating its role in *C. albicans* interaction with bacteria. Surprisingly, *CDR4* expression was only induced when *S. aureus* was present in the environment with *C. albicans*. *S. aureus*, along with several other Gram positive bacteria, is known to produce small peptide QS molecules. It may be that close contact with *C. albicans*
results in the liberation of these molecules from *S. aureus* and *C. albicans* increases its expression of *CDR4* in response to these peptides. This may also be specific to Gram positive bacteria, for example, when *C. albicans* is co-cultured with *Lactobacillus rhamnosus* or *Lactobacillus reuteri* there is a two to three fold increase in *CDR4* expression [50]. Since we did not observe any major change in *CDR4* expression when *C. albicans* was grown with *E. coli* or *P. aeruginosa*, may indicate that these organisms don’t produce a peptide required for *CDR4* induction. The fact that mutants lacking *CDR4* or have reduced expression of the protein may indicate that Cdr4p is playing a role as an importer of bacterial peptides and other molecules as well as *C. albicans* inability to block filamentation in response to the presence of bacteria is a direct result of Cdr4p’s absence.

A second categorical group identified from the screen is the four genes (*ALS6, EAP1, PGA28*, and orf19.5813) that are associated with adhesion. Previous work had found that the attachment of *C. albicans* to *S. gordonii* involves multiple interactions between several components of the bacterial cell wall and *C. albicans* components [51]. Recently, it had been shown the *EAP1* and *ALS3* genes, when expressed in *S. cerevisiae*, conferred the ability of *S. gordonii* to attach to *S. cerevisiae* [52]. The *ALS3* gene has also been shown to be involved in mediating aggregation and, more specifically, directing the attachment of *S. aureus* to the hyphal form of *C. albicans* [53]. The identification of the *eap1* mutant in our screen may indicate that the protein plays not only a role in adherence to bacterial surfaces but may somehow be linked to *C. albicans* ability to “sense” other organisms in its environment. *ALS6* belongs to the *ALS* family of adhesins involved in attachment to biotic and abiotic surfaces, biofilm
formation, and virulence, however the specific role of ALS6 is unknown at this time [52–54]. Though we did not identify an als3 mutant, the identification of only als6 in our screen may also signify some importance with regards to attachment and “sensing” of bacteria in the environment. It is possible that other members of the ALS gene family could play some role in attachment and “sensing” of bacteria in the environment but due to the nature of this screen, the reduction in gene expression of one copy of other ALS genes may be insufficient to create a haploinsufficient phenotype. To our knowledge, no implication of the ALS gene family in signal transduction has been suggested in the literature. The ability of these proteins to “sense” bacteria would also add a new functional role for these adhesion proteins with a relation to signal transduction. As with CDR4 we did confirm our original screen results with heterozygous and homozygous null mutants of ALS6 further lending credibility to its involvement in Candida-bacterial interactions. However unlike CDR4 we did not see any induction of ALS6 in the presence of the bacteria. Regardless, it is clear that ALS6 plays some role in either the adherence to bacteria or “sensing” bacteria in the environment and is somehow able to convey that response by inhibiting Candida filamentation.

We were quite surprised to identify multiple insertions within the RPS and RB2 regions of the MRS. In the original use of the haploinsufficiency screen by Uhl et al [34], they also identified insertions within these regions from four clones that either increased or decreased filamentation under their experimental conditions. They identified one ORF they termed FGR6 (fungal growth regulator) within the RB2 region, though as to its actual function nothing is currently known. There are eight copies of this gene in the genome that all reside within the RB2 region of the MRS elements
It has been suggested that mutations affecting the *FGR6* family of genes phenotypically are not believed to be due to haploinsufficiency but some type of dominant regulatory mechanism [55]. Though we have no evidence, it is also possible that insertions of the Tn7 elements into the MRS regions could locally destabilize a region of a chromosome, inadvertently causing a chromosomal translocation/rearrangement thereby affecting the observed phenotype in this screen.

The identification of 18 non-coding/unannotated transcripts also was unexpected. These transcripts were previously identified using RNA-seq and gene tiling studies on the *C. albicans* genome [39,40]. These non-coding or unannotated transcripts may represent small protein coding genes with fewer than 90 amino acids or could possibly be new types of regulatory RNAs that may represent an additional level of control on the morphogenic process. Currently, little to nothing is known about the role of non-coding transcripts or small ORFs below 90 amino acids and their role in *C. albicans* morphogenesis. However, the idea that several of these transcripts could code for regulatory RNAs is intriguing. None of the transcripts appear to be transcribed as antisense with other known ORFs so it is possible that they could play a role in RNA silencing. Nevertheless, there has been some controversy about whether RNA silencing actually occurs in *C. albicans*. Initially, using cell extracts, it had been shown that *C. albicans* does have a Dicer-like activity [56]. A second study demonstrated that *in vivo* production of a dsRNA hairpin to interfere with the *ADE2* gene resulted in no observable gene silencing [57]. Furthermore, characterization of the *DCR1* dicer gene of *C. albicans* demonstrated that this activity is primarily associated with rRNA and and snRNA processing [58]. So, it appears that if the transcripts we have reported here...
have any impact on RNA silencing of RNA pol II transcribed genes the mechanism is likely unknown or does not exist in *C. albicans*.

This study has allowed us to develop the framework for future delineation of the genetic and signaling events that occurs between *Candida* and bacteria. We postulate that this genetic overlap with response to different bacterial species involves several common pathways that *C. albicans* utilizes for communication. However, several questions remain. For example, do the mutants we identified show a lack of response to bacteria in their environment due to a single secreted bacterial metabolite or QS molecule, or is it a combination of several molecules that exert the inhibitory action on *C. albicans*? It is well documented that single molecules from bacteria can inhibit *C. albicans* filamentation under laboratory conditions [11,12,14,16,17], but it is not well understood if these molecules are present in the environment at all times, which could indicate a combination of effectors may be required in the environment. Also, do the mutants that we have identified in this study also play a role in *C. albicans* QS regulation of filamentation or is that a separate phenomenon? We believe that it is likely that *C. albicans* uses some of these genes to regulate its own QS response as it doesn’t make sense that *C. albicans* would have developed separate molecular machinery to respond to both types of stimuli. We hope to address these questions in future studies. We believe that in understanding the mechanisms of action of both bacterial QSMs and other metabolites on *C. albicans* biology may lead to development of novel ways to control *C. albicans* growth in critically ill patients in addition to current antifungal therapies.
Acknowledgements

