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MECHANISM OF CANDIDA ALBICANS BIOFILM AND VIRULENCE INHIBITION BY A BACTERIAL SECRETED FACTOR

Carrie Graham

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MECHANISM OF *CANDIDA ALBICANS* BIOFILM AND VIRULENCE INHIBITION BY A BACTERIAL SECRETED FACTOR

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MECHANISM OF CANDIDA ALBICANS BIOFILM AND VIRULENCE INHIBITION BY A
BACTERIAL SECRETED FACTOR

A
DISSERTATION
Presented to the Faculty of
The University of Texas
MD Anderson Cancer Center UTHealth
Graduate School of Biomedical Sciences
in Partial Fulfillment
of the Requirements
for the Degree of
DOCTOR OF PHILOSOPHY

by
Carrie Elaine Graham, M.S.
Houston, Texas
December 2017
Dedication

To my son, Emiliano Carlos Diaz-Graham,
who is the light in my life and one of my greatest endeavors.
I know you can break boundaries and accomplish all of your dreams. Never give up.

“If anything is worth doing, do it with all your heart.”

- Eknath Easwaran
Acknowledgement

First, I would like to thank my advisors Dr. Danielle Garsin and Dr. Michael Lorenz, who have consistently encouraged me and have offered endless support in both my academic and personal life. Pursing my Ph.D. has been one of the greater challenges in my life and has made me face many of my shortcomings. However, at every challenge both Danielle and Mike have offered words of advice and encouragement. They set a great example for me to follow and I hope to reach their level of expertise and scientific accomplishment. In spite of all the difficult challenges, pursuing my Ph.D. has also been one of the greatest parts of my life and I have grown not only as a scientist, but also as a person. That would not have been possible without both Danielle and Mike. Many people have asked me how it is with two mentors and I will admit at time I did felt a little overwhelmed, but it was worth the challenge and I have been able to be part of two wonderful scientific communities working on such a unique and multifaceted project. Thank you both so much for all of your advice and support throughout my time as your student it has been a great pleasure to have such high caliber scientist as mentors.

I would also like to acknowledge my committee members, Dr. Ambro van Hoof, Dr. Barrett Harvey, and Dr. Mike Gustin, for their guidance and support throughout my Ph.D. and during my candidacy exam. Thank you all for your feedback, encouragement, and brilliant ideas that kept my project moving forward and helped optimize time. I have thoroughly enjoyed sharing my research with you all in an environment that fosters growth and love for science.

Next, I would like to thank all of the faculty, students, and administration in the Microbiology and Molecular Genetics department. It has been a great pleasure to be a part of this wonderful department. I have also made many lifelong friends in the department that I would like to thank for their friendship and support. Yi Liu, who was one of my first friends in the department, has always shown me love and caring. Without you I might not have made it in so many different ways. I thank my good friend Norah Owiti for all of her advice and support, thank
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MECHANISM OF CANDIDA ALBICANS BIOFILM AND VIRULENCE INHIBITION BY A BACTERIAL SECRETED FACTOR

Carrie Elaine Graham, M.S.

Advisory Professors: Danielle A. Garsin, Ph.D. and Michael C. Lorenz, Ph.D.

The human microbiome is a diverse polymicrobial population comprised of both fungi and bacteria. Perturbations of the normal microbiome can have a profound impact on health, including the development of infections. Exploitation of these polymicrobial interactions has the potential to provide novel treatment and prevention strategies for infectious diseases. Enterococcus faecalis, a Gram-positive bacterium, and Candida albicans, a polymorphic fungus, occupy overlapping niches as ubiquitous constituents of the gastrointestinal and oral microbiome. Both species are also amongst the most important and problematic, opportunistic nosocomial pathogens and are often co-isolated during infection. Surprisingly, these two species antagonize each other’s virulence in both nematode infection and in vitro biofilm growth.

Herein we identify the E. faecalis secreted bacteriocin, EntV, as both necessary and sufficient for the reduction of C. albicans virulence and biofilms through the inhibition of hyphal formation, a critical virulence trait of C. albicans. Furthermore, we demonstrate that the EntV propeptide is proteolytically processed by the metaloprotease, GelE, in E. faecalis, resulting in production of a highly active peptide of 68-amino acids, EntV^{68}. The mature peptide effectively blocks biofilm development in multiple media conditions and disrupts pre-formed biofilms, which are resistant to current antifungal treatments. Moreover, EntV^{68} is efficacious against clinical strains of C. albicans, including azole resistant strains. Biofilm development of other
pathogenic *Candida* species is also impeded by EntV$^{68}$ treatment, demonstrating the target spectrum of EntV$^{68}$ is not limited to *C. albicans*. EntV$^{68}$ is protective in the murine macrophage and oropharyngeal candidiasis (OPC) infection models at nanomolar concentrations. Epithelial invasion, inflammation, and fungal burden in the OPC model were significantly reduced in response to treatment with EntV$^{68}$. Collectively, *C. albicans* cells present in the hyphal form were greatly reduced in all models examined. Despite these profound effects, EntV$^{68}$ has no effect on *C. albicans* viability, even in the presence of significant host-mimicking stresses. EntV$^{68}$ associates with cell surface of both yeast and hyphal cells of *C. albicans* and deletion of genes involved in hyphal morphogenesis and cell wall composition abrogate the inhibitory activity in *C. albicans* biofilms. These findings demonstrate that EntV has potential as a novel antifungal agent that targets virulence rather than viability.
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CHAPTER 1:

Background and Significance
**C. albicans as an Opportunistic Pathogen**

*Candida* spp. rank 3rd among nosocomial pathogens, with *C. albicans* being the prevalent species and there is a 30% rate of morbidly in device-associated infections in the U.S. (1-4). There are two-main types of infections caused by *C. albicans* that differ very drastically in severity: superficial infections of the mucosa, such as oral or vaginal candidiasis and life-threatening systemic infections that occur when *C. albicans* enters the blood stream and then infects organs throughout the body (5-7). *C. albicans* has specialized virulence and fitness attributes that allow it to survive and adapt in diverse environments in the host (5). The transition between yeast and hyphal morphotypes, expression of cell surface adhesins, and biofilm development are among these adaptive traits that are also considered important virulence factors (6).

As a commensal *C. albicans* can live in equilibrium with the normal bacterial flora on mucosal surfaces (3, 4, 8). *C. albicans* is transmitted at birth from mother to infant and no external environmental reservoirs exist, which suggests that *C. albicans* has coevolved with the mammalian host (9) However, these commensal populations are frequently the source of infection that arise from transmigration through the mucosal barrier or colonization of implant devices (10). Several factors have been identified that influence the switch from commensalism to pathogenesis in *C. albicans* such as: (1) changes in the normal microbial flora, (2) damaged epithelial barriers, and (3) immune dysfunction (4, 8, 11). After the onset of *C. albicans* superficial infections more severe systemic infections can occur (11-13). These blood-borne disseminated infections often have considerable mortality rates, near 40%. Severe *Candida* infections are most prevalent in immunocompromised individuals, such as those with HIV/AIDS or being treated with immunosuppressive therapy (14-17). Healthy individuals with implanted medical devices are also commonly plagued with *Candida* infections that stem from colonization of the medical devices (18, 19).
Clinical Importance of Biofilms

*C. albicans* has the ability to produce highly structured biofilms on abiotic and biotic surfaces, which promotes the high morbidity and mortality correlated with device-associated infections (20, 21). These highly structured biofilms are composed of different cell types that form a striated pattern with round, budding yeast-form cells and/or oval pseudohyphal cells at the bottom and elongated, cylindrical hyphal cells at the top encased in an extracellular matrix (Fig. 1.1) (22-25). Of the bloodstream infections in the clinical setting 15% are caused by *C. albicans* or closely related species; however, *C. albicans* is the predominant fungal species found on medical devices (26-28). Different medical devices used throughout the body are susceptible to *C. albicans* biofilm, such as mechanical heart valves, pacemakers, central venous and urinary catheters, dentures and joint prosthetics to name a few (2, 29-31).

Biofilms formed on medical devices have the potential to seed disseminated bloodstream infections that can lead to systemic infections of tissue and organs. Astonishingly, 50% of the over five million central venous catheters placed annually in the U.S. become infected with biofilms, both bacterial and fungal (2, 32). Routinely, once a medical device becomes infected it is removed and the patient will undergo antimicrobial therapy. Since fungal biofilms are resistant to traditional antifungal treatments, increased concentrations and longer durations of antifungal treatment are required (33-37). However, in some cases removal of the infected medical devices is costly or unsafe and the extremely elevated antifungal concentrations used to treat the infection can lead to further complications such as kidney or liver failure (15, 37, 38). Thus, due to the complicated nature of device-associated infections in the U.S. alone, $6.5 billion is spent annually to treat these types of infections (39). Not only do these infections have an immense economic impact, but also a significant health impact resulting in an estimated 100,000 deaths per year (39).
Figure 1.1: Stages of *C. albicans* biofilm development over time.

1) Adherence: yeast cell attach to an abiotic or biotic substrate. 2) Initiation: biofilm development starts with cell-cell adhesion and hyphal morphogenesis. 3) Maturation: hyphal cells form an extensive network encased in a secreted, extracellular matrix. 4) Dispersal: yeast cells bud off the biofilm and disperse and adhere at new locations. Modified from: Finkel JS, and Mitchell AP. Genetic control of Candida albicans biofilm development. Nature Reviews Microbiology; 2010;9:109–18 (40-42). Permission to use this figure was granted by Copyright Clearance Center (License #: 4242690280081).
Role of Hyphal Morphogenesis During Biofilm Formation

Biofilm formation can be divided into four distinct stages composed of different populations of yeast, psuedohyphal, and hyphal cells (Fig. 1.1) (23, 39, 40). C. albicans yeast-hyphal transition is essential for maximum virulence potential and the biofilm growth cycle (13, 20, 21, 43). The early stages of biofilm formation are adherence and initiation, which consist of yeast cell attachment to a substrate followed by development of psuedohyphae and then hyphae. During maturation of the biofilms, cells are encased in an extracellular matrix, biomass increases, and striated layers of yeast (bottom) and then hyphal cells (top) are observed. The final stage of biofilm formation is dispersal where yeast cells bud off and disperse, allowing them to form biofilms in other locations (Fig. 1.1) (23, 39, 40). The stages of biofilm growth are interdependent with regulation of hyphal morphogenesis and controlled by a complex signaling network.

Hyphal morphogenesis is regulated by intricate interconnected pathways in response to environmental signals that induce or repress hyphal morphogenesis, giving rise to the distinct morphologies that have been observed in C. albicans (13). Morphological variability allows C. albicans to adapt to different environmental changes. Intriguingly, many of the distinct morphologies can be observed during biofilm growth at distinct stages of development, indicating differences in gene expression of biofilm cells in response to the biofilm microenvironment. To form the striated biofilm structure described above cells must be able to regulate hyphal morphogenesis (40, 42, 44, 45).

Three major signaling cascades have been identified to date that regulate hyphal morphogenesis in C. albicans: (1) the cyclic AMP-protein kinase A pathway (cAMP-PKA), (2) mitogen-activated protein kinase pathway (Cph1-MAPK), and (3) pH pathway (Rim101) (46-48). The cAMP-pKA pathway is the central signaling pathway that responds to an array of different conditions and regulates hyphal morphogenesis during biofilm growth via activation of Ras1 (GTPase) by an unknown signal and receptor (49) (Fig. 2.1). Upon Ras1 activation,
cAMP is synthesized from ATP by adenylyl cyclase (Cyr1) (50, 51). cAMP then activates PKA, which consists of one of two redundant catalytic subunits, Tpk1 and Tpk2, and a regulatory subunit, Bcy1. PKA then phosphorylates the downstream transcription factor, Efg1, which regulates hyphal-specific genes (52, 53).

Efg1 is a key regulator of hyphal morphogenesis and has various functional roles in both the repression and the induction of hyphal morphogenesis. This multi-functionality is owed impart to the multiple functional domains of the protein (53). Efg1 is able to bind at least two different DNA binding motifs (E-box and MCB). It interacts with the terminal transcription factors of the other hyphal signaling cascades and modulates their functions. Post-translational modifications are also thought to modulate the regulatory behavior of Efg1 (53, 54). Efg1 is one of the core master regulators (Flo8, Rfx2, Gal4, Brg1, Bcr1, Rob1, Efg1, Ndt80, and Tec1) that positively regulate biofilm development and form an intricately regulated transcriptional network that controls biofilm-associated genes (Fig. 1.2) (23, 42, 55). Recently, an even larger set of genes has been identified that encompasses this subset of core master regulators to include a total of 51 transcription factors that regulate different aspects of biofilm development (56).

Additionally, other key transcriptional regulators of interest include the negative regulators Nrg1 and Rfg1 that with co-repressor Tup1 negatively regulate hyphal-specific genes and allow dispersal of yeast cells in the final stage of biofilm development (57). The positive regulator of hyphal-specific genes, Ume6, is important for hyphal extension by maintaining expression of hyphal-specific genes and is important for the later stages of biofilm growth (54, 57, 58). Downstream from every hyphal morphogenesis signal cascade, Ume6 is thought to be a global positive regulator of hyphal-specific genes and is negatively regulated by Nrg1-Tup1 and to a lesser extent by Rfg1-Tup1 (59).
Figure 1.2: *C. albicans* hyphal and biofilm development signaling cascade.

Ras1-cAMP-PKA (purple) is responsible for transducing the environmental signal to the terminal transcription factor Efg1 (blue). Activated Efg1 is part of the biofilm core regulators and positively regulates the biofilm transcriptional network (green), hyphal specific and biofilm-associated genes. The positive regulator Ume6 regulates hyphal elongation (brown) and is important for developing mature biofilms. Repressors Nrg1 and Rfg1 with the co-repressor Tup1 (red) negatively regulate the signaling cascade and are important for the late biofilm stage, dispersal. Modified from Sudbery PE.
Growth of *Candida albicans* hyphae. Nature Reviews Microbiology; 2011;9:737-748 . Permission to use this figure was granted by Copyright Clearance Center (License #: 4242730485058).
Biofilm-Associated Genes

Nobile et al. (42) identified a subset of genes that are differentially regulated during biofilm development. This subset of genes is regulated by different combinations of the six-biofilm master regulators and includes cell wall proteins, metabolic proteins, and transporter proteins. Functional analysis of these genes during biofilm growth revealed that cell wall proteins play a direct role in biofilm development (42). Considering that the cell wall interacts most directly with surface substrates and other cells it is no surprise that the cell wall and surface proteins play a major role in biofilm formation. The cell wall of *C. albicans* is comprised of primary carbohydrates and glycoproteins (8, 12). Chitin, β-1,3-glucan, and β-1,6-glucan comprise the inner wall just above the cell membrane, while cell wall proteins and mannans that are in the form of N- and O-linked glycosylation on the cell wall proteins constitute the outer wall (Fig. 1.3) (8, 13). Adhesins directly mediate adherence to a substrate or other cells. Several different adhesins have been identified in *C. albicans* by deletion of the gene resulting in loss of adherence or due to homology to known adhesins (60). The content of the cell wall and surface proteins can affect adhesin activity that results in masking or exposing adhesins to the cell surface (8, 13, 60).

Two different classes of cell wall proteins have been identified in *C. albicans*: proteins glycosphatidylinositol (GPI) anchored to β-1,6-glucan and proteins with internal repeats (Pir) linked to β-1,3-glucan (60). The GPI anchored cell wall proteins are the most abundant and many are important for biofilm formation. One such protein is hyphal wall protein 1 (Hwp1), a well-characterized GPI-anchored cell wall protein that is covalently linked to β-glucan. Deletion of *hwp1* reduces virulence in a mouse infection model and results in biofilm defects (42, 55, 61, 62). The ALS (agglutinin like sequence, Als1-7, 9) family of proteins is a family of GPI-anchored cell wall proteins, of which several have been identified as being important for biofilm formation. Als1 and Als3 have both been shown to play important roles in hyphal
Figure 1.3: The *Candida albicans* cell wall.

Two distinct layers make up the cell wall. The outer layer composed of O- and N-linked mannans covalently linked to proteins to form glycoproteins. The inner layer is comprised of polysaccharides β-1,3-glucan, and β-1,6-glucan and chitin. Modified from Gow NA, Hube B. Importance of the Candida albicans cell wall during commensalism and infection. Current Opinion in Microbiology; 2012;15:406–12 (13). Permission to use this figure was granted by Copyright Clearance Center (License #: 4242731391290).
morphogenesis and biofilm formation; deletion mutants have defects in biofilm growth (62, 63). Over expression of the other ALS genes rescues the in vivo and in vitro biofilm growth defects of an als1Δ/Δ als3Δ/Δ mutant, indicating redundancy in the function of the Als family of proteins (62).

Both HWP1 and ALS1 are regulatory targets of BCR1 and over expression of either rescues a bcr1 biofilm defect (42). However, overexpression of HWP1 does not rescue biofilm growth defects of an als1Δ/Δ als3Δ/Δ mutant suggesting they have distinct roles during biofilm formation (62). When biofilms of mixed mutant strains (als1Δ/Δ als3Δ/Δ and hwp1Δ/Δ) are grown together the biofilm defects are overcome indicating Hwp1 and Als1/Als3 have complementary functions (62). Heterologous expression of HWP1 in S. cerevisiae mediates adherence to wild-type C. albicans but not the als1Δ/Δ als3Δ/Δ mutant (62). Taken together these results suggest Hwp1 and Als1/Als3 have distinct complementary roles during biofilm growth by mediating cell-cell attachment via interactions together on the cells surface.