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References


CANDIDA ALBICANS CDR4 AND ALS6 ARE INVOLVED IN THE RESPONSE TO FARNESOL AND SEVERAL BACTERIAL MOLECULES

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Keywords: Candida albicans, polymicrobial interactions, CDR4, ALS6, quorum sensing, morphology
Abstract

Numerous organisms inhabit the human body living out a life of intimate relationships and unique interactions. Among these interactions are those involving the opportunistic polymorphic fungi, *Candida albicans*, with bacteria. The ability of *C. albicans* to control morphology, communicate via quorum sensing, attach to numerous surfaces, and form complex biofilms are all important virulence factors of this fungal pathogen. Research within the last decade has highlighted numerous bacterially secreted molecules that can influence the dimorphic transition of *C. albicans*, yet little is known about how these interactions function at a genetic level. In the present study, we characterized the involvement of two genes, *CDR4* and *ALS6*, previously identified by our lab for their involvement in *Candida*-bacterial interactions. Strains lacking either *CDR4* or *ALS6* have increased filamentation as compared to wild-type strains when grown in the presence of the *C. albicans* quorum sensing molecule farnesol as well as several bacterial molecules known to inhibit filamentation. *CDR4* and *ALS6* null strains have increased adhesion and biofilm growth when grown in the presence of farnesol and bacterial molecules. Our data indicates that both *CDR4* and *ALS6* are part of the machinery that defines how *C. albicans* responds to not only its own quorum sensing molecule, but also bacterial products it can encounter in the environment. Delineating the roles of *CDR4* and *ALS6* regarding *Candida*-bacteria interactions may aid in the understanding of polymicrobial infections as well as identify new targets for antifungal development.
Introduction

With the recent findings of the Human Microbiome Project the impact microorganisms have on human health is only beginning to be appreciated. For the first time, we now have a snapshot of what constitutes a “healthy” individual. However, little is understood about how members of the human microbiota interact and govern their behavior within and on the host [1-5]. With the number of microorganisms constituting the microbiome in the trillions there undoubtedly are a multitude of interactions, influences, and competitiveness for a small amount of space and resources. This includes the interactions between bacteria and the polymorphic opportunistic fungus Candida albicans. Indeed, C. albicans is part of the normal flora for about 75% of the human population and is particularly dangerous when it transitions from a member of the normal flora to an opportunistic pathogen [6]. Among the populations that are at increased risk for C. albicans infections are those of extreme age (both the young and the elderly), immune suppressed, patients undergoing cancer treatment, and those on long term antibiotic use [7,8]. In the hospital setting, C. albicans is quickly emerging as an important healthcare concern as it is the most common fungal pathogen, the fourth most common pathogen in systemic infections, and exhibits an increase in resistance to current antifungal treatments [9-11].

A distinguishing virulence factor of C. albicans is its unique ability to control morphology ranging from budding yeast to pseudohyphae to invasive hyphal filaments. While the yeast form governs attachment and dissemination, it is the hyphal form that is associated with severe infections [12,13]. Morphological control is mediated in C. albicans through quorum sensing (QS) by the quorum sensing molecule (QSM)
farnesol, an isoprenoid alcohol [14]. This phenomenon works in a density dependent manner and allows *C. albicans* to coordinate morphology in response to the surrounding environment. In addition to QS, filamentation can be manipulated in *C. albicans* by a number of physiological factors such as pH, temperature, media, nutrients, or serum [15,16]. The ability of *C. albicans* to control morphology is imperative for survival, particularly in the human body where it is constantly in competition with bacteria for space and resources as well as fending off host immune responses.

Numerous physical and chemical interactions have been identified between *C. albicans* and bacteria. Perhaps the most studied of the physical interactions is that of *Pseudomonas aeruginosa* and *C. albicans* and the antagonistic relationship they share. *P. aeruginosa* can bind to and kill the hyphal-filamentous form of *C. albicans*, but not the yeast form [17]. Alternatively, *Streptococcus gordonii* and *Staphylococcus epidermidis* appear to have the ability to attach to both the yeast and hyphal forms of *C. albicans* [18,19]. More recently, research has focused on small molecules secreted by bacteria that impact *C. albicans* morphology. Specific molecules or spent media from a variety of bacteria including *Lactobacillus*, *Streptococcus*, *Pseudomonas*, and *Burkholderia* have been shown to interrupt *C. albicans* ability to transition between morphological forms and many times these small bacterial molecules cause *C. albicans* to become locked into the yeast form [1,20-22]. Alternatively, *S. gordonii*, and bacterially derived molecules, such as peptidoglycan, appear to enhance hyphal formation in *C. albicans* [18,23]. These analogous and conflicting effects that bacteria exert on *C. albicans* demonstrate the dynamic relationships involved in a shared environment in an attempt to achieve a competitive advantage.
Previously, our lab performed a large scale library screen to identify genes that \textit{C. albicans} utilizes to control filamentation in the presence of bacteria [24]. Two of these genes, \textit{ALS6} and \textit{CDR4}, have shown potential to play participating roles in this process. In this study, we demonstrate the involvement of \textit{ALS6} and \textit{CDR4} in \textit{C. albicans} response to farnesol as well as bacterial molecules as it pertains to morphogenesis, attachment, biofilm formation, and gene expression.

**Methods**

**Strains, media, and growth condition.** \textit{C. albicans} strains were routinely cultured on Yeast Peptone Dextrose (YPD) medium (20g dextrose, 20g peptone, 10g yeast extract, 20g bacto agar in 1 liter) at 30°C. \textit{C. albicans} wild-type SC5314 [25], \textit{CDR4} deletion strain (\textit{\Delta}cdr4/\textit{\Delta}cdr4) [26], and \textit{ALS6} deletion strain (\textit{\Delta}als6/\textit{\Delta}als6) [27] have previously been described. In all subsequent experiments, \textit{C. albicans} strains were inoculated into YPD and grown overnight for 16 hours at 30°C with shaking (155 rpm). Cells were harvested by centrifugation, washed twice with sterile ultrapure water, and inoculated into Medium 199 (M199) (9.5g medium 199 with Earles salts, 18.7 Tris-HCl, 20g bacto agar, pH 7.5 ,q.s. to 1L).

**Liquid filamentation assay.** Filamentation assays were performed as previously described, but with minor alterations [28]. Briefly, prior to cell addition, \textit{trans,trans}-farnesol (Sigma), \textit{trans}-2-decenoic acid (TCI America), indole (Sigma), N-3-oxo-dodecanoyl-L-homoserine lactone (HSL) (Cayman), lipopolysaccharide (LPS) from \textit{E. coli} (Sigma), Competence Stimulating Peptide-1 (CSP1) from \textit{Streptococcus mitis} (Genscript), 1-dodecanol (Acros), phenazine (Acros), and pyocyanin (Cayman) were
added to pre-warmed (37°C) M199 liquid media. Washed *C. albicans* cells were inoculated (1 x 10^6 cells/mL) and incubated under hyphal inducing conditions (M199, pH7.5, 37°C, 155 rpm) for two and a half hours. Aliquots were then examined microscopically (400x) for cell morphology. For each experimental condition 300 cells/field were counted and results were reported as the average of triplicate experiments in terms of the percent of total cells displaying hyphal morphology.

**Cell attachment assay.** The cell attachment assay was performed as previously described with some modifications [29]. Briefly, 96 well plates were pre-coated with 5% serum and incubated overnight at 4°C. Excess serum was then removed from the wells and *trans,trans*-farnesol, *trans*-2-decenoic acid, indole, HSL, or LPS were diluted into either methanol, water, or DMSO and added to pre-warmed (37°C) M199 media at varying concentrations in the 96 well plates. Cells were inoculated at 1 x 10^5 cells/well and plates were incubated at 37°C for 90 minutes to allow for attachment. Plates were then washed with sterile dH2O, to remove non-bound cells. They were air dried, stained with 100μL crystal violet, washed with dH2O after 10 minutes, treated with 033% acetic acid to release the stain and transferred to a second plate where the absorbance (600nm) was measured using a Turner Biosystems modulus microplate reader. Attachment assays were carried out in three independent trials with each individual trial carried out in triplicate.

**Biofilm assay.** The biofilm assay was carried out similarly to the attachment assay with minor modifications [29]. Again, 96 well plates were pre-coated with 5% serum and incubated overnight at 4°C. Excess serum was removed from the wells and strains were inoculated (1x10^6 cells/mL) into pre-warmed M199 media, added to each well, and
incubated at 37°C for 90 minutes to allow for attachment. The attached cells were then washed to remove non-bound cells and fresh media containing trans,trans-farnesol, trans-2-decenoic acid, indole, HSL, or LPS were added and the plates were incubated for 48 hours at 37°C. Plates were then assayed as described above [30]. Biofilm assays were carried out in three independent trials with each individual trial carried out in triplicate.

Results and Discussion

In this study, we chose to examine two genes previously identified by our lab for their role in Candida-bacterial interactions regarding the C. albicans virulence traits of filamentation, attachment, and biofilm formation. The first gene, ALS6, belongs to a family of cell surface glycoproteins that are associated with adhesion to surfaces [31]. The ALS1 and ALS3 family members have also been demonstrated to be involved in Candida-bacterial interactions by mediating aggregation [32] and particularly in the adherence of C. albicans to Staphylococcus aureus [33]. Deletion of ALS6 has been shown to cause an increase in adhesion to human cells [27] so there is some question as to the specific role of ALS6. This increase in adhesion by the als6−/− strain may indicate that there is some sort of coordinated regulation within the ALS gene family for adherence. It is entirely possible that since ALS6 is not a highly expressed protein it may play a specialized role in the ALS gene family, possibly it functions to “see” and “sense” the environment for contacts with bacterial and host cells rather than a direct role in adherence. The second gene we examined, CDR4, belongs to a family of ABC transporters [26]. While CDR1 and CDR2 are involved with C. albicans resistance to fluconazole and transport of phospholipids [34], CDR4 does not appear to play a role in
fluconazole resistance and the cellular function of \( CDR4 \) is unknown at this time [26]. In regards to \( CDR4 \) involvement in polymicrobial interactions, co-cultures of \textit{Lactobacillus rhamnosus} or \textit{L. reuteri} [35] with \textit{C. albicans} causes a 2 to 3 fold increase in \( CDR4 \) expression. As these are Gram positive bacteria that produce peptide QSMs it is possible that \( CDR4 \) induction is in relation to uptake of these peptide molecules. \textit{S. aureus} has been shown to regulate its QS response in a similar fashion using ABC transporters in the import of secreted peptides [36], however, ABC transporters are known to import and export a large variety of compounds into and out of the cell [37].

\textbf{\textit{C. albicans} \( cdr4^{-/-} \) and \( als6^{-/-} \) strains have increased filamentation in the presence of farnesol and several bacterial molecules.} We have previously identified mutants of \( CDR4 \) and \( ALS6 \) that were able to filament in the presence of high levels of bacteria. To determine the involvement of \( CDR4 \) and \( ALS6 \) in the response to farnesol, bacterial QSMs, and other bacterial metabolites, SC5314 wild type and null strains were grown in the presence of a panel of molecules known to affect morphogenesis. SC5314, \( cdr4^{-/-} \), and \( als6^{-/-} \) strains were grown under hyphal inducing conditions for 2.5 hours with or without the addition of CSP1, 1-dodecanol, \textit{trans,trans}-farnesol, HSL, indole, LPS, phenazine, pyocyanin, or \textit{trans}-2-decenoic acid. We also performed this assay with reconstituted heterozygous strains of \( CDR4 \) and \( ALS6 \), which exhibited phenotypes identical to the homozygous nulls (data not shown). We chose these compounds as they are known to be secreted into the environment by bacteria, with the exception of farnesol, which is the QSM of \textit{C. albicans} and 1-dodecanol. Dodecanol has previously been used as a surrogate for HSL when inhibiting hyphal filamentation [38]. However, it has been demonstrated that dodecanol exerts its effect on \textit{C. albicans}
differently than HSL [39]. We chose farnesol to determine if the mutant strains would respond to the QSM. Using increasing amounts, we attempted to identify concentrations of the various molecules that demonstrated a measureable effect, but not completely inhibitory due to potential toxicity (Supplementary Figure 3.1). Figure 3.1A is a representation of the concentrations where a measureable effect was observed on the cells by the molecules, but without toxicity. The addition of these molecules did not alter growth rates and the solvents (methanol, water, or DMSO) used to dilute the molecules had no significant effect on morphology (data not shown). Also, solvents never constituted more than 1% of the total volume. Under control conditions, after 2.5 hours 93% of the wild-type SC5314 strain cell population displayed the typical hyphal-filamentous dominant morphology while cdr4 -/- and als6 -/- strains show similar morphology to the wild-type SC5314 control with about 91% of cell populations producing hyphae. Farnesol served as a control for filamentation with the wild-type strain, but also as a test for determining whether the als6 -/- and cdr4 -/- strains respond to the molecule. The addition of farnesol at 50µM, 150µM, and 300µM concentrations greatly reduced wild-type SC5314 hyphal morphology populations to 32%, 15%, and 11% respectively. At 50µM, 150µM, and 300µM concentrations of farnesol, the cdr4 -/- strain had 57%, 40%, and 19% hyphal morphology while als6 -/- had 56%, 30%, and 15% hyphal morphology, thus both deletion strains had significantly increased hyphal morphology over wild-type SC5314 (Figure 3.1A and Supplementary Figure 3.1).