Additional cell wall associated proteins have been shown to play important roles in biofilm formation. Eap1 (enhanced adherence to polystyrene 1) is a GPI anchored protein with a similar structure to the Als family and is important for biofilm initial adherence. Deletion of eap1 causes a reduction in adherence to polystyrene and causes defects in both in vivo and in vitro biofilm growth (64, 65). Rbt1 (repressed by Tup1) is also a GPI anchored protein that belongs to the same family of adhesins as Hwp1 (60). Deletion of RBT1 results in mild biofilm defects in vitro although additional deletions of HWP1 and HWP2 have enhanced biofilm defects (66, 67). Expressed at high concentrations in C. albicans yeast cells, Ywp1 (yeast wall protein 1) is an adhesin-like cell wall protein that is thought to act as an anti-adhesin by an unknown mechanism (68, 69). Deletion of YWP1 results in loss of dispersal and increased adherence suggesting that Ywp1 is important for dispersal, the final stage of biofilm development (69). Most of the multiple families of adhesins are associated with hyphal growth and play a major role in biofilm development. However, a balance of cell-substrate and cell-cell adherence is important for biofilm integrity. Cabral et al. (70) found that overexpression of
certain cell wall adhesins, such as PGA22, disrupted biofilm growth. Furthermore, upregulation of cell adhesins had different consequences in mono-culture biofilms compared to polymicrobial. Thus, regulation of cell adhesions is dependent on the nature of the biofilm and substrate.

The secreted aspartyl proteases, (Sap1-10), are secreted proteases that are differentially expressed in hyphal and yeast cells and important for adhesion, invasion and tissue degradation during infection (11, 71). During biofilm formation the SAP-encoding genes are significantly upregulated in both in vitro and in vivo growth conditions (42, 71). In denture stomatitis, inflammation of the palatal mucosa in denture wearers is induced by biofilm growth on the surface of the top of denture. Under these conditions, SAP8 expression is highly unregulated followed by SAP5, 6, 2 and 1. Overall a positive correlation between proteinase activity and biofilm formation associated with infection have been observed in C. albicans oral isolates (71, 72). Bloodstream isolates of C. albicans have upregulated gene expression of SAP5 and 9 during in vitro biofilm growth compare to isolates from other sites (73). Deletion of SAP5 and 6 causes in vivo and in vitro biofilm defects (74).

The Saps have been shown to be hyphae-specific genes important for induction of hyphae-infiltration (4, 75). However, the observations during biofilm growth suggest that the Saps have functions distinct from hyphal invasion during infection (71-74). Defects in biofilm adhesion suggest that these proteases are required for proper adhesion during the initiation stage (74). Thus, the Saps may also play a role in both seeding new biofilm after dispersal and maintaining adhesion during biofilm maturation (71, 73, 74). The dynamic expression of the SAP genes over time suggests they play different roles during the stages of biofilm development such as nutrient acquisition, cell-cell interactions (adhesion, quorum sensing), and extracellular matrix production and remodeling (42, 71-74, 76).
Antifungal treatment strategies

Traditional antifungal drug development is limited because humans and fungi are evolutionarily closely related resulting in a limited number of probable drug targets and greater potential for toxicity due to off target affects (77-79). Currently, three classes of traditional antifungals (polyenes, azoles, echinocandins) are commonly used to treat *C. albicans* infections (80). These antifungals have different advantages and limitations with toxicity and cross reactivity being the main challenges (78).

Polyenes were the first antifungals discovered and are natural derivatives of antifungals from *Streptomyces* spp. (77, 81). Polyenes generate pores in the cell membrane by binding ergosterol, leading to ion leakage and ultimately cell death (82, 83). The most successful polyene is Amphotericin B and is commonly used for serious fungal infections, but has severe toxic side affects (77, 84). Azoles disrupt the cell membrane fluidity by disrupting biosynthesis of ergosterol and are extensively used because of their low toxicity (85). Fluconazole, itraconazole, voriconazole, and posaconazole are the most commonly used azoles and are often used as a first measure against fungal infections (77). Azole resistance has increased with the widespread use of azoles in the clinical setting (83). Echinocandins are the newest class of antifungal and inhibit cell wall biosynthesis by inhibiting β-1,3-glucan synthase (Fks1) (86). Caspofungin, anidulafungin, and micafungin are the most commonly used echinocandins and have little to no side effects (87).

Antifungal Resistance in *C. albicans* Biofilms

The ability of *C. albicans* to form biofilms influences antifungal drug resistance due, in part, to the inability to disrupt or permeabilize the biofilms (88, 89). The inability to disrupt *C. albicans* biofilms correlates with the hypha-yeast ratio, where biofilms with more than 50% hyphae have a significant increase in compression strength thus increasing resistance to disruption (90). Therefore, the structure of the biofilm allows *C. albicans* to persist even in the
presence of antifungals (20, 21, 88). Biofilms of *C. albicans* are up to 1,000-fold more resistant to antifungals compared to planktonic cells (20, 88, 91, 92).

One key characteristic of biofilm growth is the encasement of the biofilm in the protective extracellular matrix that protects the biofilm integrity and acts as a physical barrier against antifungal agents. The extracellular matrix of *C. albicans* biofilms is comprised of proteins, polysaccharides, lipids, and nucleic acid. Although each of the extracellular matrix components augment the architecture and integrity of the biofilm, only polysaccharides and nucleic acid have been shown to enhance antifungal resistance (93-97).

The extracellular matrix also contains polysaccharides, mostly mannan-glucan complexes (98). The β-1,3-glucan synthase gene, *FKS1*, produces cell wall β-1,3-glucan that is deposited into the ECM of *C. albicans* biofilms. Nett *et al.* (20, 99) demonstrated that β-1,3-glucan sequesters antifungals, impeding diffusion of the antifungal and conferring resistance of the biofilm to antifungals. Heterozygous deletion of *FKS1* (*GSC1*) reduced β-1,3-glucan synthesis and deposition in the extracellular matrix, which increased biofilm susceptibility to fluconazole (99). Moreover, depletion of β-1,3-glucan by β-1,3-glucanase treatment increased sensitivity to fluconazole. Whereas, addition of exogenous β-1,3-glucans increases resistance to fluconazole in planktonic cells (20).

Another component of the extracellular matrix that contributes to antifungal resistance is extracellular DNA (eDNA). DNase treatment enhances caspofungin and amphotericin B disruptive activity of mature biofilms (100). eDNA contributes to the maintenance and stability of biofilms and is not required for the early biofilm stages (adherence and initiation). DNase treatment of mature biofilms disrupts biofilm integrity, but does not affect establishment of biofilm (101). Moreover, addition of eDNA to biofilms increases biofilm biomass and does not affect adherence (101). eDNA is also present, to a lesser extent, in the supernatant of planktonic cells and can be enhanced by addition of chitinase in both planktonic and biofilm cells suggesting that the eDNA source is from cell lysis (97, 100, 101).
Efflux pumps also contribute to the intrinsic resistance of *C. albicans* biofilms. There are two major classes of efflux pumps in *C. albicans*: ATP-binding cassette transporters (*CDR1* and *CDR2*) and major facilitator class pumps (*MDR1*) (80, 102, 103). In the presence of antifungal drugs these efflux pumps are upregulated in planktonic cells (80, 102). However, during biofilm growth these efflux pumps are upregulated in response to surface adherence, which further influences resistance to antifungal agents (42, 104, 105). Independent of efflux pumps, persister cells contribute to antifungal resistance in biofilms (88). Biofilms are metabolically heterogeneous, with cells at the base more dormant than those at the surface. Persister cells are metabolically dormant yeast cells found in biofilms of *C. albicans* that are exceedingly resistant to antifungals and can survive very high concentrations of antifungal drugs to seed new biofilms once antifungal treatment ceases (88, 106). Most antifungal are fungistatic and not fungicidal and are more effective against actively growing cells, which explains the resistance observed in persister cells.

Due to the rise in antifungal resistance the discovery of novel antimicrobial agents is imperative. The rise in resistance to traditional antimicrobials is due impart to targeting essential processes that increase the selective pressure for mutations that confer resistance (3, 91, 92). One promising alternative is the use of antimicrobials that target virulence factors, such as hyphal morphogenesis and the biofilm matrix in *C. albicans* (3, 4, 92). Although immense advances have been made recently in understanding the molecular details of hyphal morphogenesis and biofilm formation, clinical application of virulence targeting therapeutics has remained underexplored.

**Bacterial and *C. albicans* Interactions and Health Related Prevalence**

In the host *C. albicans* encounters hostile environments with antagonism from the host and commensal bacterial flora. As a result *C. albicans* has developed a multitude of strategies to overcome these challenges, making *C. albicans* a resilient pathogen (107). However, in a
healthy host *C. albicans* is for the most part benign. Although the switch from commensal to pathogen is largely dependent on the status of the host, the microflora and presence of other pathogens also play a role in this switch. As both a commensal and pathogen of the human host *C. albicans* encounters numerous bacterial species, which include both pathogenic and commensal species (11, 108-111). Complex interactions have been previously documented that include both synergistic and antagonist interactions where bacteria and *C. albicans* can have direct or indirect interactions (108, 110). These interactions can influence the growth and physiology of both microbes, often modulating the virulence of *C. albicans* (108, 112, 113). Physical interactions where bacteria adhere to *C. albicans* has been demonstrated with specific affinity for hyphal cell wall adhesins (112-114). Chemical exchange between *C. albicans* and bacteria that involves crosstalk between quorum sensing molecules has also been demonstrated in previous studies (114, 115).

Apart from the studies described here, there are only three bacterial species whose interactions with *C. albicans* have been significantly characterized, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Streptococcus gordonii*. As the complexity of these types of interactions are being uncovered, two main types of interactions have been observed: antagonism between pathogens (*P. aeruginosa–C. albicans*) and synergism between pathogens (*S. aureus–C. albicans, S. gordonii–C. albicans*) (110, 113, 116).

**Antagonistic Interactions**

Antagonistic interactions between *P. aeruginosa* and *C. albicans* have been the most extensively examined and reveal a complex dynamic between two pathogens that are commonly co-isolated from the lungs of cystic fibrosis (CF) patients (117-119). A Gram-negative bacterium, *P. aeruginosa* is an opportunistic pathogen with a multitude of virulence factors (120). In clinical cases where *C. albicans* is found during pulmonary infection, such as pneumonia and cystic fibrosis-related infections, it is usually co-isolated with bacteria (121, 122). Furthermore, *C. albicans* and *P. aeruginosa* are commonly co-isolated from burn wounds. Burn victims often have primary infections of *P. aeruginosa* that are followed by severe life-
threatening candidemia (108). Similarly, increased virulence was observed in a murine pseudomonal burn wound infection model during co-infection with *P. aeruginosa* (123). In vitro investigations in rats found that pre-colonization with *C. albicans* in lung tissue increased rates of *P. aeruginosa* associated pneumonia (122). This suggests that *C. albicans* mucosal biofilms promotes secondary bacterial infections, which has also been supported by a clinical study of ventilator patients who had an increased rate of bacterial infection with prior *C. albicans* colonization (124).

Conversely, in vitro examination of *C. albicans* and *P. aeruginosa* demonstrated antagonistic interaction in a wide range of environmental conditions. During biofilm growth *P. aeruginosa* preferentially adheres to *C. albicans* hyphal cell and forms biofilms on the hyphae, which leads to killing of *C. albicans*. This hyphal specific killing is due to the production of pseudomonal virulence factors, secreted haemolytic phospholipase and phenazines (125-127). Furthermore, *P. aeruginosa* inhibits the yeast-hyal transition by production of a 3-oxo-C12 homoserine lactone, a bacterial quorum sensing signaling molecule that mimics farnesol (126, 128). Farnesol is a quorum sensing signal molecule produced by *C. albicans*, which represses hyphal morphogenesis and plays an important role during biofilm dispersal (40, 129). Farnesol and 3-oxo-C12 homoserine lactone inactivate the Ras1-cAMP-PKA pathway and increase *TUP1* expression, thus blocking expression of hyphal-specific and biofilm genes that are positively regulated by Efg1 (129-133). Homoserine lactones and similar compounds are produced by other Gram-positive bacteria that similarly repress hyphal morphogenesis in *C. albicans*, suggesting cross-talk between bacteria and *C. albicans* might be important in polymicrobial interactions (134, 135).

In addition to the effects *P. aeruginosa* has on *C. albicans*, *C. albicans* does exert changes in *P. aeruginosa*. Farnesol inhibits the *Pseudomonas* quinolone signal (PQS), which responds to the production of 3-oxo-C12 homoserine lactone in *P. aeruginosa* and controls the production of phenezine products (136). During co-culture of *P. aeruginosa* and *C. albicans*, a reduction in PQS and thus phenezine products, suggests that the production of farnesol from
C. albicans is great enough to have an influence on P. aeruginosa (136). Taken together these results suggest an antagonistic relationship exists. However, these pathogens are frequently found during co-isolation in the clinical setting suggesting that a synergistic relationship might exist in certain settings. It is possible that the antagonistic behaviors observed in vitro allow P. aeruginosa to use C. albicans biofilms as a sort of scaffold or foundation, thus increasing the survival and colonization rates. There is apparent antagonism in vitro, but synergism in vivo, similar to our observation with E. faecalis and C. albicans.

Antagonist interactions between C. albicans and Streptococcus mutans have also been documented. S. mutans are Gram-positive bacteria found in the oral cavity and are associated with dental caries. They are resistant to highly acidic environments and produce acids that are associated with dissolution of the tooth surface, which leads to dental caries (137, 138). S. mutans and C. albicans are commonly co-isolated from early childhood dental caries and denture plaques of healthy individuals (139, 140). Similar to P. aeruginosa, S. mutans produces quorum sensing signaling molecules that inhibit C. albicans hyphae development.

The competence-stimulating peptide (CSP) was found to inhibit hyphal development in C. albicans (141). CSP is necessary for natural competence of S. mutans. Additionally, trans-2-decenoic acid, a diffusible signaling molecule inhibits hyphal development of C. albicans (142). SigX, an alternative sigma factor in S. mutans, is the master regulator of quorum sensing (143). Strong induction of sigX is observed in S. mutans biofilms grown with C. albicans compared to monoculture biofilms suggesting that C. albicans stimulates quorum sensing signaling in S. mutans (144).

**Synergistic Interactions**

Adhesion is an important factor for C. albicans colonization, pathogenesis and polymicrobial interactions. The ALS proteins in particular play an important role in the biofilm lifecycle and polymicrobial interactions. Many of the clinical conditions where C. albicans is co-isolated with bacteria are considered biofilm-associated and adherence and/or colonization to a surface are a requirement for infection (145). The ALS (agglutinin-like sequence) genes encode
eight different glycoproteins that contain an N-terminal domain with adhesive function that are found diffusely over the cell surface of \textit{C. albicans} (146).

A vital component in inter-kingdom interactions, coaggregation is essential for polymicrobial communities found in the oral cavity and facilitates the complex architecture found in dental plaques. \textit{C. albicans} is capable of adhering to various oral streptococcus species (\textit{S. mutans}, \textit{S. salivarius} and \textit{S. gordonii}). The ALS proteins mediate these interactions with streptococci during polymicrobial biofilm development. Specifically, Als3 in \textit{C. albicans} is necessary for biofilm formation with \textit{S. gordonii} on a salivary pellicle (147). Furthermore, \textit{S. gordonii} was able to bind \textit{Saccharomyces cerevisiae} heterologously expressing \textit{C. albicans} Als3p (147). Als1 was also found to bind to the surface of \textit{S. gordonii}; the Als proteins have similar structures, and thus redundant function is feasible. The presence of \textit{S. gordonii} enhanced hyphal morphogenesis and biofilm formation in human saliva mediated by SspA and SspB bacterial surface proteins that interact with Als1 and Als3 (146, 148). Additionally, quorum sensing signaling via \textit{luxS} regulation of autoinducer-2 (A-II) is necessary for enhanced hyphal morphogenesis and biofilm formation (149).

Synergistic interactions between \textit{Staphylococcus aureus} and \textit{C. albicans} have been observed. \textit{S. aureus} has an affinity for binding to hyphal cells of \textit{C. albicans} that is mediated by Als3 on the surface of \textit{C. albicans} (113). \textit{S. aureus}, a Gram-positive opportunistic pathogen found commonly on the skin, possesses a series of virulence factors that contribute to its significant prevalence in nosocomial infections in immunocompromised individuals (150). \textit{S. aureus} was unable to bind to the adhesin-deficient mutant strains of \textit{C. albicans ALS3} (113). In addition, \textit{S. aureus} was able to bind the recombinant purified N-terminal domain of Als3 and \textit{Saccharomyces cerevisiae} heterologously expressing \textit{C. albicans} Als3p (113). In an \textit{ex vivo} murine co-infection model, tongues excised from mice where inoculated with \textit{C. albicans} and \textit{S. aureus} in a 12-well plate, \textit{S. aureus} was found in the outer layers of the tissue when tongues were infected with \textit{S. aureus} only or with the ALS3 mutant strain. Conversely, tongues infected
with *S. aureus* and wild-type *C. albicans* had *S. aureus* in epithelial tissue with invasive hyphae (113).

The interactions between *C. albicans* and *S. aureus* have also been examined extensively in multiple animal models that demonstrate a significant increase in virulence of both microorganisms. During intraperitoneal infections of mice with *C. albicans* and *S. aureus*, mice have increased mortality rates and both microorganisms are found together in the spleen, pancreas, and esophagus. This demonstrates synergistic behavior during dissemination from the intraperitoneal site of infection, similar to in vitro observations (151-153). Co-infection with *C. albicans* and *S. aureus* in a peritonitis murine model significantly increased mortality compared to mono-infection and increased fungal and bacterial burden was observed during co-infection. Increased proinflammatory cytokines (IL-6 and G-CSF) and neutrophil infiltration was observed during co-infection. Treatment with nonsteroidal anti-inflammatory drugs (NSAIDs) increased mouse survival and lowered fungal and bacterial burdens in the kidneys and spleen (154).

*C. albicans* has complex interactions with both pathogens and commensal bacteria found in the human host in various niches. These complex interactions can be physical interactions where bacteria adhere with specific affinity to hyphal cells, as has been demonstrated to occur with *C. albicans* hyphal cell wall ahesions (112, 113). Chemical exchange between *C. albicans* and bacteria that involves crosstalk between quorum sensing molecules has also been observed (133, 155). Furthermore, a combination of these types of interactions can transpire between *C. albicans* and bacterial species, alluding to the complexity of the inter-kingdom interactions that occur.

**Co-isolation of *C. albicans* and *E. faecalis***

The most well studied niches of *C. albicans* are the gastrointestinal (GI) and urogenital tracts and it is well documented to come into contact with the bacterial flora, such as
Enterococcus faecalis. Interestingly, co-isolation of *C. albicans* and *E. faecalis* is common, of which the frequency is likely due to their biological similarities, specifically colonization of overlapping niches (156-158). Both are opportunistic pathogens causing serious infection in the presence of immunological dysfunction.

The prevalence of *C. albicans* and *E. faecalis* co-isolation from sites throughout the human host is effectively demonstrated by a large-scale retrospective examination of 67,765 different clinical samples (109). The samples were collected at a large teaching hospital in Germany over a two-year period. *Candida spp.* were detectable in about 7.7% of the samples that included blood, CSF, feces, sputum and skin. The frequency of *E. faecalis* isolation increased 2-fold in the *Candida*-positive samples compared to *Candida*-negative samples. This trend was further increased in *Candida*-positive samples from blood and ICU patients, demonstrating the prominence of co-infection in more severe infections and individuals with pre-existing medical conditions (109).

Commonly co-isolated from the oral cavity of individuals with secondary root canal infections, *C. albicans* and *E. faecalis* are typically resistant to antimicrobials, a probable cause for failed root canal treatment strategies (159). During root canal treatment, the infected root canals are mechanically removed and irritants are chemically removed followed by blocking the root canal system with dental filling (gold, porcelain, silver amalgam, polymethyl methacrylate resin, PMMA) to eliminate microbial activity thus preventing reinfection. However, absolute removal of all microorganisms from the root canal is not always achieved and the oral cavity is abundant with microorganisms that can colonize microspaces between the dental filling acrylic and tooth surface (160-162).

Multifaceted microbial communities that consist of anaerobic bacteria (strict and facultative) and fungi reside in the root canals of infected teeth (163, 164). Of these microbes, *E. faecalis* and *C. albicans* are equipped to resist various endodontic treatment strategies that allow them to survive and cause secondary root canal infections. (165-167).
Many studies demonstrate the importance of *C. albicans* and *E. faecalis* separately during secondary root canal infection. Yet, only a handful of studies investigate the co-isolation of these microorganisms. Given their pathological similarities during periodontal infections it is plausible that the occurrence of co-infection rates is underrepresented. A study investigating single-rooted teeth with pulp necrosis, found that samples collected after root canal treatment had increased populations of enterococci co-isolated with *Candida* spp. and/or enteric bacteria (168). However, the majority of samples tested had enterococci mono-infections, suggesting that interactions with other microorganisms are not necessary for this type of periodontal infection (168).

Although secondary root canal infections are treatable with additional surgical removal and debridement of the infected root canal and filling, if left untreated can become a more severe systemic infection. *C. albicans* and *E. faecalis* that survive the root canal system can spread to the periodontal tissues and invade the bloodstream, which can lead to bloodstream infections (BSI) and followed by development of endocarditis. BSIs with at least two different microorganisms are considered polymicrobial BSI and are on the rise with rates ranging from 5% to 32% of all BSI reports (169-172). With increased mortality rates (21%-63%) compared to mono-microbial mortality rates, polymicrobial BSIs are a growing concern (169, 170).

Of more than 8000 reported incidences of candidemia (from 1965 to 2007) 23% were polymicrobial, suggesting that high rates of polymicrobial BSI occur with candidemia (157). An extensive study examining patients with candidemia found that of 372 patients, 141 patients from 8 Veterans Affairs hospitals and 231 patients from a tertiary care hospital, 27% had polymicrobial BSI (24% with synchronous bacteremia and 3% with more than one *Candida* spp.) (157). Of the patients with synchronous bacteremia (88 patients) 27% where infected with *Enterococcus* spp. Furthermore, the most frequent source of polymicrobial BSI is from intra-abdominal infections that are frequently the result of loss of GI integrity allowing
microorganisms, such as *C. albicans* and *E. faecalis*, from the GI tract to infect the peritoneal cavity (170, 173-175).

Most populations of *C. albicans* and *E. faecalis* are located in the GI tract where they act as commensals and are for the most part benign. However, if the host becomes immunocompromised these microbes can become pathogenic and the populations in the GI tract are a common reservoir for more severe types of infections. Intra-abdominal infections (IAI) are commonly caused by infection from the GI tract microbiota and include many pathological conditions that can range from uncomplicated, where only one organ is infected and the infection does not progress to the peritoneum, to complicated, where the infection progresses past the organ to the peritoneum causing localized or diffuse peritonitis (176, 177).

Peritonitis is further classified into primary, secondary or tertiary peritonitis (176). Primary peritonitis is a disseminated infection with no loss of GI tract integrity and mainly occurs in pediatric patients. The most common form, secondary peritonitis is an acute peritoneal infection that results from loss of GI integrity due to perforation of the GI tract or from intra-abdominal viscera from infected organs (e.g. gangrenous appendicitis). Recurrent infections of the peritoneal cavity that follow primary or secondary peritonitis are classified as tertiary infections. These types of infections can be acquired from the community or healthcare setting. The latter is associated with increased mortality due to the status of the host and multidrug resistance of the infections (178). Additionally, increased mortality rates (~30%) are associated with severe sepsis or toxic shock in secondary infections (179-181).

A retrospective study of 163 adult patients with intra-abdominal candidiasis (IAC) compared to 161 patients with candidemia found increased rates of bacterial co-infections in patients with IAC (67%) compared to polymicrobial BSI (6%) (182). Different types of IAC were evaluated: intra-abdominal abscesses (55%), secondary peritonitis (33%), primary peritonitis (5%), infected pancreatic necrosis (5%), and cholecystitis/cholangitis (3%). The most common
Candida spp. isolated were C. albicans (56%) and C. glabrata (24%). Consistent with previous studies, enteric pathogens (Enterococcus and Enterobacteriaceae) were co-isolated with Candida spp (183, 184). Of the 110 patients with bacterial co-infection, enterococci were most abundant (29%), followed by Escherichia coli (19%) and Klebsiella spp. (15%) (182). However, the mortality rates for IAC mono-infection and IAC bacterial co-infections are comparable in these investigations, suggesting that Candida spp. are capable pathogens and not significantly modulated by co-infecting bacteria in IAI (182, 185).

**E. faecalis Pathogenesis**

Enterococci are the initial colonizers of the GI tract in infants and were once only considered as commensal colonizers with nominal clinical relevance. However, over the last four decades, with increased use of antibiotics, enterococci have become prominent opportunistic pathogens responsible for causing numerous types of infections: urinary tract infections (UTI), IAI, bacteremia, and endocarditis (186). Antibiotic resistance is directly related to the emergence of enterococci as a clinically important pathogen and the most clinically significant species are Enterococcus faecalis and Enterococcus faecium (186, 187). Since enterococci are intrinsically resistant to many antibiotics, have the ability to acquire resistance genes, and are tolerant to antibiotics that target the cell wall, treatment of enterococcal infections is often problematic (187, 188). Thus, the use of combinational therapy is required to synergistically acquire bactericidal effects (189).

As part of the microflora enterococci colonize the GI tract of mammals (including humans) and insects. Although both E. faecalis and E. faecium colonize humans, E. faecalis colonizes the GI tract at a higher density (10^7 CFU/μg) than E. faecium [191]. E. faecalis is also the predominant species of enterococci found in the urogenital tract. The endogenous microflora was previously thought to be the predominant source of enterococcal infections. Nevertheless with increased investigations in the clinical setting, transmission of pathogenic
enterococci from nosocomial sources (e.g. hands of healthcare provider) has been extensively documented (190, 191). The ability of enterococci to persist for extended periods on hands (60 minutes) and inanimate objects (4 months) creates a reservoir for continual transmission in the hospital setting (192). Necessary for the numerous types of infections they cause, *E. faecalis* has a multitude of virulence factors, such as: cytolysin, gelatinase, serine protease, aggregation substances, Esp (enterococcal surface protein), Ace (adhesin to collagen of *E. faecalis*), EfaA (*E. faecalis* antigen A), EbpABC (endocarditis and biofilm-associated pili), and enterococcal capsule (193). Acquisition of extrachromosomal elements plays an important role in adaptation and survival of enterococci (194). Extrachromosomal elements are usually acquired by conjugation, where conjugative plasmids are transferred from a donor to recipient controlled by peptide sex pheromones (194). Antibiotic resistance, elevated resistance to UV light, virulence factors and bacteriocins are frequently plasmid-determined traits. Thus, significant genetic diversity in response to environmental selection pressure is observed in enterococci (195, 196).

Part of the core genome, the proteases, gelatinase (GelE, a matrix metalloprotease) and a serine protease (SprE), in *E. faecalis* are important for degradation and damage of host tissues during infection (197). These proteases affect a broad range of host functions by degradation of host connective tissues and proteins, and interference with host cell signaling and immune response (198, 199). Included in a single operon, GelE and SprE gene expression is regulated by the *fsr* (*E. faecalis* regulator) genes that encode a canonical two-component system, a homolog to the *agr* (accessory gene regulator) operon in *S. aureus* (200, 201).

The response regulator (FsrA) positively regulates the *fsr* genes (*fsrABCD*) and downstream virulence factors (*gelE, sprE*). A quorum sensing two-component system, Fsr responds to increased concentrations of a peptide pheromone encoded by *fsrD*, gelatinase biosynthesis-activating pheromone (GBAP) that is secreted and processed by the transmembrane protein FsrB. FsrA is constitutively expressed at basal levels, when threshold
concentrations of extracellular GABP have accumulated the histidine kinase, FsrC is phosphorylated followed by phosphorylation of the response regulator, FsrA. This signal transduction via the Fsr system results in upregulated expression of the fsr genes and downstream virulence factors, gelE, sprE (197, 202).

Although both GelE and SprE, to a lesser extent, are important virulence factors, only GelE activity and role in virulence has been extensively examined. The gelE gene encodes a 509-amino acid protein of which 29-amino acids at the N-terminus serve as a Sec secretion signal sequence. This signal sequence is cleaved upon secretion resulting in release of the proenzyme of 480-amino acids. After secretion the proenzyme is further proteolytically processed at the N-terminus and C-terminus by an autocatalytic process resulting in the mature protein of 33kDa (203, 204).

GelE is a member of the M4 family of proteases and is similar to thermolysin from Bacillus thermoproteolyticus and aureolysin from Staphylococcus aureus (205, 206). With broad substrate specificity, GelE has the ability to hydrolyze gelatin, cleave enterococcal conjugative sex pheromones, and has several host-derived substrates important during infection (insulin B chain, endothelin, hemoglobin, fibrinogen, fibronectin, collagen, and laminin) (207, 208). Furthermore, GelE interferes with the innate immune response by inactivating human antimicrobial peptides LL-37 and β-defensins and is pertinent for E. faecalis dissemination (209-211).

A serine glutamyl endopeptidase of 25kDa in size, SprE is similar to staphylococcal glutamyl endopeptidases V8 and GluSE (212, 213). Similar to GelE, insulin, fibrinogen, and casein have been observed as substrates of SprE and disruption of sprE results in decreased virulence in different infection models, mouse peritonitis, Caenorhabditis elegans, and a rabbit endophthalmitis (201, 214-217). SprE enzymatic activity is modified in the presence of GelE similar to reports in S. aureus, where the GelE homolog, aureolysin, inhibits autocatalytic processing of the SprE homolog, SspA (V8), by proteolytically processing the N-terminus of
SspA by aureolysin (218, 219). Purification of SprE proenzyme from gelE mutant supernatant resulted in purification of several forms of SprE that were proteolytically processed at both the N-terminus and C-terminus (218, 220). Three different mature forms of SprE were purified that had enzymatic activity (Leu\(^1\)-Ala\(^{237}\), Ser\(^1\)-Glu\(^{227}\), Leu\(^1\)-Glu\(^{227}\)). However, only one form of SprE (25kDa) was purified from wild-type supernatant (218, 220). Although GelE processes SprE, the reverse is not apparent because a sprE insertion mutant produces gelatinase (201).

Considering the interconnected gene regulation and proteolytic processing of GelE and SprE, it no surprise that they function in combination. While GelE and SprE play independent roles in different infection models (mouse peritonitis, Caenorhabditis elegans, rabbit endophthalmitis) disruption of both proteases had an additive effect (201, 214-217). Moreover, roles for both GelE and SprE have been observed in biofilm development and regulating autolysis in E. faecalis (221). Deletion of gelE resulted in a significant reduction in biofilm biomass compared to wild-type biofilms. Whereas deletion of sprE resulted in accelerated biofilm development and increased autolysis, suggesting that interplay between these proteases controls biofilm development in E. faecalis (221).

**E. faecalis Commensalism**

Enterococci colonize the GI tracts of mammals, reptiles, birds and insects, which suggests that enterococci have been part of the GI microflora since the early Devonian period, (~412 MYA), making them one of the earliest colonizers of the GI tract (222-224). In the human GI tract, enterococci are predominantly located in the small and large intestine (jejunal, ileal, cecal, and recto-sigmoidal sections) (225). E. faecalis and E. faecium are the predominant enterococci found in human feces, and E. faecalis is most common species found in the GI tracts of mammals and insects (226, 227).

*E. faecalis* is a member of the lactic acid group of bacteria (LAB), which are low-GC gram-positive bacteria that produce lactic acid as a major end product of carbohydrate
fermentation [232,233]. Many of the LAB are used as probiotics, specifically *Lactobacilli* spp., which have been shown to benefit GI health (228, 229). In spite of the fact that enterococci are as abundant in the human GI tract as lactobacilli, they are commonly associated with being a multidrug resistant (MDR) pathogen (228). Although commensal enterococci can act as opportunistic pathogens by translocating across the mucosal barrier causing systemic infections in immune-compromised individuals, more commonly infections stem from colonization, overgrowth, and translocation of hospital-adapted strains that have enhanced pathogenicity and antibiotic-resistance (230, 231).

Enterococci are commonly used in food fermentation and preservation, traditionally used in cheeses, fermented meats and vegetables manufactured in Mediterranean countries (232-234). *E. faecium* and *E. faecalis* are the most commonly used and impact the ripening and aroma development in these food products (229, 233). Enterococci are not purposely added as starter cultures to these types of food. However, they occur in high numbers and their natural habitat is probably not fermented food, but an outside environmental source that has yet to be identified (235).

Enterococci as probiotics are used in countries (European Union) other than the US (229, 236, 237). *E. faecium* and *E. faecalis* are the species most commonly used as probiotics. The *E. faecium* strain SF68 probiotic, was demonstrated to be clinically effective in children for treatment of diarrhea and prevention of diarrhea due to antibiotic treatment (238). The efficacy of *E. faecium* SF68 in treatment of intestinal infections is likely due to the strain being part of the natural intestinal microflora and having a short doubling time in optimal conditions. Growth inhibition of *E. coli*, *Salmonella* serovars, *Shigella* spp. and *Enterobacter* spp by *E. faecium* SF68 was demonstrated in vitro and shown to tolerate the extreme conditions of the GI tract (239). Other probiotic preparations containing *E. faecium* have been shown to reduce irritable bowel syndrome, dysbiosis as a result of foodborne pathogens, and lowering of serum cholesterol (240, 241).
Bacteriocins Produced by Gram-positive Bacteria

Bacteriocins are produced by Gram-positive and Gram-negative bacteria and are ribosomally synthesized proteinaceous secreted compounds that have antimicrobial activity against other bacteria, typically those that are closely related to the bacteriocin producing species. Production of bacteriocins in Gram-positive bacteria, especially the lactic acid bacteria (LAB), has been increasingly studied with the growing interest of probiotic LAB. Bacteriocins produced by Gram-positive bacteria can be organized into four major groups: (1) lantibiotics, (2) small non-modified peptides, (3) large proteins, and (4) cyclic peptides. The classification of bacteriocins from Gram-positive bacteria is continually modified with the discovery of new types of bacteriocins. However, Heng et. al. (242) have formulated a systematic classification plan based on previous literature that encompasses recently characterized bacteriocins (243-245).