Figure 3.1B demonstrates the observed hyphal phenotypes of the cdr4 -/- and als6 -/- clearly indicating they are refractory to the molecule and are involved in C. albicans QS response. The cdr4 -/- and als6 -/- strains were refractory to a number of bacterial
molecules as well. At 50µM and 200µM concentrations of HSL, *cdr4/-* had significantly more hyphal cells (79% and 15%) than wild type (65% and 9%) at both concentrations while *als6/-* (14%) only displayed increased hyphae over wild-type SC5314 at the 200µM concentration (*Figure 3.1A and Supplementary Figure 3.1*). At 1mM and 2mM concentrations of indole, *cdr4/-* (81% and 65%) and *als6/-* (81% and 72%) strains were also able to produce more hyphae than wild-type SC5314 (72% and 56%), however this was not enhanced above 2mM with almost all cells in all three strains having yeast morphology at 4mM concentrations (*Figures 3.1A and Supplementary Figure 3.1*). *trans*-2-decenoic acid required relatively small concentrations to observe hyphal morphology population differences. The addition of 1µM of *trans*-2-decenoic reduced SC5314 to near 54% hyphal morphology and 5µM greatly exacerbated this effect to 19% hyphae. The *cdr4/-* and *als6/-* strains still retained the ability to produce significantly more hyphal cells at 1µM, 69% and 63% respectively, and this was also observed at 5µM with both *cdr4/-* and *als6/-* at around 30% hyphae. At 25 µM of *trans*-2-decenoic acid concentrations all three strains produced negligible amounts of hyphal cells (*Figure 3.1A and Supplementary Figure 3.1*). LPS has also been demonstrated to inhibit filamentation of *C. albicans*. We observed that at 1ug/mL *cdr4/-* (47%) had the most refractory phenotype while the *als6/-* strain (20%), surprisingly, appeared to be more sensitive than the wild-type SC5314 (30%) with more yeast cell phenotypes (*Figure 3.1A and Supplementary Figure 3.1*). Adding CSP1, phenazine, and pyocyanin decreased the percentage of hyphal cells, but did so at similar and insignificant levels when *cdr4/-* and *als6/-* strains were compared to wild-type SC5314, indicating that the activity of these molecules is not dependent on *ALS6* or *CDR4*. 
Interestingly, 1-dodecanol concentrations at or above 50µM, almost completely ablated the ability of all three strains to produce hyphae indicating that response to dodecanol is not through the same mechanism, but due to some other stress on the cells (Figure 3.1A and Supplementary Figure 3.1).

Of the molecules tested, the reduced response to trans,trans,-farnesol was the greatest when comparing cdr4/- and als6/- deletion strains to the wild type control. While the deletion strains did have fewer hyphal cells in the presence of exogenously added trans,trans-farnesol, they still produced twice the number of hyphal cells as the control. The addition of indole, trans-2-decenoic acid or HSL reduced hyphal cell numbers in all strains, but the cdr4/- and als6/- deletion strains maintained increased numbers of hyphal cells. This could indicate that there is a global signal-response cascade that C. albicans utilizes in the control of morphogenesis in reaction to its own QS molecule as well as bacterially secreted factors. Numerous bacterial molecules that have been identified to inhibit C. albicans morphogenesis share similar carbon backbone molecular structures to farnesol. HSL from P. aeruginosa and varying forms of decenoic acid from S. mutans, B. cenocepacia, and X. campestris all share structural similarities to farnesol and appear to inhibit hyphal morphogenesis. The fact that the deletion strains in the presence of inhibitors did not restore hyphal cell populations to control levels could indicate that CDR4 and ALS6 play participating or co-factor roles in the genetic pathway regarding small molecule regulation of morphogenesis. It is also possible that CDR4 and ALS6 are not critical regulators of this pathway and can be compensated in part by other genetic factors, but the fact that deletion of these factors causes a significant increase in hyphal morphology cannot be
disregarded. Perhaps the most interesting result of the filamentation assay was that \textit{cdr4-/-} and \textit{als6-/-} were not responsive to farnesol and HSL, but were to dodecanol. Dodecanol has been used in many publications as a substitute to HSL and as a control in experiments as a hyphal formation inhibitor [1,38]. However, recent research has shown that dodecanol acts along a different mechanism than that of farnesol and HSL [39]. This could indicate, when evaluated with our results, that \textit{CDR4} and \textit{ALS6} act along a separate signaling system and accounts for the increase in hyphal morphology of the deletion strains to farnesol and HSL, but not dodecanol. It is important to note, of the bacterial compounds tested, some did not produce significant differences between the control and deletion strains. CSP1, phenazine, and pyocyanin reduced hyphal cell numbers equally in both the wild-type control strain as well as the \textit{cdr4} and \textit{als6} deletion strains. It is worth mentioning that the CSP1 used in this study is different from the CSP1 used in the original paper identifying these peptides as inhibitors of morphogenesis [41] which may explain the inability of it to elicit a reaction in the control and mutant strains we tested.

\textbf{Figure 3.1 (a)} The effects of \textit{trans,trans}-farnesol and bacterial molecules on morphology of \textit{C. albicans} SC5314 wild-type, \textit{cdr4-/-}, and \textit{als6-/-} strains. Strains were grown under hyphal inducing conditions (pH7.5, 37°C, M199 media) for 2.5 hours with or without the addition of bacterial molecules: competence stimulating peptide-1 (CSP), 1-dodecanol (DOD), \textit{trans,trans}-farnesol (FRN), 3-oxo-C12-homoserine lactone (HSL), indole (IND), lipopolysaccharide (LPS), phenazine (PHN), pyocyanin (PYO), or \textit{trans}-2-decenoic acid (TDA). Morphology was determined microscopically by counting 300 cells and expressed as the percentage of cells displaying hyphal morphology. Asterisk denotes statistical significance (p < 0.05) compared to matched SC5314 wild-type control as determined by student's t-test. The mean and standard deviation of triplicate trials are shown. \textbf{(b)} Effects of \textit{trans,trans}-farnesol on morphology of \textit{C. albicans} wild-type, \textit{cdr4-/-} and \textit{als6-/-} strains. Strains were inoculated (1x10^6 cells/mL) into hyphal promoting conditions (pH7.5, 37°C, M199 media) with or without the addition of 150µM of \textit{trans,trans}-farnesol. After 2.5 hours morphology was examined. (Top panel) wild-type SC5314 control, (Middle panel) \textit{cdr4-/-} deletion strain, (Lower panel) \textit{als6-/-} deletion strain.
**C. albicans cdr4-/- and als6-/- cells have increased attachment and biofilm formation in the presence of farnesol and known bacterial inhibitors.** Farnesol has been known to inhibit or reduce the ability of *C. albicans* to adhere to abiotic surfaces [42]. Taking the molecules that were found to be significant in the filamentation assay, the next step was to measure the ability of *C. albicans* to adhere to polystyrene surface in the presence of *trans,trans*-farnesol, indole, *trans*-2-decanoic acid, HSL, or LPS. *C. albicans* SC5314, *cdr4-/-*, and *als6-/-* strains were inoculated into M199 with the addition of farnesol or bacterial molecules and incubated under hyphal inducing conditions for 1.5 hours to allow for attachment. The effects of increasing concentrations of these molecules on *C. albicans* wild type and null strains in regards to attachment are shown.
in Figure 3.2 and Supplementary Figure 3.2. Since values were obtained in respect to optical density the untreated SC5314 control was designated as 100% attachment and all other percent values were determined in relation to this optical density value. SC5314, cdr4/-, and als6/- strains, without added molecules, served as a control baseline and all three strains had near equal ability to attach to the 96 well plates. The addition of trans,trans-farnesol reduced the attachment ability of all three strains, but significant differences were not found until the concentration of farnesol exceeded 150µM. At 300µM concentrations of trans,trans-farnesol, both cdr4/- and als6/- strains were able to significantly attach with a higher percentage, 77% and 83%, than that of the SC5314 control which only had about 69% of attached cells (Figure 3.2). HSL, at both 50µM and 200µM, caused a decrease in wild-type SC5314 attachment, while both cdr4/- and als6/-, surprisingly, had equal or greater attachment than that of the wild-type SC5314 as well as near the levels of the molecule free media only controls (Figure 3.2). Indole, at all concentrations, decreased attachment for all three strains with no significant difference in their ability to adhere to polystyrene (Figure 3.2). LPS had minimal effect on all three strains with each retaining their ability to attach near the levels of the untreated controls (Figure 3.2). Finally, trans-2-decenoic acid, at the highest concentration of 25µM, resulted in the SC5314 having about 63% of the attached cells while cdr4/- and als6/- had 78% and 85% respectively (Figure 3.2). These results indicate that trans,trans-farnesol, HSL, trans-2-decenoic acid do not dramatically inhibit the ability of cdr4/- and als6/- strains to adhere to polystyrene. However, the reduction in adherence is slightly above wild type suggesting that the initial stage of biofilm adherence is minimally compromised by these molecules. The
similar molecular structure of trans, trans-farnesol, trans-2-decenoic acid, and HSL could indicate that attachment to abiotic surfaces is impacted by Cdr4p and Als6p in response to molecules with the shared 12 carbon backbones and not the lipid polysaccharide structure of LPS or the aromatic structure of indole.

Figure 3.2. Effects of farnesol and bacterial molecules on attachment of C. albicans SC5314 wild-type, cdr4-/-, and als6-/strains to abiotic surfaces. Strains were grown in 96 well polystyrene plates under hyphal inducing conditions (pH7.5, 37°C, M199 media) for 1.5 hours with or without the addition of: trans,trans-farnesol (FRN), 3-oxo-C12-homoserine lactone (HSL), indole (IND), lipopolysaccharide (LPS), and trans-2-decenoic acid (TDA). Attachment was determined by crystal violet staining and quantified by absorbance. Asterisk denotes statistical significance (p<0.05) compared to matched SC5314 wild-type control as determined by student’s t-test. Control SC5314 without added bacterial
molecules represents 100% attachment and all other values are determined in proportion to this value. Mean percentage and standard deviation are shown. Each condition was performed in triplicate and is the result of three independent trials.

After determining the effects on attachment by trans,trans-farnesol, indole, trans-2-decenolic acid, HSL, and LPS, we tested the ability of cdr4--/ and als6--/ strains to form biofilms in the presence of these molecules. The effect of increasing concentrations of these molecules on C. albicans wild type and null strains in regards to biofilm growth are shown in Figure 3.3 and Supplementary Figure 3.3. Again, because values were obtained in respect to optical density, the untreated SC5314 control was designated as 100% biofilm formation and all other percent values were compared in relation to this optical density value. All three strains had near equal ability to form dense biofilms under control conditions by 48 hours. No statistical difference was found between the wild-type SC5314 control and cdr4--/ and als6--/ strains at trans,trans-farnesol concentrations of 50 and 150µM. However, at the 300µM concentration of trans,trans-farnesol there was an increase in total biofilm mass by the cdr4--/ (81%) and als6--/ (80%) strains over wild-type SC5314 (65%) (Figure 3.3). At the 200µM concentration of HSL wild-type SC5314 was reduced to 85% while cdr4--/ (98%) and als6--/ (95%) had significantly more biofilm mass which were near the untreated control levels. (Figure 3.3). Indole added at the highest concentration, 4mM, was able to suppress biofilm formation of wild-type SC5314 to 77% and only als6--/ (83%) was able to significantly increase biofilm formation over the wild-type SC5314 control (Figure 3.3). LPS minimally reduced biofilm mass of all three strains, but cdr4--/ (98%) and als6--/ (98%) had an increased biofilm mass over wild-type SC5314 (91%) (Figure 3.3).
Lastly, there was no statistical difference among the three strains when \textit{trans}-2-decenoic acid was added with all three strains having equal reduction in biofilm mass (Figure 3.3).