Class I bacteriocins are the lantibiotic bacteriocins that contain modified amino acids that are not genetically encoded (246). Lantibiotics are produced ribosomally as precursor peptides that undergo post-translational modifications to produce the non-genetically encoded amino acids lanthionine (Lan), 3-methylanthionine (MeLan), dehydroalanine (Dha), and dehydrobutyrine (Dhb) (247). Class II bacteriocins are small (<10 kDa), unmodified (non-lantibiotic and non-cyclic) peptides that encompass bacteriocins from diverse origins making it the largest class of characterized bacteriocins (248). Although most characterized bacteriocins from Gram-positive bacteria are small (<10 kDa), class III contains larger (>10 kDa) bacteriocins that are typically heat liable, but not always (243, 249). The most distinctive bacteriocins produced by Gram-positive are the cyclic bacteriocins of class IV, which are post-translationally modified to produce the mature cyclic peptide (250).

Bacteriocin Production in *E. faecalis*
Enterococci that produce bacteriocins have been isolated form a wide-range of environments. Bacteriocin-producing enterococci are commonly found in cheeses, fermented vegetables and meats, thus, have been implicated in food preservation (229, 233, 250). The high frequency of bacteriocin-producing enterococci isolated from healthy individuals stool samples suggests that they are important part of the healthy microflora and may benefit the host and microbe (251). Although bacteriocin production in LAB is commonly associated with probiotics and benefiting the health of the GI tract, enterococcal bacteriocins have also been associated with infection (252, 253).

In *E. faecalis* bacteriocin encoding genes are often carried on mobile genetic elements, such as conjugative plasmids, suggesting that efficient dissemination of bacteriocin-producing traits may be important for survival and virulence. Cytolysin is a secreted lantibiotic class of bacteriocins with cytotoxic effects on prokaryotic and eukaryotic cells (252, 253). Epidemiological data support a role for the cytolysin as a toxin in human infection. Enrichment for cytolysin expressing *E. faecalis* has been observed in the hospital-acquired populations compared to community acquired populations of isolates derived from infection (254). These findings suggest that selection pressure for cytolysin activity is prevalent in the hospital environment, which corresponds to experimental and clinical evidence that cytolysin activity plays an important role during infection (255).

On the other hand, the heat-stable circular bacteriocin AS-48 produced by *E. faecalis* strains that are commonly isolated from cheese, milk, and fermented food products has potential as food biopreservative (250, 256). This bacteriocin is encoded on a plasmid that contains ten biosynthetic genes (257). Arranged into five alpha helices giving it a compact globular structure, this 70 amino acids bacteriocin is circularized head to tail by a peptide bond. AS-48 has broad inhibitory spectrum that includes both Gram-positive and Gram-negative species (250). AS-48 is active against foodborne pathogens, toxigenic bacteria and bacteria associated with food spoilage (258).
The role of bacteriocins production in natural environments is debatable. However, one logical and common idea is that bacteriocins provide the producing organism with a fitness benefit that allows them an ecological advantage over competitors (259). The occurrence of bacteriocins as a beneficial probiotic trait in the human GI tract and as a virulence trait during infection, suggests that bacteriocin production allows enterococci the ability to adapt to different environmental conditions. Moreover, bacteriocins enriched during infection associated with antibiotic resistance are different from bacteriocins associated with commensal enterococci from healthy individuals (260).

**Bacteriocins as Treatment Strategies**

Bacteriocins produced by LAB have mainly been used and extensively studied in biopreservation. However, more recently the interest for using bacteriocins for biomedical applications has increased with investigations for the use of bacteriocins against pathogenic microorganisms still gaining significance (261). Studies examining biomedical application of LAB bacteriocins include efficacy analyses against systemic infections caused by *S. aureus* and *P. aeruginosa*, oral infections caused by *Streptococci*, and vaginal infections caused by *Gardnerella vaginalis* (262, 263).

One of the most extensively characterized bacteriocins is the lantibiotic nisin that is produced by LAB (*Lactococci* and *Streptococci*) (264, 265). First characterized in *Lactococcus lactis* isolated from fermented milk cultures, nisin is approved by the Joint Food and Agriculture Organization/ World Health Organization (FAO/WHO) and the Food and Drug Administration (FDA) for use as biopreservative (244, 265). Nisin A from *Lactococcus lactis* is a small (34 amino acids) cationic peptide that contains five lanthionine rings that are formed by dehydration of precursor serines and threonines that react with neighboring cysteines to give lanthionine or 3-methyllanthionine that contain a thioether group (266).
Nisin A targets lipid II, which is essential for bacterial cell wall biosynthesis and growth, (267, 268). The high affinity for binding lipid II initiates pore formation in the membrane leading to depletion of the membrane potential due to leakage of cellular content followed by cell death. Nisin A has broad spectrum of activity against Gram-positive bacterial pathogens (foodborne and non-food related) and Gram-negative bacteria when used in conjunction with antibiotics (269, 270). Other natural variants of nisin have been identified from different *Lactococcus spp.* and *Streptococcus spp* (270). Although, these natural variant share similar structure, slight changes in the amino acid sequence alter the efficacy and target spectrum of these bacteriocins, as seen with Nisin Z (271, 272). Moreover, bioengineered nisin variants have enhanced activity against Gram-negative pathogenic bacteria, demonstrating the potential of bacteriocins based therapeutic strategies (273).

**Bacteriocins Active Against Candida spp.**

Bacteriocins have traditionally only been tested for their antibacterial properties. However, the prominence of nosocomial fungal infections resistant to traditional antifungals has amplified the need for alternative treatment strategies, such as bacteriocin-based therapeutics. A similar natural-variant of nisin A, nisin Z is produced by *L. lactis* NIZO22186 and significantly inhibits in vitro growth and hyphal morphogenesis of *C. albicans* (271, 274). Additionally, nisin Z inhibits hyphal morphogenesis and adhesion of *C. albicans* to oral gingival cell in an *ex vivo* oropharyngeal candidiasis (OPC) model (275). Although nisin A and Z have similar antimicrobial properties, there is a single residue change (H27N) that confers a superior diffusion rate and increases solubility at neutral pH (272). This suggests that subtle changes in bacteriocins can yield increased efficacy against fungal pathogens. However, other natural or bioengineered variants of nisin have not been reported to have activity against *C. albicans*.

*Lactobacillus plantarum*, another LAB associated with fermented food, produces multiple bacteriocins (class II) that comprise the plantaricin C11 complex, components of which
have been reported to have fungicidal activity against *C. albicans* (276, 277). The plantaricin C11 locus includes five operons: *plnABCD*, the three-component signal transduction system; *plnEFI*, two bacteriocins (PlnE and F) and immunity factor; *plnJKLR*, two bacteriocins (PlnJ and K) and immunity factors; *plnMNOP*, unknown function; *plnGHSTUV*, bacteriocin export machinery (278). The mechanism of action for plantaricin C11 is similar to other bacteriocins of its class, PlnEF and PlnJK target the membrane, create pores that disrupt the membrane potential and lead to bacterial cell death (269, 279). Given the difference in cell wall anatomy of Gram-positive bacteria and *C. albicans* the mechanism of action could vary. Treatment of *C. albicans* with the different bacteriocins disrupted the membrane potential, however, PlnJ had the greatest effect and caused release of potassium ions (K+) from cells. Additionally, all plantaricin peptides increased reactive oxygen species (ROS) in *C. albicans*, suggesting that plantaricins induce apoptotic cell death resulting in toxin-induced necrosis (277).

**Antimicrobial Peptides Active Against *C. albicans***

Part of the innate immune response, antimicrobial peptides (AMPs) are differentially expressed in response to pathogen-associated molecular patterns (PAMPs) that are recognized by pattern recognition receptors (PRRs) present on host cells. AMPs from the major classes, including cathelicidins, histatins, and defensins are demonstrated to be active against *C. albicans*. Interestingly, AMPs have common characteristic found in bacteriocins (cationic, amphipathic, positive net charge, structure stabilized by thiols), suggesting mechanisms of action may overlap between AMPs and bacteriocins (266, 280).

The antimicrobial activity of histatin-5 (Hst-5) against *C. albicans* is the most extensively examined of the AMPs that target *C. albicans* (281-283). Hst-5 is a cationic peptide found in saliva providing the first line of defense against oral pathogens such as *C. albicans*. The mechanism of action against *C. albicans* involves binding cell wall β-glycans and the cell wall protein, Ssa1 and 2 (Stress-Seventy subfamily A), that allow for translocation of Hst-5 into the
cell via polyamide transporters (Dur3 and 31, Degradation of URea) (284-287). Inside the *C. albicans* cell, Hst-5 has multiple intracellular targets that lead to disruption of mitochondrial function, production of ROS, osmotic stress due to loss of potassium ion (K+), and then ultimately cell death (288, 289).

LL-37 is a human cathelicidin that is derived by proteolysis of the CAP18 protein C-terminus and is produced by macrophages, neutrophils, keratinocytes, and mucosal epithelial cells (290-292). Shown to have fungicidal activity against *C. albicans*, LL-37 associates with the cell wall and/or membrane and binds to the cell wall protein Xog1 (β-1,3-exoglucanase) that causes remodeling of the cell wall of *C. albicans* (293). Increased concentrations of LL-37 disrupt the cell membrane causing segregation of the membrane into small vesicles disrupting the membrane potential due to loss of intracellular contents. The mechanism suggests that LL-37 is able to remodel the membrane via Xog1 binding, thereby allowing for interaction with the cell membrane and eventually disrupting the cell membrane and causing cell death (293-295).

Defensins are small (3.5 to 6 kDa) with three disulfide bridges and are grouped in two classes, α-defensins and β-defensins (296, 297). Found in neutrophil granulocytes, α-defensin (HNP-1) is active against *C. albicans* by non-lytic ATP efflux that depletes intracellular ATP leading to cell death (298). β-defensins are larger and share little primary structure similarity, but have very similar tertiary structures (297). Expressed in environmentally exposed epithelia, β-defensin (hBD-3) has candidacidal activity against *C. albicans* by binding Xog1 which causes cell wall remodeling similar to LL-37, without depletion of ATP (295).

AMPs are generally positively charged (+2 to +9), small peptides of 10 to 50 amino acids that are frequently hydrophobic residues (≥30%). The structure of AMPs is important for antimicrobial activity and many are amphipathic helices that form pores on bacterial membranes (299). As reported, the mechanisms of action against *C. albicans* of these AMPs are different and often require remodeling or transient interactions with the cell wall of *C. albicans*. However, the structural conformation of these AMPs is important for interactions
with the cells wall and membrane that initiate the mechanism of action (294, 296, 300). Interestingly, sub-lethal concentrations of human LL-37, hBD-3 and Hst-5 reduce adhesion of C. albicans to polystyrene (293, 295, 301, 302). LL-37 and hBD-3 also inhibit hyphal morphogenesis at sub-lethal concentrations, suggesting that these AMPs could potentially have clinical relevance in biofilm prevention (295, 302).
CHAPTER 2:

Materials and Methods

Portions of this chapter are based on my first author publication. I have received permission to reproduce data or text from this article. I have contributed significantly to this publication. The article is listed for reference: Graham, C. E., M. R. Cruz, D. A. Garsin, and M. C. Lorenz. 2017. Enterococcus faecalis bacteriocin EntV inhibits hyphal morphogenesis, biofilm formation, and virulence of Candida albicans. Proc. Natl. Acad. Sci. U.S.A. 114: 4507–4512 (303).
Strains and Culture Conditions

Bacterial and fungal strains used in this study are listed in Table 2.1. Media was purchased from DIFCO and chemicals from Sigma, unless otherwise stated. *Escherichia coli* strains were cultured in Luria Bertani broth overnight at 37°C with antibiotics where appropriate using the following concentrations (µg/ml): kanamycin, 50; spectinomycin, 100; erythromycin 300. *E. faecalis* strains were cultured overnight at 37°C in Brain Heart Infusion (BHI) medium or M9HY (304) with 100 µM glucose. Addition of antibiotics where applicable were at the following concentrations (µg/ml): erythromycin, 50; and rifampicin, 100. Fungal strains were propagated in yeast extract-peptone-dextrose (YPD) (305). All *C. albicans* biofilms were grown in artificial saliva (YNB-AS) modified from Wong *et al.* (306) (0.17 % YNB (w/v), 0.5 % casamino acids (w/v), 0.25 % mucin (w/v), 14 mM potassium chloride, 8 mM sodium chloride, 100 µM choline chloride, 50 µM sodium citrate, 5 µM ascorbate).

Strain Construction

The *entV* mutant strain was constructed as previously described to create an in-frame markerless deletion using a *P*-pheS*⁺* counterselection system (307). Briefly, sequences flanking the 5’ and 3’ ends of *entV* were amplified by PCR. The 2 products were fused together by PCR with primer-introduced NotI and Pst1 sites. The resulting fragment was ligated into NotI/PstI digested pCJK47 vector, resulting in pCEG1. pCEG1 was electroporated into CK111 cells, and counterselection was completed to create strain CEG1F. Deletion of *entV* was confirmed by PCR and sequencing. The *entV* complement strain was constructed using a previously described strategy in which the wild type locus is regenerated with a silent mutation in the gene to distinguish it from the original wild type strain (308). To generate *entV*⁺, a mutation (CTT) was introduced into the leucine codon TTA corresponding to amino acid position 50. The hyphal specific reporter strain (*HWP1)p::GFP) was transformed with plasmid
pADH1-mcherry (ADH1_p::mcherry) to construct a C. albicans strain expressing both hyphal specific GFP and constitutive mcherry (309).

C. albicans Biofilm Assay

The C. albicans biofilm assay was developed using a similar technique as described previously with the following modifications (42, 44, 310). C. albicans strains were grown overnight at 30°C in YPD and washed three times with phosphate-buffered saline (PBS), and the final OD_{600} was adjusted to 0.2 (~1 x 10^7 cell/ml) in PBS. Tissue culture treated 96 well polystyrene plates (Corner) or chambered polystyrene slides (Ibidi) were inoculated with C. albicans in PBS and incubated at 37°C for 90 minutes. Inoculated plates and slides were then gently washed with PBS. After removal of PBS, YNB-AS was added with 50% bacterial supernatant or sterile M9HY and incubated at 37°C for 24 to 48 hours. The supernatant of E. faecalis (OG1RF, ΔfsrB, ΔentV, ΔentV/entV) was collected as previously described (115). Biofilms grown with rEntV^{136} or sEntV^{68} were grown similarly with the peptide added to the YNB-AS medium at the appropriate concentrations and compared to HEPES or DMSO vehicle controls, respectively. After 24 to 48 hours of growth biofilms were washed three times with PBS and hyphal morphogenesis and biofilm biomass were assessed.

Hyphal morphogenesis was quantified in C. albicans (HWP1::pGFP) biofilms grown in 96 well microplates by measuring GFP fluorescence, fluorescence intensity (excitation 488 nm, emission 520 nm) was determined using a fluorescence microplate reader (SynergyMX, Biotek). The relative fluorescence was calculated per well as a ration of GFP fluorescence to cell density (OD_{600}) and normalized to control treated biofilms (sterile medium, HEPES, or DMSO). Biofilm biomass was determined by resazurin staining of C. albicans biofilms as previously described (311), using 0.015mg/ml of resazurin for up to 1 hour in the dark.

For microscopic imaging, C. albicans biofilms were gently washed in PBS, as stated above. Wild-type C. albicans (SC5341) were stained using 35 g/ml of calcofluor white for 5
minutes in the dark and imaged using 4',6-diamidino-2-phenylindole (DAPI), Fluorescein isothiocyanate (FITC), and differential interference contrast (DIC) filters on an Olympus IX81 automated inverted microscope with Slidebook software (version 6.0) or Nikon A1R Confocal Laser automated inverted microscope system with NIS Elements (version 4.5).

All biofilm assays were done in triplicate for three independent experiments. The different treatment types per experiment were compared by pairwise by Student’s t-test or One-Way ANOVA with a P value of <0.05 considered statistically significant using GraphPad Prism (version 6.0).

**Expression and Purification of Recombinant EntV**

Hexahistidine-tagged EntV\(^{136}\) was expressed as described previously (312) with the following modifications. The Coding regions of EntV (residues 35 to 171) were cloned in the pET28a vector and transformed in to E. coli BL21 (DE3) cells. Cells were lysed in lysis buffer (25 mM TrisHCl pH 8.0, 0.1 M sodium chloride, 1% (v/v) NP40, 10% (v/v) glycerol and 1 mM PMSF) by sonication for 10 minutes on an ice water bath followed by centrifugation at 10,000 x g at 4°C for 20 minutes.