\textbf{Figure 3.3.} Effects of farnesol and bacterial molecules on biofilm formation of \textit{C. albicans} SC5314 wild-type, \textit{cdr}4-/-, and \textit{als}6-/- strains. Strains were grown in 96 well polystyrene plates under hyphal inducing conditions (pH7.5, 37°C, M199 media) for 1.5 hours then fresh media was added with or without the addition of: \textit{trans},\textit{trans}-farnesol (FRN), 3-oxo-C12 homoserine lactone (HSL), indole (IND), lipopolysaccharide (LPS), and \textit{trans}-2-decenoic acid (TDA). Biofilm formation was determined by crystal violet staining and quantified by absorbance. Asterisk denotes statistical significance \((p<0.05)\) compared to matched SC5314 wild-type control as determined by student’s t-test. Control SC5314 without added bacterial
molecules represents 100% biofilm formation and all other values are normalized to this value. Mean percentage and standard deviation of triplicate trials are shown.

In both the attachment and biofilm assays, with a few exceptions, only the highest concentrations of trans, trans-farnesol and bacterial molecules were able to elicit a statistically significant difference. This result is supported by a previous study that looked at attachment time, inhibition of biofilm formation, and increasing concentrations of farnesol [42]. This earlier study determined that high concentrations of farnesol (300μM) were required to inhibit C. albicans biofilm formation and that attachment time also played a factor in this process [42]. This is likely due to the community lifestyle where C. albicans biofilms have been shown to be up to 2000 times more resistant to antifungals [43]. With the exception of farnesol, none of the molecules tested were able to dramatically decrease attachment or biofilm formation among any of the strains tested. This may be the result of two factors. First, due to the complexity that communities of C. albicans encompass, it may be that higher concentrations are required to produce a reduction in attachment and biofilm formation. It has been well documented that planktonic cells are more susceptible to antifungals than stationary cells [43]. Secondly, it has been shown that C. albicans cells locked into the yeast form still retain the ability, albeit at a reduced capacity, to attach and form biofilms [44]. The study of C. albicans in the context of polymicrobial interactions is definitely one with numerous factors to consider. In a series of studies on the effect of LPS on C. albicans attachment and biofilm growth, Bandara et al. [45-47] established that the bacterial species the LPS was derived from, the amount of time, the concentration of LPS, the
species of *Candida*, and even a *Candida* strain dependency were all factors that influenced the increase, decrease, or no effect on attachment and formation of *Candida* biofilms.

**Conclusions**

Overall, we have begun to explore the impact that *C. albicans* genes play in *Candida*-bacterial interactions and their potential role in *C. albicans* quorum sensing. Though this has been a limited study with two genes of interest, there are several that we identified previously [24] that remain to be characterized in the future. Furthermore, this lays the groundwork into deciphering *C. albicans*-bacterial interactions at the genetic level. For example, can we alter the bacterial molecules that inhibit *C. albicans* filamentation and use them for new antifungal therapies alone or in addition to current treatments? Further work needs to establish the mechanisms of action that bacterial molecules have on *C. albicans* as well as further characterization of their production when *C. albicans* and different bacteria are present in co-culture.

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**Conflicts of Interest**

The authors declare no conflict of interest.
References


31. Hoyer, L. L.; Hecht, J. E. The *ALS6* and *ALS7* genes of *Candida albicans*. *Yeast* 2000, 16 (9), 847-855.


34. Smriti; Krishnamurthy, S.; Dixit, B. L.; Gupta, C. M.; Milewski, S.; Prasad, R. ABC transporters Cdr1p, Cdr2p and Cdr3p of a human pathogen *Candida albicans* are general phospholipid translocators. *Yeast* 2002, 19 (4), 303-318.


CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTION

Use of Genetic Library Screening in Candida-Bacterial Interactions

The use of genetic library screens in microbiological research has been instrumental in determining numerous attributes of microorganisms and C. albicans is no exception. More specifically, the use of a Tn7 transposon as an insertional mutagen to identify genes instrumental in specific functions of C. albicans is becoming more prevalent as a research tool. The original Tn7 library implemented in our research was derived from a study that screened for genes involved in C. albicans filamentous growth (Uhl et al. 2003). Tn7 library screenings have also been used to identify genes involved in the C. albicans RAM signaling pathways of filamentation (Bharucha et al. 2011), and haploinsufficiency has aided in observable changes in phenotypes for other studies including drug susceptibility targets (Oh et al. 2010). The results from our study demonstrate that a Tn7 transposon insertion library can successfully be used to identify genetic elements involved in Candida-bacterial interactions.

Defining Signaling Pathways in Candida-Bacterial Interactions

The signaling cascade of events involved in C. albicans regulation of morphology in response to bacteria and bacterially derived molecules is relatively unknown. Presumably, these signaling pathways could coincide with some of the same elements that control morphology as directed by the autoregulatory molecule farnesol. However, given the diverse array of bacteria that C. albicans contacts within the shared habitat of the human body a number of questions arise: 1) Are the genetic pathways that control
morphology the same, share components of, or completely different between farnesol response and bacterial response? 2) Are there global signaling pathways in *C. albicans* in response to all bacteria or are they specialized? For example, do Gram-positive bacteria use the same pathways as Gram-negative or entirely different pathways? 3) Do those bacteria that have antagonistic relationships with *C. albicans* activate separate pathways than those that share mutualistic or symbiotic relationships? 4) Are there human host factors that influence or exacerbate these *Candida*-bacterial interactions? 5) How do these polymicrobial interactions alter the host immune response? Bacteria exposure elicits a different immune response from that of *Candida*, and evidence exists showing that combined *Candida*-bacterial exposure causes a host response directed at one microorganism while the other may benefit from a less specific host response (Allard et al. 2009). Polymicrobial exposure also causes distinct proinflammatory responses compared to monomicrobial exposure as well as numerous documented examples of increased mortality of coinfection where single species infection cause no mortality (Carlson 1982; Klaerner et al. 1997; Peters and Noverr 2013).

**Polymorphism as a Target for *C. albicans* Treatments**

The interest of *C. albicans* polymorphism as a virulence trait and the role it plays in pathogenesis is evident in the escalation of research involving the understanding of the regulation of this shift, as well as investigation of therapeutics aimed at inhibiting this trait. Understandably, development of pharmological interventions that could block this transition is very attractive in that they could hinder *Candida* overgrowth, reduce invasiveness, and diminish biofilm capacity. The importance of the 2 dominant morphologies, yeast and hyphae, and their role in infection has been and continues to
be ardently debated. Typically, yeast cells are associated with moderate attachment ability and little to no invasiveness and host damage. Thus, yeast cells are associated with initial attachment and dissemination. Alternatively, the hyphal form is believed to have strong adhesive ability, invasiveness, increased damage, dissemination across epithelial cells, and host immune evasion. While hyphal cells can be found in tissue, there are reports that yeast cells can be found as well. Likewise, commensal *C. albicans* are believed to be of yeast morphology, while the invasive hyphae are believed to be involved in infections (Cheng et al. 2012). In reality, it is the ability to express both morphologies that leads to full virulence. This is evident by 2 lines of evidence. First, biofilms of *C. albicans* that are locked into either morphological form are severely attenuated in their growth and drug resistance (Baillie and Douglas 1999). Second, *Candida* locked into either morphological form are avirulent (Baillie and Douglas 1999). Inhibiting polymorphism has been successfully used to treat *Candidiasis* in mouse models (Ramage et al. 2002). Targeting the virulence factors of *C. albicans* may prove more effective and sustainable as a treatment option given that *C. albicans* cells remain viable, thus, there are limited selective pressures that would promote or favor drug resistance. However, only in obtaining a thorough understanding of the signaling events and genetic controls of polymorphism and QS combined will there be effective treatment options based on such.

**Quorum Sensing as a Target for *C. albicans* Treatment**

Farnesol appears to be a tempting mode of treating *Candida* infections as it governs the morphogenic transition. However, the application of farnesol *in vivo* has garnered mixed results. Farnesol has been shown in mouse models to provide
protection in oral candidiasis (Hisajima et al. 2008). This has also been observed in human cells where farnesol exposure increased host immune response as well as inhibited morphogenesis (Saidi et al. 2006; Décanis et al. 2009). The effects of farnesol on systemic candidiasis were less favorable as a treatment option. Mice in a systemic candidiasis model that were administered farnesol had increased mortality and there was an inhibition of immune function that made the mice more vulnerable to candidiasis (Navarathna et al. 2007). In vitro, farnesol is able to inhibit Candida biofilms as well as cause apoptosis in Candida cells (Ramage et al. 2002; Shirtliff et al. 2009).

**Quorum Sensing as a Method of Controlling Polymicrobial Infections**

Locking Candida into the yeast form could prove valuable in combating polymicrobial infections. It is well documented that a number of bacteria preferentially associate with the hyphae of C. albicans and in some instances are unable to attach to the yeast form (Hogan and Kolter 2002; Tampakakis et al. 2009). Locking C. albicans into the yeast form could reduce the colonization of bacteria and possibly diminish the development of mixed species biofilms. Again, locking C. albicans into the yeast form produces minimal biofilms that would also reduce the ability of Candida-bacterial biofilms from becoming established (Baillie and Douglas 1999). Candida-bacterial biofilms are more resistant to antifungals and antibiotics so this would reduce the burden of long courses of treatment with high concentrations of antimicrobials, lead to fewer instances of polymicrobial biofilms, as well as reducing the reservoir of infection. In vitro testing of farnesol on bacterial biofilms has shown to work synergistically with antibiotics, inhibit biofilm formation, and compromise cell integrity (Meiller et al. 2006; Cugini et al. 2007; Peleg et al. 2008). Farnesol appears to have the ability to inhibit
both the attachment and maturation phases of bacterial biofilm growth as well as reduction in preformed biofilms and causes biofilm detachment (Unnanuntana et al. 2009; Cerca et al. 2012).

The targeting of *C. albicans* virulence factors, such as polymorphism, holds great potential in both the treatment of monospecies *C. albicans* infections as well as polyspecies *Candida*-bacterial infections. With the increases in drug resistance, the large percentage of polymicrobial infections affecting patients, and the limited number of antifungals there is a recognizable need for new applications to combat *C. albicans*. A significant key to advancing new therapeutics may be the molecules and metabolites microorganisms use, such as QS, during their own plight to survive when challenged by other microorganisms.
REFERENCES


Gaddy Ja, Tomaras AP, Actis L a. 2009. The *Acinetobacter baumannii* 19606 OmpA protein plays a role in biofilm formation on abiotic surfaces and in the interaction of this pathogen with eukaryotic cells. Infection and immunity 77:3150–60.


factors impair biofilm development in *Candida albicans*. Microbiology 156:1476–86.


Köhler G a, Assefa S, Reid G. 2012. Probiotic interference of *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 with the opportunistic fungal pathogen *Candida albicans*. Infectious Diseases in Obstetrics and Gynecology 2012:636474.


Li A, Nair SK. 2012. Quorum sensing: how bacteria can coordinate activity and synchronize their response to external signals? Protein Science 21:1403-17


Neely AN, Law EJ, Holder IA. 1986. Infections in burned mice preinfected with Increased Susceptibility to Lethal *Candida* Infections in Burned Mice Preinfected
with *Pseudomonas aeruginosa* or Pretreated with Proteolytic Enzymes. Infection and Immunity 52:200-4.