The clarified lysate was then loaded on to a pre-equilibrated TALON affinity resin (Clontech) and washed three times with 10 column volumes each of wash buffer (50 mM sodium phosphate, 100 mM sodium chloride, 10 mM imidazole). The hexahistidine-tagged protein was eluted in elution buffer (50 mM sodium phosphate, 100 mM sodium chloride, 150 mM imidazole) and elution fractions were collected. Samples with purified protein were combined and dialyzed against buffer containing 5 mM HEPES pH 7.0. The collected fractions were then subjected to Tricine-SDS-PAGE and analyzed by western blot using anti-His antibodies (THE™, GenScript).
Murine Macrophage Infection

The activity of sEntV\textsuperscript{68} against \textit{C. albicans} was evaluated in the murine macrophage infection model using the end point dilution assay (313, 314). Briefly, RAW264.7 murine macrophages were seeded in a 96-well plate with RPMI 1640 with phenol red supplemented with 10% fetal bovine serum (RPMI-10) at a concentration of $2.5 \times 10^5$ cells/ml per well and incubated overnight at 37°C with 5% CO\textsubscript{2}. After overnight growth at 30°C in YPD, \textit{C. albicans} cultures were washed twice with PBS and resuspended at a concentration of $5 \times 10^5$ cells/ml in RPMI minus phenol with 100 nM of sEntV\textsuperscript{68} or DMSO and incubated at room temperature (RT) with shaking for 1.5 hours. The macrophage seeded 96-well plate was inoculated with serial dilutions of the pretreated \textit{C. albicans} cell suspension. Media only controls were prepared in the same manner. The plates were incubated at 37°C with 5% CO\textsubscript{2} for 15 hours. Fungal biomass was determined by quantification of \textit{C. albicans} microcolonies using the XTT assay as previously described (315). Absorbance was read at 451 nm in a microplate reader (SynergyMX, Biotek). Assays were performed with replicates in three independent experiments.

Protection of macrophages by sEntV\textsuperscript{68} was examined by monitoring cytotoxicity of \textit{C. albicans} on macrophages using the CytoTox96 Non-Radioactive Cytotoxicity assay (Promega) that measures release of lactate dehydrogenase (LDH) release (316). RAW264.7 murine macrophages in RPMI-10 were seeded in a 96-well plate at a concentration of $2.5 \times 10^5$ cells/ml and incubated overnight at 37°C with 5% CO\textsubscript{2}. \textit{C. albicans} cell suspensions were prepared at $1.5 \times 10^6$ cells/ml in RPMI minus phenol with 100 nM of sEntV\textsuperscript{68} or DMSO and incubated at RT with shaking for 1.5 hours. Media only controls were also prepared in the same manner. The macrophage seeded plates were inoculated with the pretreated \textit{C. albicans} cell suspension and incubated at 37°C with 5% CO\textsubscript{2} for 5 hours. LDH release was normalized to chemically lysed macrophages and assays were performed with replicates in three independent experiments.
Hyphal morphogenesis of *C. albicans* in phagocytes was quantified after infection of RAW264.7 murine macrophages as described above with the following modifications. Macrophages were seeded on glass coverslips in 12-well plates at a concentration of 2.5 x 10^5 cells/ml and incubated overnight at 37°C with 5% CO₂. Overnight cultures of CEG1C (*HWP1*<sub>p</sub>::*GFP*; *ADH1*<sub>p</sub>::*mcherry*) were subcultured in YPD for 5 h at 30°C. Cells were collected and washed with PBS, and diluted in RPMI minus phenol with 100 nM of sEntV<sup>68</sup> or DMSO to a concentration of 1.5 x 10^6 cells/ml and incubated at RT with shaking for 1.5 hours. Media only controls were also prepared in the same manner. The macrophage seeded glass coverslips were inoculated with the pretreated *C. albicans* cell suspension and incubated at 37°C with 5% CO₂ for 2 hours. The media was aspirated and cells were fixed in 2.7% paraformaldehyde (pH 7.5) for 15 minutes at 37 °C followed by washing twice with PBS. Images were captured using Tetramethylrhodamine (TRITC), Fluorescein isothiocyanate (FITC), and differential interference contrast (DIC) filters on an Olympus IX81 automated inverted microscope with Slidebook software (version 6.0). Cell enumeration was performed using at least 10 different fields of view and at least 200 cells per treatment from replicates in three independent experiments.

**sEntV Toxicity Assay**

Toxicity of sEntV<sup>68</sup> on murine macrophages (RAW 264.7) and human cervical epithelial cells (HeLa) was examined by monitoring cytotoxicity affects of *C. albicans* on macrophages using the CytoTox96 Non-Radioactive Cytotoxicity assay (Promega). This assay measures release of lactate dehydrogenase (LDH) release (32). RAW264.7 murine macrophages in RPMI-10 and HeLa cells in Dulbecco’s Modified Eagle Medium with high glucose and 10% fetal bovine serum (DMEM-HG-10) were seeded in a 96-well plate at a concentration of 2.5 x 10^5 cells/ml and incubated overnight at 37°C with 5% CO₂. Media from the seeded plates was replaced with media containing 10 µM sEntV<sup>68</sup> and incubated at 37°C with 5% CO₂ for up to 72
hours. LDH release was measured every 24 hours and the percentage of maximum LDH was calculated as a ratio of LDH from treated cells to LDH from chemically lysed cells.

**Oropharyngeal Candidiasis Model**

The efficacy of sEntV$^{68}$ was tested in the oropharyngeal candidiasis (OPC) model as previously described (317). Mice were immunosuppressed by injecting cortisone acetate subcutaneously in the dorsum of the neck at a concentration of 225mg/kg of body weight one day before inoculation, and subsequently on days 1 and 3 of the infection. Before inoculation, with *C. albicans* mice were anesthetized with an intraperitoneal injection of 0.1 ml/10g of body weight with 10mg/ml ketamine and 1 mg/ml xylazine and placed on pre-warmed hot water blankets. *C. albicans* was grown at 30°C in YPD broth overnight and sub-cultured twice before washing with PBS twice and preparing cell suspensions in Hanks’ balanced salt solution (HBSS) at 1 x 10$^6$ cells/ml. Calcium alginate swabs were immersed in the *C. albicans* suspension with 100 nM sEntV$^{68}$ or DMSO for 5 minutes and then used to inoculate the mice sublingually for 75 minutes.

The mice were monitored continuously for signs of waking up (movement of extremities and/or blinking of the eyes) and were given additional doses of ketamine, as necessary (without xylazine, 50 mg ketamine/kg of body weight). After inoculation mice were treated with 100 nM of sEntV$^{68}$ or DMSO in the drinking water for the remainder of the experiment. Mice were euthanized at 3 and 5 days post-inoculation. The tongues were excised for tissue histology and assessment of the fungal burden.

Tongues were fixed in zinc-buffered formalin followed by paraffin embedding. Thin sections of the tongues stained with periodic acid–Schiff (317). Tissue histology was observed using a light microscope. Images were captured at 40X and histology quantified by measuring the percentage of infected epithelium relative to the entire epithelial area (318). The complete epithelial area and infected epithelium were measured from 40X images taken of the entire
tissue section of each sample. For each image the total area of epithelium and infected epithelium were measured using Image J (version 1.5). Measurements were totaled and expressed as a percentage of total infected epithelium relative to the entire totaled epithelial area.

For examination of fungal burden, quantitative PCR (qPCR) was utilized by amplifying a 269 basepair fragment of internal transcribed spacer 2 (ITS2) between the 5.8S and 28S ribosomal RNA genes of *C. albicans* using 5.8S forward primer and 28S-1 reverse primer (Table 2.1) (319, 320). DNA was extracted using the Yeast DNA Extraction Kit (Thermo Scientific) according to the protocol with the following modifications. A portion of tongue from each mouse was homogenized in 300μl of Y-PER reagent for 8–10 second increments with incubation on ice until samples were completely homogenized followed by incubation at 65°C for 10 minutes. Samples were centrifuged at 13,000 x g for 10 minutes, supernatant discarded, 400ul of Reagent A and 400ul of Reagent B was added, vortexed, and incubated at 90°C for 3 hours. After treatment protein removal agent DNA was precipitated with isopropyl alcohol and washed with 70% ethanol and resuspended in sterile ultra pure water.

The ITS2 fragment was amplified from the DNA extractions and qPCR was performed with a CFX96™ Real-Time System with a C1000 Touch™ thermal cycler (BioRad). A standard curve was generated using *C. albicans* genomic DNA ranging from 0.5–5000 pg. *C. albicans* DNA was quantified using FastStart Universal SYBR Green master mix with ROX (Roche). Reactions were set-up according to the manufacturer’s recommendations. To screen for contamination and background fluorescence during qPCR amplification, no template controls were used. Non-infected mouse tongues and extraction-negative controls were processed in parallel with tissue samples to test for possible contamination during homogenization and extraction.
Proteolytic Cleavage of rEntV\textsuperscript{136} by GelE

Since EntV\textsuperscript{136} is larger than expected based on size exclusion centrifugation of the inhibitory activity and GelE and SprE cleave \textit{E. faecalis} surface and secreted proteins, proteolytic cleavage of rEntV\textsuperscript{136} by GelE was examined. Proteolytic activity of GelE was examined as previously described by Pinkston \textit{et al.} (321). rEntV\textsuperscript{136} (30 µg/ml) was incubated with increasing concentrations of purified GelE (1.5, 4.5, 15, and 30 µg/ml) in PBS at 37°C for one hour. Samples were boiled at 100°C for five minutes in SDS-PAGE sample buffer with BME to terminate the reaction. To evaluate cleavage of rEntV\textsuperscript{136}, reactions were subjected to Tricine-SDS-PAGE and stained with SYPRO Ruby (Lonza) protein gel stain and visualized using a 300 nm UV transilluminator (322).

Homozygous Deletion Mutant Library Screens

To identify possible inhibitory targets of the morphogenesis pathways and stress response pathways a \textit{C. albicans} mutant library of homozygous deletions in non-essential transcription factors was screened for resistance to \textit{E. faecalis} supernatant. Biofilms were grown in a 96-well microtitered plates in YNBM as described above. Biofilm biomass was quantified using resazurine (Sigma), as a measure of biofilm biomass and was quantified using a fluorescence plate reader. \textit{E. faecalis} supernatant was harvested as described above and 50µl added to 96 well plates with 1µl of mutant culture and 50µl YNBAS. Biofilms were grown for 24 hrs at 37°C with shaking (200 rpm) (42). The 50µl of \textit{E. faecalis} supernatant was replaced for 50µl of sterile bacterial medium as non-treatment controls for each mutant strain. Resistance to supernatant was determined by comparing treated cells to non-treated controls. Wildtype (SC5314) and the respective parent strains (SN152 and Day286) were tested in the same manner. Statistically significance differences between treated cells and non-treated controls was determined by Student’s t-test. Mutant strains that had no statistically significance
differences between treated cells and non-treated controls were tested in a secondary screen using the in vitro biofilm protocol described above.

**sEntV\textsuperscript{68}-FITC localization on *C. albicans***

*C. albicans* SC5314 expressing constitutive mcherry (ADH\textsubscript{1}p::mCherry) were grown overnight at 30°C in YPD with aeration. Overnight cultures were sub-cultured in YPD for 5 h at 30°C and washed three times with phosphate-buffered saline (PBS). Washed cells were transferred to chambered polystyrene slides (Ibidi) at a final OD\textsubscript{600} of 0.2 (~1 x 10\textsuperscript{7} cell/ml) in YPD with or without 100nM of sEntV\textsuperscript{68}. Cells were grown for 1 hour at 30°C or 37°C with aeration. To observe sEntV\textsuperscript{68} localization on hyphal cells, cells were grown at 37°C for 1 hour prior to addition of sEntV\textsuperscript{68}. sEntV\textsuperscript{68} localization was observed by fluorescence microscopy (Olympus IX81) using slidebook 6 (3i, Intelligent Imaging Innovations).
### Table 2.1: Strains and Plasmids

#### Candida albicans Strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Designation</th>
<th>Relevant genotype/characteristic</th>
<th>Parent</th>
<th>Source or reference</th>
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<td>Wild-type</td>
<td>Prototroph</td>
<td>-</td>
<td>(323)</td>
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<td>HWP1-GFP</td>
<td>ura3::ENO1/eno1::HWP1p-GFP-URA3</td>
<td>SC5314</td>
<td>(324)</td>
</tr>
<tr>
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<td>HWP1-GFP ADH1-mCherry</td>
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<td>DHC271</td>
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<td>Parent</td>
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<td>BWP17</td>
<td>(325)</td>
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<td>SN152</td>
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<td>JC50</td>
<td>hog1Δ/Δ</td>
<td>ura3::ura3/ura3::his1::his1::hog1Δ::LoxP/hog1Δ::LoxP-HIS1::LoxP::Clp20::HOG1</td>
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<td>(327)</td>
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<tr>
<td>JC52</td>
<td>hog1Δ/HOG1</td>
<td>ura3::ura3/ura3::his1::his1::hog1Δ::LoxP/hog1Δ::LoxP-HIS1::LoxP::Clp20::HOG1</td>
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#### Other Fungal Strains

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<td>Candida glabrata</td>
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<td>(328)</td>
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<td>J941367</td>
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#### Enterococcus faecalis Strains

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<td>Wild-type</td>
<td>Gel· Spr· Rif· Fa·</td>
<td>OG1</td>
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<td>TX5266</td>
<td>ΔfsrB</td>
<td>fsrB in-frame deletion mutant (bp 79 to 684); Gel· Spr· Rif· Fa· Kan·</td>
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<td>CK111</td>
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<td>upp4::P23repA4, Sp·</td>
<td>OG1Sp</td>
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<td>CEGF1</td>
<td>ΔentV</td>
<td>Markerless deletion of entV; Rif· Fa·</td>
<td>OG1RF</td>
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<td>CEGF2</td>
<td>ΔentV/entV</td>
<td>Reconstituted entV with a silent nucleotide change, Δent1097 [entV*], Rif· Fa·</td>
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#### Escherichia coli Strains and Plasmids
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<td><em>E. coli</em> cloning host for pCJK47-based plasmids; provides RepA in trans</td>
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<td>DH5α</td>
<td><em>E. coli</em> cloning host for routine cloning</td>
<td>Invitrogen</td>
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<td>BL21 (DE3)</td>
<td><em>E. coli</em> cloning host for routine cloning, suitable for protein expression</td>
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<td>CEGE1</td>
<td>BL21 (DE3) with pET28a containing entV&lt;sup&gt;136&lt;/sup&gt;</td>
<td>This study</td>
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<td>pCJK47</td>
<td>Plasmid for markerless exchange, oriT-pCF10 and P-&lt;i&gt;pheS&lt;/i&gt;·&lt;i&gt;Em&lt;/i&gt;·&lt;i&gt;R&lt;/i&gt;</td>
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<td>pET28a</td>
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<td>Novagen</td>
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CHAPTER 3:

The *Enterococcus faecalis* bacteriocin EntV inhibits hyphal morphogenesis, biofilm formation, and virulence of *Candida albicans*

Portions of this chapter are based on my first author publication. I have received permission to reproduce data or text from this article through license number. I have contributed significantly to this publication. The article is listed for reference: Graham, C. E., M. R. Cruz, D. A. Garsin, and M. C. Lorenz. 2017. Enterococcus faecalis bacteriocin EntV inhibits hyphal morphogenesis, biofilm formation, and virulence of Candida albicans. *Proc. Natl. Acad. Sci. U.S.A.* 114: 4507–4512 (303).
Introduction:

The mucosal surfaces, such as the orogastrointestinal tract of mammals, are ideal niches for microorganisms and it is here where the balance between microbe-microbe interaction and the host play an important role (331-333). Most of the microorganisms in the orogastrointestinal tract are benign or beneficial to the host. However, the status of the host is a major determinant of microbial pathogenesis. As discussed previously, microorganisms typically found as part of orogastrointestinal tract microbiome can cause life-threatening infections. In order for this to occur the microorganisms must cross host protective barriers and/or evade the immune response and must be capable of colonizing other host niches throughout the body (9).

Although *E. faecalis* and *C. albicans* are part of the orogastrointestinal microbiome, they are among the most important and problematic, opportunistic nosocomial pathogens (186, 235, 334). When the host defenses are compromised and/or the microbiome is altered, both are capable of crossing anatomical barriers and evading the host immune response (335). Furthermore, the ability to form biofilms is an important virulence trait of both *E. faecalis* and *C. albicans*, as both are capable of forming biofilms on biotic or abiotic surfaces that complicate treatment strategies (39, 336). Specifically, *C. albicans* forms formidable biofilms that can exceed 200µm in depth and is often found in polymicrobial biofilms with bacteria that have enhanced resistance to anti-microbial drugs and immune surveillance (39, 42, 149, 337).

Since *C. albicans* and *E. faecalis* occur in overlapping niches as commensals and are commonly co-isolated during infection, it is plausible that diverse interactions exist between these microbes. Using a *Caenorhabditis elegans* model infections model, we demonstrated antagonistic interactions between *C. albicans* and *E. faecalis* (115). Attenuation of virulence is observed in both microorganisms during co-infection of *Caenorhabditis elegans*. As illustrated in Figure 3.1, these findings demonstrate a commensal-like interaction within this model host; co-colonization with both microbes does not cause disease unlike individual colonization.
Further characterization of the activity by which *E. faecalis* inhibited *C. albicans* virulence, identified a secreted factor that exerts its protective effect by inhibiting *C. albicans* hyphal morphogenesis and biofilm formation (115, 338).

Taking into account the interactions observed in the *C. elegans* model, environments where these commensal-like interactions might occur in a more relevant mammalian host were considered. *C. albicans* infections of the oral cavity occur in immunocompetent and immunosuppressed individuals (339). Oropharyngeal candidiasis (OPC), a superficial fungal infection of the oral cavity, is one such infection (339-341). OPC most commonly occurs in immunocompromised individuals, such as those that on immunosuppressant drugs, chemotherapy, or suffering from HIV/AIDS (342-344). In immunocompetent individuals, some common risk factors include use of dentures, corticosteroid inhalers, cigarettes and broad-spectrum antibiotics (345-347).