### Supplementary Table 2.1. Genetic elements identified from the C. albicans Tn7 insertion library screen

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orf19.3001  TEM1  ORF  134c1  Cell cycle  Spindle pole body
orf19.3087.2  ------  ORF  161e5  Unknown  Unknown
orf19.3100  ------  3’ of ORF  156f1  Unknown  Unknown
orf19.3124  ------  ORF  131a2  RNA binding  Ribosome
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orf19.3519  SUA72  ORF  145c6  Transcription  Unknown
orf19.3643  ------  ORF  131b1  Unknown  Intracellular
orf19.3730  ------  ORF  126g4  Protein processing  Endoplasmic Reticulum
orf19.3767  ------  ORF  158c10  Protein processing  Golgi
orf19.3791  FGR10  ORF  143h2  Proteolysis  Cytosol
orf19.4018*  ------  ORF  116b7  RNA binding  Ribosome
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orf19.4054  CTA24  ORF  125c9  Transcription  Unknown
orf19.4086  ------  3’ of ORF  126g4  Protein processing  Cytoplasm
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orf19.4112  ------  3’ of ORF  135c1  Enzymatic activity  Cytosol
orf19.4119  SPO72  ORF  175e7  Autophagy  Membrane
orf19.4176  ------  ORF  147h9  Translation  Ribosome
orf19.4246  ------  ORF  68d3  Enzymatic activity  Unknown
orf19.4261  TIF5  5’ of ORF  127f6  Translation  Ribosome
orf19.4263  ------  ORF  173g2  Unknown  Unknown
orf19.4412  ------  ORF  120d11  DNA repair  Chromatin
orf19.4610  ------  ORF  161g5  Proteolysis  Unknown
orf19.5076  CDR4  5’ of ORF  35d10  Transport  Membrane
orf19.5101*  CCR4  5’ of ORF  133d6  Transcription  DNA binding complex
orf19.5144  PGA28  5’ of ORF  97h8  Adhesion  Cell surface
orf19.5169*  ------  5’ of ORF  94b8  Enzymatic activity  Nucleus
orf19.5212  ------  ORF  81c7  Protein processing  Unknown
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orf19.5485  MEC3  ORF  134f5  DNA repair  DNA binding complex
orf19.5506  PLC1  ORF  134g3  Enzymatic activity  Cytoplasm
orf19.5519  GCV1  5’ of ORF  162d1  Enzymatic activity  Mitochondria
orf19.5665  ------  ORF  134d9  Enzymatic activity  Unknown
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orf19.5813  ------  3’ of ORF  125h3  Adhesion  Nucleus
orf19.5897  ------  5’ of ORF  168h5  Unknown  Cytosol
orf19.5902*  RAS2  ORF  109b6  Signaling  Membrane
orf19.5938  SEN1  ORF  134b8  RNA processing  RNA binding complex
orf19.6082  ------  ORF  175a5  Protein processing  Endoplasmic Reticulum
orf19.6323  HPA2  5’ of ORF  88a4  Amino acid synthesis  Nucleus
orf19.6488*  ------  ORF  72d12  Unknown  Unknown
orf19.6592  ------  3’ of ORF  176f2  Transport  Membrane
orf19.6722*  ------  ORF  78e8  DNA repair  Cytosol
orf19.6736  ------  5’ of ORF  78g8  RNA repair  Mitochondria
orf19.6747  ------  ORF  175c8  Transport  Cytoplasm
orf19.6785  RPS12  ORF  189h2  Ribosomal associated  Ribosome
Supplementary Table 2.2. Major repeat sequences identified from the *C. albicans* Tn7 library screen

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Supplementary Table 2.3. Non-coding/unannotated transcripts identified in *C. albicans* library screen

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*denotes transcript was identified in multiple candidates
Supplementary Figure 2.1. Phenotypes observed when SC5314, library transposon candidates, heterozygous and homozygous deletion strains grown in liquid culture (37°C) with bacteria or spent media. A) *C. albicans* strains with M199 filamentation control and coculture with *E. coli*, *P. aeruginosa*, and *S. aureus*. Magnification 400x; B) *C. albicans* strains with M199 filamentation control and culture in spent media *E. coli*, *P. aeruginosa*, and *S. aureus*. Magnification 400x.
Supplementary Figure 2.2. *C. albicans* CDR4 gene expression when cocultured with a media only control, *E. coli*, *P. aeruginosa*, or *S. aureus*. ENO1 control is also shown.
Supplementary Figure 3.1. Individual graphs of SC5314 wild type, cdr4/-, and als6/- cell morphology in the presence of varying concentrations: (A) competence stimulating peptide-1 (CSP1), (B) 1-dodecanol, (C) trans, trans-farnesol, (D) 3-oxo-C12-homoserine lactone (HSL), (E) indole, (F) lipopolysaccharide (LPS), (G) phenazine, (H) pyocyanin, and (I) trans-2-decenolic acid.
**Supplementary Figure 3.2.** Individual graphs of SC5314, *cdr4-/-*, and *als6-/-* attachment in the presence of varying concentrations: (A) *trans,trans*-farnesol, (B) 3-oxo-C12-homoserine lactone (HSL), (C) indole, (D) lipopolysaccharide (LPS), and (E) *trans*-2-decenoic acid.
Supplementary Figure 3.3. Individual graphs of SC5314, cdr4-/-, and als6-/- biofilm formation in the presence of varying concentrations: (A) trans,trans-farnesol, (B) 3-oxo-C12-homoserine lactone (HSL), (C) indole, (D) lipopolysaccharide (LPS), and (E) trans-2-decenoic acid.
VITA

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Sean J. Fox and Michael D. Kruppa. Candida albicans CDR4 and ALS6 are involved in the response to farnesol and several bacterial molecules. Microorganisms. 2013 (In submission)


Presentations:

Sean Fox. 2012 ETSU Quillen College of Medicine Department of Biomedical Sciences (Johnson City, TN). Seminar: “It’s a Zoo in Here!: The Human Microbiome and Implications on Human Health.”


Sean Fox and Michael Kruppa. 2011 Appalachian Research Forum (Johnson City, TN). Oral Presentation: “Fungi are from Mars, Bacteria are from Venus: A Screen for Genetic Mediators of Polymicrobial Interactions.”

Sean Fox. 2011 ETSU Quillen College of Medicine Department of Biomedical Sciences (Johnson City, TN). Seminar: “Look Who’s Talking: Characterizing the Genetic Determinants in Candida-Bacteria Interactions.”


Sean Fox and Michael Kruppa. 2010 Appalachian Research Forum (Johnson City, TN). Poster Presentation: “A Genetic Screen to Identify Genes in C. albicans that are involved in Cross Species Interactions.”

Sean Fox and Uday Kumaraguru. 2009 Appalachian Research Forum (Johnson City, TN). Poster Presentation: “TH1 Skewing of the Neonatal Immune Response Upon Early Microbial Exposure.”

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2012 2nd Place Oral Presentations, Appalachian Research Forum
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