Formation of thick white plaques (biofilms) of *C. albicans* on the surface of the tongue, buccal mucosa, soft palate and pharynx are the clinical manifestations of OPC (347). While superficially infecting the oral cavity, *C. albicans* invades the oral epithelial cells by inducing endocytosis of *C. albicans* by the epithelial cells and active penetration of epithelial cells (348-351). Invasion of the oral epithelial by *C. albicans* causes cell damage that elicits production of the proinflammatory response that in turn stimulates production of AMPs and recruitment professional phagocytes (339, 341). Professional phagocytes, neutrophils and macrophages, are crucial elements of the innate immune response to *C. albicans* during oral infections (352, 353). Depletion of neutrophils and elimination of macrophages in a murine OPC model increased susceptibility to *C. albicans* infection (352).
Figure 3.1: *E. faecalis-C. albicans* interactions during infection of *Caenorhabditis elegans.*

During mono-infection (top, left) *C. albicans* has pathogenic association with the host, primarily through the production of hyphal cells. As seen in the scanning electron (SEM) image (bottom, left) *C. albicans* forms hyphal cells that are capable of disseminating throughout the nematode. In the presence of *E. faecalis* during co-infection (top, right), *C. albicans* has commensal like association with the nematode. Apparent in the SEM image (bottom, right) *C. albicans* and *E. faecalis* are able to colonize the GI tract of the nematode and hyphal morphogenesis is inhibited. Hyphal morphogenesis is inhibited by the presence of *E. faecalis* via production of a secreted factor(s) (115).
Here I identify the *E. faecalis* secreted inhibitory factor as EntV, a secreted bacteriocin with reported antibacterial activity. When synthetically produced, this peptide inhibited *C. albicans* hyphal morphogenesis and biofilm formation at sub-nanomolar concentrations. Furthermore, EntV was active against mature biofilms, reducing the depth and biofilm biomass. EntV significantly protected murine macrophages against *C. albicans* infection and was protective in a murine model of OPC. As the rise of drug-resistant fungal infections threatens to undermine the current small arsenal of available treatments, I propose that EntV, or similar compounds, may offer a viable therapeutic alternative, either alone or in combination with existing antifungal agents.

**Results:**

**E. faecalis** supernatant inhibits *C. albicans* hyphal morphogenesis and biofilm formation

*E. faecalis* secretes an inhibitor of *C. albicans* virulence and biofilm formation (115). To facilitate identification of this inhibitor, I developed a *C. albicans* in vitro biofilm model in which cells are grown on a polystyrene substrate in an artificial saliva medium (YNBAS, adapted from (306)). This media supported biofilm growth over 24 hours (Fig. 3.2A and B), while supplementation with supernatant from *E. faecalis* cultures decreased both biofilm biomass and hyphal morphogenesis as compared to biofilms grown in medium alone (Fig. 3.2). The *E. faecalis*-mediated inhibition was observed microscopically in biofilms stained with calcofluor white (Fig. 3.2A and B) and quantitated using the redox reactive dye resazurin to measure biomass (311) (Fig. 3.2C). The reduction in hyphal formation was assayed using a reporter strain expressing a transcriptional fusion of the hyphal-specific *HWP1* gene to GFP (*HWP1*<sub>p</sub>::GFP) (Fig. 3.2D-F) (324). A direct correlation between decreases in *HWP1*-induced fluorescence (Fig. 3.2F) and biofilm biomass (Fig. 3.2C) was
Figure 3.2: *E. faecalis* supernatant inhibits *C. albicans* hyphal morphogenesis and biofilm formation.

*C. albicans* biofilms grown for 24 hours in YNBAS. Representative images of *C. albicans* (strain SC5314) biofilms in the (A) absence and (B) presence of *E. faecalis* supernatant and stained with calcofluor white. (C) Biofilm density was quantified by measuring resazurin fluorescence. Representative images of *C. albicans* hyphal reporter strain (*HWP1*::GFP) in the (D) absence or (E) presence of *E. faecalis* supernatant. (F) Hyphal morphogenesis was quantified by measuring GFP fluorescence as a ratio to the OD$_{600}$. Experiments were performed three times and analyzed using Student's t-test (***, p<0.0001).
observed, which is in agreement with previous studies demonstrating that hyphal deficient mutants have reduced biofilm biomass (354, 355).

EntV inhibition of hyphal morphogenesis and biofilm formation in *C. albicans*

Our previous studies in the *C. elegans* infection model suggested that the secreted inhibitor is regulated by the Fsr two-component quorum sensing system mediated by the Gelatinase Biosynthesis Activating Pheromone (GBAP) (115, 338). The Fsr system regulates a number of virulence-related traits, including secreted proteases GelE and SprE, as well as a bacteriocin identified systematically from the genome of strain V583 as *ef1097* (356, 357). *ef1097* encodes a 170 amino acid pre-pro-peptide, and cleavage of the secretion leader sequences results in the export of a 136-residue pro-peptide that has bactericidal activity against Gram-positive bacteria (245, 358). Bacteriocins are typically named after the strain in which they are found, and thus the *ef1097* gene product was called enterococcin V583 and enterocin O16 in previous publications (245, 358). However, because the gene is present in all *E. faecalis* strains sequenced to date (245), we propose to name the gene product EntV, for enterocin originally found in V583.

I hypothesized that EntV is the secreted inhibitor of *C. albicans* hyphal morphogenesis and biofilm formation and tested this genetically. I constructed a deletion mutant of *entV* and tested the supernatant for activity against *C. albicans* biofilms using the hyphal specific reporter strain (HWP1::GFP). Indeed, the biofilm inhibitory activity was lost in supernatants derived from the *entV* mutant strain relative to those from the WT *E. faecalis* strain (Fig 3.3A), confirming that EntV is necessary for the inhibition of biofilm formation. The mutant strain was complemented by introducing the gene back into the endogenous locus with a silent mutation to distinguish it from the original wild type strain (308). Supernatant from the complemented strain inhibited biofilm formation as well as that of wild type *E. faecalis.*
Figure 3.3: EntV inhibition of hyphal morphogenesis and biofilm formation in *C. albicans*.

(A) *C. albicans* SC5314 biofilms grown for 24 hours in YNBAS with sterile media (M9HY) or spent supernatant from wild-type and mutant *E. faecalis* strains. Hyphal morphogenesis was quantified by measuring GFP fluorescence as a ratio to the OD$_{600}$. The experiment was performed three times and analyzed using one-way ANOVA (***, p<0.0008). (B) Biofilms were grown with increasing concentrations of rEntV$_{136}$ or sEntV$_{68}$ and the IC$_{50}$ calculated at 1000 nM and 0.3 nM, respectively.
To test if EntV$^{136}$ was sufficient for the inhibitory activity, rEntV$^{136}$ was expressed and purified from *E. coli* with a hexa-histidine tag (Table 2.1). I detected rEntV$^{136}$ biofilm inhibitory activity with an IC$_{50}$ of ~1000nM (Fig. 3.3B). Thus, the genetic and biochemical evidence suggests that EntV is both necessary and sufficient for inhibition of *C. albicans* biofilm formation. The 136-amino acid EntV is larger than the 3-10 kDa indicated by our preliminary characterization. However, recent work from Dundar et al. suggests that EntV$^{136}$ is further processed in a GelE-dependent manner to a 7.2 kDa peptide encompassing the 68 carboxy-terminal amino acids (EntV$^{68}$) (245). This mature form has a predicted disulfide bond that encompasses nearly the entire length of the active protein (from amino acid 4 to 65 of the 68 amino acid peptide) that is necessary for its anti-bacterial activity (245, 358). Attempts to express and purify EntV$^{68}$ were unsuccessful due to apparent toxicity in *E. coli*. To surmount this obstacle, the peptide was synthetically produced (sEntV$^{68}$) in a form that included a disulfide between the cysteine residues. As seen in Fig. 3.3B, sEntV$^{68}$ inhibited biofilms with an IC$_{50}$ of 0.3nM, and therefore was ~3,000-fold more active than the unprocessed form of the peptide.

**Characterization of sEntV$^{68}$ inhibitory activity**

To investigate the dynamics of *C. albicans* biofilms in the presence of sEntV$^{68}$ I turned to confocal microscopy, with which I could generate a 3-D projection allowing me to measure the overall biofilm depth. When formed in the nutrient-poor artificial saliva medium, control biofilms were 25-30 µm thick after 24 hours, but only 5-10 µm thick in the presence of sEntV$^{68}$, roughly the length of a *C. albicans* yeast cell (Fig.3.4A and B). I next examined the effectiveness of sEntV$^{68}$ against mature biofilms grown for 24 hours. As viewed by confocal microscopy, when biofilms that had developed for 24 hours were treated with sEntV$^{68}$, a reduction in biofilm depth from ~30µm to ~15µm (Fig. 3.4A) was observed, indicating
Figure 3.4: Characterization of sEntV\textsuperscript{68} inhibitory activity.

*\textit{C. albicans} SC5314* biofilms were grown with 0.01% DMSO or 100 nM sEntV\textsuperscript{68} added at different time points during biofilm formation (‘-’, not added; ‘0hr’, added at the beginning of the experiment. \textbf{(A)} Representative image of 24 hour and 48 hour biofilms observed by confocal microscopy. \textbf{(B)} Hyphal morphogenesis was quantified by measuring GFP fluorescence as a ratio to the OD\textsubscript{600} for three separate experiments (**, p=0.0058; ***, p=0.0005; ****, p<0.0001).
that this peptide could dismantle mature biofilms. Correspondingly, when the \textit{HWP1p::GFP} reporter was used to measure hyphal morphogenesis, a decrease in signal was observed at 48 hours following treatment with sEntV\textsuperscript{68} at 24 hours compared to biofilms grown with the vehicle control after 24 and 48 hours of growth (Fig. 3.4B).

**Protection of Murine Macrophages by sEntV\textsuperscript{68} during \textit{C. albicans} infection**

\textit{C. albicans} is readily phagocytosed by murine macrophages, whereupon it activates a complex transcriptional and morphogenetic program that promotes fungal survival and results in hyphal-dependent lysis of the macrophage (316, 359, 360). I asked whether sEntV\textsuperscript{68} could protect phagocytes from fungal-dependent killing. In these experiments, \textit{C. albicans} was incubated with sEntV\textsuperscript{68} for 1.5 hours before being added to the macrophages. sEntV\textsuperscript{68} was also added to the macrophage cell culture medium immediately prior to infecting with \textit{C. albicans}. A decrease in \textit{C. albicans} survival was observed when the macrophage infection was carried out with sEntV\textsuperscript{68} as compared to the control infections (Fig. 3.5A), suggesting that sEntV\textsuperscript{68} works in synergy with murine macrophages to make \textit{C. albicans} more sensitive to macrophage attack. Additionally, I observed a significant decrease in macrophage cytotoxicity in the presence of sEntV\textsuperscript{68} compared to the controls (Fig. 3.5B), suggesting that sEntV\textsuperscript{68} protects murine macrophages during \textit{C. albicans} infection. To assess whether sEntV\textsuperscript{68} inhibits hyphal morphogenesis within macrophages I used a hyphal specific reporter strain (\textit{HWP1p::GFP}) constitutively expressing mCherry (\textit{ADH1p::mCherry}). Cells treated with sEntV\textsuperscript{68} had a decrease in hyphal growth and GFP fluorescence as compared to control treated cells (Fig. 3.5C and D). No toxicity of the peptide towards macrophages or HeLa cells was observed (Fig. 3.6A and B). Taken together these results suggest that sEntV\textsuperscript{68} protects murine macrophages by inhibition of \textit{C. albicans} hyphal morphogenesis.
**Figure 3.5: Protection of Murine Macrophages by sEntV<sup>68</sup> during *C. albicans* infection.**

(A) Murine derived macrophages (RAW264.7) were infected with *C. albicans* (*HWP1<sub>p</sub>::*GFP, *ADH1<sub>p</sub>::*mCherry) at an MOI of one for one hour with 0.01% DMSO or 100nM sEntV<sup>68</sup> followed by fixation with paraformaldehyde and visualized by fluorescence microscopy. (B) Filamentation of phagocytosed cells was scored after 2 hours of co-culture. At least 200 cells in at least 10 different fields of view were counted per replicate and the experiment was repeated three times. (C) Macrophage killing was evaluated using the LDH cell toxicity assay and the percentage of killed macrophages calculated. (D) *C. albicans* survival in murine macrophages was assessed using the XTT cell viability assay and the percent survival was calculated. Statically significant differences were calculated using one-way ANOVA (* p<0.02).
Figure 3.6: Activity of sEntV<sup>68</sup> in mammalian cells.

Lactate dehydrogenase (LDH) was measured from (A) Murine macrophages (RAW 264.7) and (B) Human cervical epithelial cells (HeLa) exposed to 10 µM sEntV<sup>68</sup> or 1% DMSO for up to 72 hours. Cell toxicity was evaluated using the LDH release assay and the percentage LDH calculated for replicates of three separate experiments.
Efficacy of sEntV$^{68}$ in the Murine Oropharyngeal Candidiasis Model

Recall, that \textit{C. albicans} grows on the mucosal surfaces of the oral cavity as part of the normal microflora. However, in patients with defective immune responses it can cause a superficial infection and invade the epithelial layer causing oropharyngeal candidiasis (OPC) that leads to increased morbidity (348-351). To test the efficacy of sEntV$^{68}$ in host relevant infection model, I used a murine OPC model (317). In this model, mice were given a sublingual inoculation of \textit{C. albicans} and then given sEntV$^{68}$ or DMSO in sterile drinking water for up to 5 days. The mice were euthanized and tongues excised at day 3 and day 5 to examine the histology and fungal burden. Control mice showed classical signs of OPC, including extensive invasion of the epithelium by fungal hyphae, disruption of the outer layers of epithelial cells, and infiltration of neutrophils (Fig. 3.7A). In contrast, mice that were treated with 100 nM sEntV$^{68}$ has significantly reduced invasion; most fungal cells were in the yeast or pseudohyphal form, indicating that the peptide can inhibit morphological differentiation in vivo as well (Fig 3.7A). To provide a quantitative measure of the epithelial invasion, I scored the proportion of the epithelium infected in multiple tongues from control or sEntV$^{68}$-treated mice, as recently described (318). This data demonstrates a clear difference in the extent of epithelial damage between treated and control animals (Fig. 3.7B). Finally, I assessed fungal burden on the tongues. Because of the significant morphological differences in the two conditions, I considered that colony forming unit plating would not accurately reflect differences in burden between the primarily yeast morphology in the sEntV$^{68}$-treated animals and the multinucleate and highly adherent hyphae in the controls. Instead, I quantitated fungal DNA using qPCR as described (319, 320), which showed a significant decrease in fungal burden in peptide-treated mice relative to control mice (Fig. 3.7C). These results indicate that sEntV$^{68}$ is effective in a complex mammalian infection model that simulates clinically relevant infection conditions.
Figure 3.7: sEntV<sup>68</sup> is protective in a murine OPC model.

Immunosuppressed mice were inoculated sublingually with *C. albicans* SC5314 with 0.01% DMSO or 100 nM of sEntV<sup>68</sup>. Water containing sEntV<sup>68</sup> (100 nM) or vehicle (DMSO) alone was provided ad libitum. After three or five days, mice were euthanized and the tongues were excised for histological examination of (A) DMSO control or (B) sEntV<sup>68</sup>-treated mice. Red arrows indicate hyphal cells and black arrows indicate yeast cells. (C) The percentage of the epithelial surface showing evidence of fungal invasion was calculated for control and treated tongues. (D) DNA was extracted from the tongues and the fungal burden was estimated from qPCR amplification of the 5.8S ITS2 region. Statistically significant differences were calculated using one-way ANOVA (**, p<0.01)
Discussion:

I observed a distinct antagonistic interaction between *E. faecalis* and *C. albicans*, in which hyphal morphogenesis, an important virulence factor, is inhibited by a secreted bacteriocin, EntV. Hyphal morphogenesis is important for many aspects of *C. albicans* pathogenicity, including biofilm formation, resistance to phagocytes, and dissemination through tissues (102, 361, 362). The implications of this inhibitory activity are clearly seen in the decreased virulence of *C. albicans* cells exposed to this peptide in murine macrophages, and in the mouse OPC model.

Biofilm-related infections pose numerous clinical challenges. Biofilms provide a measure of protection to the microbes encased within them from phagocytic attack and antibiotics (20). Protection of persister cells within the biofilm allows *C. albicans* to disseminate to distant sites, seeding relapses and systemic infections (88). Biofilms of *C. albicans* are robust and form both on implanted medical devices (catheters, heart valves, dentures) and on epithelial surfaces (OPC) (39). Our in vitro experiments were designed to model the oral cavity, and I observed biofilms exceeding 50 µm in depth; in other media conditions, they can be greater than 200 µm thick. Often, device removal is the only effective treatment option, though this may not be feasible or desirable given a patient’s underlying conditions. Agents that directly disrupt biofilms would be very useful chemotherapeutics in conjunction with existing drugs. Our work suggests that EntV is a candidate anti-biofilm agent.

Currently, nearly all antimicrobials in clinical use work by killing or preventing the growth of the infectious agent. Certainly, this is true for *C. albicans*. There are presently only three families of commonly used antifungal drugs, polyenes, azoles and echinocandins, all of which target the cell membrane or the cell wall (80, 363). Resistance to this limited arsenal of antifungals is a growing problem (364). It has long been speculated that therapeutics that target virulence might effectively treat infection and not select for resistance as strongly as traditional
antimicrobials. In support of this idea, a small molecule also exhibiting *C. albicans* biofilm and hyphal morphogenesis-inhibiting properties did not induce resistance after repeated exposure (365).

Many clinical *Candida* infections are polymicrobial. In one survey of these polymicrobial infections, the most common bacterial co-habitant was *E. faecalis* (109). In contrast, our work suggests that these two microbes have evolved a relationship in which one inhibits a key virulence trait of the other. A precedent for this apparent contradiction is seen in the interaction between *C. albicans* and *P. aeruginosa*, species that are antagonistic in vitro (126, 128), but are associated with significantly worse outcomes when found together in patients, including the lungs of cystic fibrosis patients, during ventilator-associated pneumonia, and in burn trauma (123, 124).

I previously speculated that the presence of both *C. albicans* and *E. faecalis* might promote a commensal interaction with the host, in which virulence attributes are suppressed to facilitate colonization (Fig.1) (338). *C. albicans* ability to colonize the GI tract in a nonpathogenic state and switch to a pathogenic state upon disruption of the host immune response or microflora has been well documented (4, 8, 11). However, *C. albicans* is able to shape the bacterial microflora during antibiotic recovery. In a murine colonization model *C. albicans* reduced *Lactobacillus* spp. and promoted *E. faecalis* colonization after antibiotic treatment (366, 367). These data thus suggest a model in which *C. albicans* and *E. faecalis* promote each other’s commensalism during nonpathogenic colonization of the orogastrointestinal tract, but when the status of the host immune response is disrupted, invasive infection might be stimulated.

We are only beginning to understand the complex interactions amongst the microbiota and their varied effects on the human host, but the knowledge is likely to have important impacts on the development of novel antimicrobials. In conclusion, I report the identification of an anti-fungal, virulence-targeting compound, EntV68, which does not slow the growth of *C. albicans*, even at high concentrations, but can elicit protection in the sub-nanomolar range by
inhibiting hyphal morphogenesis. This study provides impetus for further discovery of novel antimicrobial therapeutics with virulence-targeting activity.
CHAPTER 4:

Post-Translational Processing of EntV influences the inhibition of hyphal morphogenesis and biofilm formation of Candida albicans

Portions of this chapter are based on my first author publication. I have received permission to reproduce data or text from this article through license number. I have contributed significantly to this publication. The article is listed for reference: Graham, C. E., M. R. Cruz, D. A. Garsin, and M. C. Lorenz. 2017. Enterococcus faecalis bacteriocin EntV inhibits hyphal morphogenesis, biofilm formation, and virulence of Candida albicans. Proc. Natl. Acad. Sci. U.S.A. 114: 4507–4512 (303).
Introduction:

Bacteria use quorum sensing to communicate via accumulation and detection of extracellular signaling molecules that enable them to respond to these environmental cues based on cell density (368). Gram-positive bacteria QS signaling molecules are peptides that are post-translationally processed from propeptides. These peptides bind the extracellular receptors on specific transmembrane proteins that trigger specific signal transduction pathways (369).

The Accessory gene regulator (Agr) system in Staphylococcus Spp. is one of the most-studied cyclic peptide-mediated QS systems found in Gram-positive bacteria (Novick & Geisinger, 2008). The autoinducing peptide (AIP) is produced from the propeptide AgrD that is post-translationally processed and secreted by the transmembrane peptidase AgrB. Upon accumulation of AIP, the canonical two-component system consisting of the histidine kinase (AgrC) and response regulator (Agr A) transduces the signal that results in phosphorylation of AgrA (370-374). Phosphorylation activates AgrA, which positively regulated gene expression. This type of QS system is widespread among Gram-positive bacteria, cognate gene cluster containing these four components have been identified in Enterococcus, Listeria, Clostridium, Lactobacillus, and Bacillus spp., suggesting the retention of this type of QS system is important for the survival of many Gram-positive bacteria (371, 372, 375, 376).

A homolog of the Agr system, the Fsr system in E. faecalis regulates expression of pathogenicity-associated genes and is important for virulence in different infection models (197, 201, 202, 377, 378). The fsr gene cluster contains four genes that encode a histidine kinase (FsrA), response regulator (FsrC), transmembrane peptidase (FsrB), and the cyclic signal peptide GBAP (FsrD) (376). Phosphorylated FsrA directly regulates the fsr genes, gelE, sprE, and entV and indirectly regulates genes involved in autolysis, adhesion, and biofilm formation (201, 356, 376, 379). Using microarray transcriptional profiling, Bourgogne et. al. (356) found that fsrB influences expression of numerous genes throughout the growth phases of E.
faecalis. In addition to the Fsr regulon, gelE, sprE, and entV all contain consensus sequences necessary for FsrA binding and expression is dependent on cell density where an increase in gene expression is seen in late log phase (356).

Here I demonstrate the post-translational modifications of EntV necessary for inhibition of C. albicans hyphal morphogenesis and biofilm formation. The mature inhibitory activity was 3-10 kDa and a synthetic peptide containing the C-terminal 68 amino acids was functional, indicating that the EntV protein is post-translationally processed via proteolytic cleavage. I show here that recombinant EntV is cleaved in vitro by GelE. Furthermore, EntV is predicted to have a disulfide bond and reduction and alkylation of the supernatant abolished inhibitory activity. Finally, characterization of EntV structure and activity provides useful insight for the future design of novel antifungal and antibiofilm therapeutics based on EntV inhibitory activity against C. albicans.

Results:

Characterization of E. faecalis supernatant inhibitory activity

To characterize the activity of the inhibitor, I used the C. albicans in vitro biofilm model I developed using polystyrene as the growth substrate grown in YNBAS (306). Biofilms grown for 24 hours in the presence of supernatant from E. faecalis had decreased biomass and hyphal growth as compared to biofilms grown in medium alone (Fig 4.1.). This inhibitory activity was retained in the E. faecalis supernatant fraction between 3 to 10 kDa that had been boiled for 20 minutes at 100°C (Fig. 4.1), consistent with what was seen in the C. elegans infection model (115). In addition, the inhibitory activity was abolished with proteinase K treatment, suggesting a small (3 to 10kDa), heat-stable protein is responsible for the inhibitory activity observed in both the in vitro biofilm model and the in vivo C. elegans infection model.
Figure 4.1: Characterization of inhibitory activity from *E. faecalis* Supernatants.

*C. albicans* (*HWP1*::*GFP*) biofilms grown at 37°C for 24 hours in YNBAS. Hyphal morphogenesis was quantified by measuring GFP fluorescence as a ratio to the OD$_{600}$. Inhibitory activity from *E. faecalis* supernatant was retained between 3-10 kDa after size exclusion centrifugation. A reduction of inhibitory activity was observed in supernatants treated with proteinase K and from deletion mutants of *fsrB* or *entV*. Experiments were repeated at least three times and statistically significant differences relative to treatment with the WT supernatant were calculated using a one-way ANOVA, and are indicated by asterisks (\(^*\), p<0.006; \(^*\), p<0.01).
Post-translational processing of EntV

As discussed previously (Chapter 1), the inhibitory activity was lost with disruption of the Fsr system and deletion of entV (Fig. 3.3A). Furthermore, deletion of gelE or sprE resulted in decreased inhibitory activity and a double deletion of gelE and sprE further decreased inhibitory activity, indicating that GelE and SprE are necessary for maximum inhibitory activity. According to the entV gene sequence, EntV is slightly larger than the inhibitory factor characterization based on size exclusion centrifugation (Fig. 4.2). Proteolytic processing of EntV is a plausible explanation for this size discrepancy. Since GelE and SprE cleave E. faecalis surface and secreted proteins, they are candidates for processing EntV (321, 379).

To test the hypothesis that GelE and/or SprE proteolytically process EntV, in silico and in vitro tests were performed. A member of the M4 family of proteases thermolysin, a homolog of GelE from Bacillus thermoproteolyticus, preferentially cleaves sites with position P1’ that have bulky and aromatic residues (Ile, Leu, Val, Ala, Met, Phe) and aromatic sites in position P1 [392]. Glutamyl endopeptidases V8 from Staphylococcus aureus is a homolog of serine protease (SprE) from E. faecalis and preferentially cleaves at Glu in position P1, Pro/Val or Phe in position P2, Ala/Val in position P3, and Asp in position P4 (380). Thus, proteolytic cleavage of EntV by these homologous proteases was examined in silico using PeptideCutter (ExPasy) [394].

EntV contains two cysteines that form a putative disulfide bond that is necessary for bactericidal activity (358). To examine the role of the disulfide bond for activity against C. albicans, E. faecalis supernatant was reduced followed by alkylation to permanently reduce any cysteine in the supernatant. As illustrated in Figure 4.3A, a reduction in the inhibitory activity of WT supernatant was observed after reductive-alkylation, suggesting that the disulfide bond is also necessary for the inhibitory activity against C. albicans. Disulfide bond formation in Gram-positive bacteria has been understudied and is not fully understood. On the other hand disulfide bond formation in Gram-negative bacteria has been extensively studied. Since Gram-positive
The E. faecalis Fsr system is involved in regulating the inhibitory activity.

The Fsr two component quorum-sensing system from E. faecalis regulates expression of EntV and secreted proteases (GelE and SprE) (356). C. albicans (HWP1p::GFP) biofilms grown at 37°C for 24 hours in YNBAS. Hyphal morphogenesis was quantified by measuring GFP fluorescence as a ratio to the OD₆₀₀. Disruption of gelE and/or sprE decreased inhibitory activity. Experiments were repeated at least three times and statistically significant differences relative to treatment with the WT supernatant were calculated using a one-way ANOVA, and are indicated by asterisks (**, p<0.009).
bacteria lack traditional periplasmic spaces it has been thought that these disulfide bonds are formed spontaneously upon secretion or that many Firmicutes secrete few, if any, proteins that have disulfide bonds (381). However, recent studies have demonstrated the importance of disulfide bond formation by thiol-disulfide oxidoreductases (DsbA-like proteins) in Gram-positive actinobacteria (382). Interestingly, disruption of dsbA by transposon mutagenesis also resulted in reduced inhibitory activity (Fig. 4.3A). Taken together these results demonstrate that protein oxidation is necessary for maximum inhibitory activity, which suggests that proper formation of the disulfide bond is necessary for EntV efficacy.

Disulfide bridges stabilize the formation of β-strands and α-helices, can control substrate accessibility, and protect proteins from damage. Therefore, only cleavage sites outside of the disulfide bonds are shown (383-385). As illustrated in Figure 4.3B, GelE cleaves at A68 and SprE cleaves at residues further toward the N-terminus, which corresponds with previous MS/MS analysis and N-terminal degradation sequencing of EntV from WT E. faecalis (245). Thus, these data suggest that GelE is important for cleavage of the EntV propeptide to produce the active bacteriocin. Additionally, GelE also cleaves the C-terminus of EntV in silico resulting in production of a 65 amino acid peptide of 6.9 kDa in size (Fig. 4.3B).

To investigate further the proteolytic processing of EntV by GelE, recombinant EntV136 (136 amino acid propeptide) expressed and purified from E. coli (BL21-DE3) was incubated with increasing concentrations (1.5, 4.5, 15, and 30 μg/ml) of purified GelE from E. faecalis (321). I observed increased amounts of lower molecular weight EntV (~7KDa) with increased concentrations of GelE (Fig. 4.4), indicating proteolytic processing of EntV by GelE. This supports previous reports that demonstrate the propeptide (136 amino acids, ~15kDa) is further modified by the extracellular gelatinase into the active bacteriocin of 68-amino acid (7kDa) peptide that has been shown to be active against bacteria (245). As discussed in Chapter 3, I
Figure 4.3: Protein oxidation is important for inhibitory activity.

(A) *C. albicans* (*HWP1*::GFP) biofilms grown at 37°C for 24 hours in YNBAS. Hyphal morphogenesis was quantified by measuring GFP fluorescence as a ratio to the OD$_{600}$. Reductive-Alkylation decreased inhibitory activity of WT *E. faecalis* supernatant. Disruption of *dsbA* (predicted dithiol-disulfide isomerase) by transposon mutagenesis also decreased inhibitory activity. Experiments were repeated at least three times and statistically significant differences relative to treatment with the WT supernatant were calculated using a one-way ANOVA, and are indicated by asterisks (**, p<0.01, ***p<0.001).

(B) In silico digestion of EntV by GelE homolog thermolysin (T) and SprE homolog glutamylpeptidase (G) using PeptideCutter (ExPASy) (242).
Figure 4.4: EntV is proteolytically processed by *E. faecalis* gelatinase.

GelE cleavage of rEntV\textsuperscript{136} was analyzed by SDS-PAGE. Increasing amounts of purified GelE was incubated with 30μg/ml rEntV\textsuperscript{136} N-terminally tagged with hexa-His for 1hr and the reactions terminated by addition of SDS-PAGE sample buffer and boiling. Proteins were stained with SyproRuby (Lonza) and visualized using a 300 nm UV transilluminator. Increased concentrations of GelE resulted in production of smaller molecular weight products (>10kDa).
demonstrated this processed peptide of 68-amino acid inhibited hyphal morphogenesis, biofilm formation, and virulence of \textit{C. albicans}.

**Classification and structure of \textit{E. faecalis} bacteriocin EntV**

EntV belongs to the class IIIb bacteriocins, which are the larger (>10kDa), non-lytic bacteriocins produced by Gram-positive bacteria [400]. To date only five bacteriocin have been identified and characterized in this class, dysgalactcin (DysA, \textit{Streptococcus dysgalactiae} subsp. \textit{equisimilis}), SA-M57 (\textit{Streptococcus pyogenes} M-type 57), EntV (\textit{E. faecalis}), and YpkK (\textit{Corynebacterium jeikeium}) (243, 358, 386). Each of these proteins have conserved secondary structures: (1) unstructured N-terminus, (2) helix-loop-helix motif at the C-terminus, and (3) two cysteine residues that form a putative disulfide bond (242, 243, 358, 386) (Fig. 4.5A and B). In addition, these class IIIb bacteriocins have a non-lytic mode of action against similar Gram-positive bacteria and only EntV has been test against fungi (245, 358).

I hypothesized that these other bacteriocins similar to EntV are also proteolytically cleaved by proteases of the M4 family, homologs of thermolysin, aurolysin and gelatinase (GelE). As seen in Table 4.1, M4 family proteases have been identified in these other bacterial species and proteolytic cleavage by thermolysin was observed in silico (387). Furthermore, all of the class IIIb bacteriocins gain a greater positive charge that is characteristic of efficacious antimicrobial peptides (Table 4.1, ProtParam) [403]. Using the protein structure of dysgalactcin in consort with the secondary structure consensus (PROMALS3D) of the class IIIb bacteriocins the predicted tertiary structure was assembled (I-Tasser) (242, 388). Collectively, these results suggest that the class IIIb bacteriocins are post-translational processing into small (<10kDa), positively charged, amphipathic bacteriocins.
Figure 4.5: Comparative primary and secondary structures of class IIIb bacteriocins after proteolytic processing.

Table 4.1. Computation of physical and chemical properties of class IIIb bacteriocins.

Based on the sequence analysis of the propeptide and proteolytically processed active peptide the size and theoretical pl was calculated using ProtPram (ExPASy). Identification of M4 family proteases was performed using the NCBI gene database.

<table>
<thead>
<tr>
<th>Species</th>
<th>Bacteriocin</th>
<th>Gene</th>
<th>M4 Family Protease</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. faecalis</em></td>
<td>Enterococcin V</td>
<td>entV</td>
<td>Gelatinase (gelE)</td>
</tr>
<tr>
<td><em>S. dysgalactiae</em></td>
<td>Dysgalacticin</td>
<td>dysA</td>
<td>Oligoendopeptidase F (pepF)</td>
</tr>
<tr>
<td><em>S. pyogenes</em></td>
<td>SA-M57</td>
<td>scnM57</td>
<td>Peptidase F (pepF)</td>
</tr>
<tr>
<td><em>C. jeikeium</em></td>
<td>Corynicin JK</td>
<td>ypkK</td>
<td>Zn-dependent metalloprotease (JKRS06030)</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Bacteriocin</th>
<th>Propeptide Size (kDa)</th>
<th>Theoretical pl</th>
<th>Active Peptide Size (kDa)</th>
<th>Theoretical pl</th>
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</thead>
<tbody>
<tr>
<td>Enterococcin V</td>
<td>14 (136 a.a.)</td>
<td>4.87</td>
<td>6.9 (65 a.a.)</td>
<td>9.84</td>
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<tr>
<td>Dysgalacticin</td>
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<td>4.77</td>
<td>7.0 (65 a.a.)</td>
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</tr>
<tr>
<td>SA-M57</td>
<td>14.4 (137 a.a.)</td>
<td>5.40</td>
<td>7.3 (68 a.a.)</td>
<td>9.60</td>
</tr>
<tr>
<td>Corynicin JK</td>
<td>21.5 (192 a.a.)</td>
<td>4.46</td>
<td>6.0 (58 a.a.)</td>
<td>9.24</td>
</tr>
</tbody>
</table>
**Discussion:**

Important for competition and commensal growth in the GI tract of the host, post-translational processing of bacteriocins is common for efficient production of antimicrobial peptide against bacteria and fungi (390). Here I demonstrate that EntV is processed in an Fsr-dependent manner into a mature form of 68 amino acids that has activity at nanomolar or lower concentrations in most of our assays, demonstrating that it is highly efficacious.

EntV is a bacteriocin that belongs to the class IIIb bacteriocins, which are the larger (>10kDA) bacteriocins (391). The structure of the mature form of EntV is predicted in silico to consist of two helical elements, separated by a flexible loop region. The conserved cysteines located immediately before and after these two helices are thought to form a disulfide bond (392, 393). The immature form contains an additional N-terminal segment predicted to be unstructured (245), which is removed by GelE to form the mature and active form of the peptide (245). While many bacteriocins encode an immunity protein, Dundar et al. could find no evidence of one, and *E. faecalis* appears to be intrinsically resistant (358). The bacteriocidal activity of EntV against sensitive species is not due to lysis, and the mechanism remains enigmatic (242).

The class IIIb bacteriocins that have been identified to date have a similar predicted secondary, though are not well conserved at the amino acid level. Considering the structure similarity and conserved cysteines necessary for activity and in silico cleavage, it is plausible that these other class IIIb bacteriocins, like EntV are also post-translational modified resulting in production of a smaller (<10kDA) highly active positively charged antimicrobial peptide. Thus, characterization and classification of these bacteriocins may need revision (242, 243, 358, 394). EntV-like bacteriocins have been identified in multiple host-associated bacteria suggesting these bacteriocins are important for both pathogenic and commensal association with the host (303). Furthermore, given the physiochemical similarities of these bacteriocins it is possible that they have overlapping target spectra. This remains to be further tested and
suggests that these bacteriocins may play an important role in bacterial-fungal interactions in other Gram-positive bacteria.
Introduction:

*C. albicans* biofilms are complex, structured communities that form on biotic and abiotic surfaces and are a growing concern in the clinical setting. One of the leading opportunistic fungal pathogens, *C. albicans* biofilms are often difficult to eradicate and can lead to more severe systemic infection if left untreated. Comprised of both yeast and hyphal cells *C. albicans* biofilms form via a highly regulated process that begins with adhesion, followed by initiation and formation of hyphal cells, then maturation, and ultimately dispersion (Fig. 1.1) (41).

Adherence is the first essential stage in *C. albicans* biofilms development and is a highly regulated process. Factors such as cell surface composition, extracellular matrix production, adhesins and other cell surface proteins are all coordinated during attachment to a substrate. Finkel *et al.* (395), identified 30 transcriptional regulators that are important for adherence during biofilm development, which regulate the expression of 37 surface-associated genes among other genes. Furthermore, a functional relationship between cell wall stress responses and regulation of adherence was identified recently. Transcriptional repression of ARC18, a member of the Arp2/3 complex important for regulation of endocytosis and the actin cytoskeleton, resulted in reduced adhesion and biofilm formation (395). Additionally, cell surface hydrophobicity and exposure of cell wall chitin and β-glucans was increased with depletion of the Arp2/3 complex leading to impaired cell wall integrity and activation of Rho1-mediated cell wall stress responses (50, 396-398).

*C. albicans* cell wall proteins are essential for maintaining cell wall integrity, adherence, enzymatic functions, biofilm development, and morphological transition. In *C. albicans*, a MAPK network regulates responses to environmental stresses such as changes in temperature, osmolarity, ionic stress, cell wall stress, and oxidative stress. Three interconnected MAPK pathways, Hog1, Mkc1 and Cek1, regulate this core transcriptional response to environmental stress (50, 398-400). Different membrane sensors sense environmental stress signals; the signals are transduced via the MAPK pathways, resulting in cooperative phosphorylation of
Hog1 and Cek1 followed by phosphorylation of Mkc1. Collectively, this results in generation of a core transcriptional stress response (3). While components of the core stress responses have been identified many of the sensors have not been identified or remain uncharacterized in *C. albicans*.

I hypothesize that EntV is eliciting a cell surface stress that disrupts cell surface proteins, adhesion and thus biofilm formation and hyphal morphogenesis. Here I identify *C. albicans* genes that when deleted decrease sensitivity to *E. faecalis* inhibitory activity during biofilm development. Deletion in genes involved in regulation of hyphal morphogenesis, cell wall stress, and cell wall architecture were identified. Furthermore, assessment of sEntV68 localization by fluorescence microscopy in yeast and hyphal cells demonstrated surface association in all cell types. Although sEntV68 inhibits hyphal morphogenesis, biofilm formation and virulence of *C. albicans*, sEntV68 did not kill *C. albicans*, nor inhibit its growth, even at high concentrations. sEntV68 inhibits hyphal morphogenesis and biofilm formation in clinical isolates of *C. albicans*, as well as other pathogenic *Candida* spp. EntV68, or similar compounds, may offer a viable therapeutic alternative. Understanding the mechanism of action is important for development and use of EntV68 based therapeutics. Identification of genes dysregulated by *E. faecalis* will provide insight into possible gene targets for development of therapeutic strategies that target hyphal morphogenesis and biofilm development (303).

**Results:**

**Role of hyphal morphogenesis and surface stress response**

*E. faecalis* secretes an inhibitor of *C. albicans* virulence and biofilm formation that I have identified as the bacteriocin EntV (23, 42, 49). Hyphal morphogenesis was reduced in both in vitro and in vivo assays, suggesting that the *E. faecalis* mediated inhibition *C. albicans* is due to dysregulation of hyphal morphogenesis pathways. Thus, I hypothesized that *E. faecalis* dysregulates hyphal-specific genes in *C. albicans* that are important for hyphal
morphogenesis and thus biofilm development. As mentioned previously, hyphal morphogenesis and biofilm formation of *C. albicans* are interconnected and regulated by the Ras1-cAMP-PKA signal pathway (401, 402). This is important when considering the mechanism of action of *E. faecalis*-mediated inhibition of hyphal morphogenesis and biofilm formation.

To understand the mechanism of action I assessed *C. albicans* mutant libraries of transcription factors and cell wall proteins (303) for decreased sensitivity to *E. faecalis* supernatant. Biofilms were grown as described above using the resazurin redox assay to quantify biofilm biomass (401). Resistance to WT *E. faecalis* supernatant was determined by comparing supernatant treated cells to media treated controls. Homozygous deletion mutants in non-essential transcription factors and cell wall proteins that were resistant to *E. faecalis* supernatant are listed in Table 5.1. Theses homozygous deletion mutants have known functions in hyphal morphogenesis, cell wall biogenesis, mating, cell cycle and unknown function.

24 Transcription factor mutants were identified that were less sensitive to *E. faecalis* supernatant (Table 5.1) (401, 403, 404). Some of the transcription factor mutants identified are involved in negatively regulating hyphal morphogenesis (NRG1, SSN6, RAP1), deletion of these genes results in a hyperfilamentous phenotype (400, 405). Transcription factor mutants identified involved in cell wall biogenesis genes and that are linked to the cell wall integrity (CWI) or Hog1 stress response signal cascades (CAS5, SKO1, FCR1, ORF19.2476) were also identified (398, 406, 407). 15 cell wall protein deletion mutants were identified that decreased sensitivity to *E. faecalis* supernatant (Table 2.2). Moreover, cell wall protein deletion mutants identified are also linked to hyphal morphogenesis (PDE2) and surface stress response (*WSC1, WSC2, WSC4, PGA26, CCW14, ECM4*) (107). Taken together these data suggest *E. faecalis* supernatant is triggering an adaptive stress response in *C. albicans* that leads to repression of morphogenesis (49).

The Ras1-cAMP-PKA signal pathway regulates hyphal morphogenesis during biofilm development (48). Therefore, inhibition of *C. albicans* biofilm formation would likely involve
Table 5.1: Homozygous deletion mutants in non-essential transcription factors resistant to *E. faecalis* supernatant.

*C. albicans* homzygous deletion mutants were screened for resistance to *E. faecalis* supernatant biofilm inhibition. Biofilms were grown in YNBAS for 24 hours and biofilm biomass was quantified using the resazurin redox assay comparing biofilms grown in the presence or absence of WT *E. faecalis* supernatant.

<table>
<thead>
<tr>
<th>Gene</th>
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Table 5.2: Homozygous deletion mutants in cell wall proteins resistant to *E. faecalis* supernatant.

*C. albicans* homozygous deletion mutants were screened for resistance to *E. faecalis* supernatant biofilm inhibition. Biofilms were grown in YNBAS for 24 hours and biofilm biomass was quantified using the resazurin redox assay to compare biofilms grown in the presence or absence of WT *E. faecalis* supernatant.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Systematic Name</th>
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</tr>
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<td>ECM25</td>
<td>C1_13310W_A</td>
<td>ECM25</td>
<td>Filamentation</td>
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<tr>
<td>SAP98</td>
<td>C2_03680W_A</td>
<td>PEP4</td>
<td>Phenotypic Switching</td>
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perturbation of this signal cascade (408). Interestingly, deletion of negative regulators of the signal pathway, NRG1 and PDE1, conferred resistance to E. faecalis, demonstrating the importance of the Ras1-cAMP-PKA signal pathway regulation of hyphal morphogenesis in response to E. faecalis (Fig 5.1A). This suggests that E. faecalis-mediated inhibition requires repressors of hyphal morphogenesis that are most likely induced during inhibition. Nrg1 is essential for the hyphal to yeast transition during the dispersal stage of biofilm development and negatively regulates the biofilm transcriptional network (155). Pde2 is a high affinity cyclic nucleotide phosphodiesterase that moderates signaling by cAMP in the Ras1-cAMP-PKA signal pathway (50, 397, 400).

The stress response MAPK network consists of three terminal MAPKs, Hog1, Mkci and Cek1, that are phosphorylated as a result of membrane sensors signaling in responses to environmental stress, such as surface stress (48, 399). Deletion of transcription factors regulated by the MAPK network, CAS5, SKO1, and FCR1, conferred resistance to E. faecalis, demonstrating a possible role of the MAPK pathway during inhibition hyphal morphogenesis by E. faecalis (Fig 5.1B). Furthermore, deletion of the poorly understood cell wall integrity sensors, WSC1, WSC2, and WSC4, decreased sensitivity to E. faecalis supernatant (Fig. 5.1B). At the center of the MAPK network is Hog1-MAPK, which at basal concentrations represses Cek1 and controls phosphorylation of Mkci (5, 409). Phosphorylated Hog1 acts a repressor of hyphal morphogenesis independently of Efg1 and Cph1 by regulating Sko1-Ssn6-Tup1 mediated repression. Thus, I hypothesized that the Hog1-MAPK stress response pathway is activated in C. albicans due to E. faecalis-mediated perturbation of the cell wall and membrane. Deletion of HOG1 resulted in decreased sensitivity to E. faecalis supernatant (Fig 5.2). Taken together these results suggest that the stress response MAPK network is activated in response to E. faecalis-mediated stress.
Figure 5.1: Deletion of hyphal morphogenesis and cell wall stress genes confers resistance to *E. faecalis* supernatant.

*C. albicans* homozygous deletion mutants were screened for resistance to *E. faecalis* supernatant biofilm inhibition. Biofilm biomass was quantified using the resazurin redox assay comparing no treatment biofilms (blue) to supernatant treated biofilms (orange). (A) Deletion of genes involved in repression of the Ras1-cAMP pathway resulted in decreased sensitivity to *E. faecalis* supernatant. (B) Deletion of genes involved in cell wall stress resulted in decreased sensitivity to *E. faecalis* supernatant. Statistically significant differences relative to treatment with the WT supernatant were calculated using a Student’s t-test, and are indicated by asterisks (**, p<0.007; *, p<0.04).
Figure 5.2: Deletion of the central stress response MAPK, HOG1, decreased *C. albicans* sensitivity to *E. faecalis* supernatant.

Treatment with *E. faecalis* WT supernatant did not decrease biofilm biomass of a HOG1 homozygous deletion mutant and complementation of the WT HOG1 restored sensitivity. was resistance to *E. faecalis* supernatant biofilm inhibition. Biofilm biomass was quantified using the resazurin redox assay comparing no treatment biofilms (blue) to supernatant treated biofilms (orange). Statistically significant differences relative to treatment with the WT supernatant were calculated using a Student's t-test, and are indicated by asterisks (**, p<0.006; *, p<0.03).
Host relevant environmental stresses impact on EntV activity

Functional stress response is important for *C. albicans* survival by initiating adaptation to changes in environmental conditions (401). Induction of MAPK stress response pathways has been observed in *C. albicans* in response to various external factors that lead to inhibition of hyphal morphogenesis and biofilm formation. Thus, I considered whether sEntV might synergize with host-relevant stresses, including oxidative, nitrosative, cell wall, and cell membrane stresses. The addition of sEntV<sup>68</sup> did not affect susceptibility to any of these stresses, reinforcing the idea that it has a novel mechanism of action (Fig 5.3). These results do not correspond with our previous observations in the in vitro biofilm model. There are some plausible explanations: (1) there is no stress response, (2) the stress response is specific to biofilm growth (e.g. adhesion), and/or (3) the stress growth conditions impedes the activity of EntV<sup>68</sup> (e.g. cell surface remodeling). This remains to be fully elucidated and will need to be further examined.

EntV activity on *C. albicans* in different growth conditions

To better understand the sEntV<sup>68</sup> mechanism of action, I considered whether EntV might inhibit biofilm formation as a result of a general inhibition of fungal growth. To address this, I grew *C. albicans* in the presence of increasing concentrations of sEntV<sup>68</sup> and found no reduction in growth as high as 10 µM, 30,000-fold higher than the in vitro biofilm inhibitory concentration (Fig 5.4). Although sEntV<sup>68</sup> was very effective at inhibiting biofilm formation in artificial saliva medium (YNBAS), I questioned the efficacy of sEntV<sup>68</sup> in stronger hyphal inducing conditions. As illustrated in Figure 5.5, sEntV<sup>68</sup> is highly active in a variety of media conditions, including fetal bovine serum (FBS), which is a robust hyphal inducer (Fig. 5.5)

Role of cell wall composition in response to *E. faecalis* mediated inhibition

While many of the genes identified in the homozygous deletion mutants screen have hyper-filamentous phenotypes, deletion of ZCF21 conferred resistance to *E. faecalis*
Figure 5.3: Impact of host relevant stresses on sEntV\textsuperscript{68} activity.

*C. albicans* (SC5314) was grown for 15 hours in YPD at 30° C with sEntV\textsuperscript{68} (100 nM) in different stress conditions: cell wall stress (A) calcofluor white (100 μg/ml) and (B) SDS (10%); cell membrane stress (C) sorbitol (1 M) and (D) Sodium Chloride (1 M); oxidative stress (E) hydrogen peroxide (5 mM) and (F) menadione (100 μM) in a 96 well plate. OD\textsubscript{600} was measured every two minutes in a microplate reader.
Figure 5.4: sEntV<sup>68</sup> activity on planktonic <i>C. albicans</i> yeast cells.

<i>C. albicans</i> was grown at 30°C in YPD for 15 hours with shaking in a 96 well plate with increasing concentrations of sEntV<sup>68</sup>. Growth of was quantified by measuring absorbance (OD<sub>600</sub>) every two minutes in a microplate reader (BioTek).
Figure 5.5: sEntV inhibitory activity in different media types.

*C. albicans* (*HWP1p::GFP*) biofilms grown for 24 hours at 37°C in YNBAS, RPMI, Spider, or YPD with 10% fetal bovine serum with 0.01% DMSO (blue) or 100 nM sEntV (orange). Hyphal morphogenesis was quantified by measuring GFP fluorescence as a ratio to the OD$_{600}$ for three separate experiments and statically significant differences were calculated using a Student’s t-test (*p<0.01*).
supernatant in a hyper-filamentous independent manner (Fig. 5.6, Table 2.1) (9). Zcf21p negatively regulates expression of genes that encode cell surface proteins important for C. albicans virulence (410). Bohm et al. (280), found that ZCF21 regulates cell wall structure and composition, including proteins and carbohydrates in the cell wall. Furthermore, the homozygous ZCF21 deletion mutant had decreased sensitivity to sEnV68, suggesting that deletion of ZCF21 causes changes in the cell wall structure and composition preventing sEntV68 inhibition.

**EntV localization in C. albicans**

Analysis of the primary and predicted secondary structure (Chapter 4) indicate EntV68 has similar physicochemical properties to human antimicrobial peptides active against C. albicans that elicit a surface stress response that is initiated via interactions with the cell wall and cell wall associated proteins (280, 295, 302). Similar to EntV68, the cationic properties of LL-37, β-d3, and Hst-5 are thought to promote interactions with the anionic microbial surface and the amphipathic structures allow the peptides to interact with microbial membranes (411). Thus, I hypothesized that EntV68 may interact with the cell surface of C. albicans in a similar fashion. As demonstrated in Figure 5.7, sEntV68 with a C-terminal FITC tag localized to the cell surface of C. albicans yeast and hyphal cells, suggesting that the EntV68 mechanism of action is cell surface dependent.

**EntV68 activity target spectrum**

Since sEntV68 is efficacious in both in vitro and in vivo conditions and has therapeutic potential, I tested the efficacy of sEntV68 against clinical isolates of C. albicans and other pathogenic Candida species to gain further insight into the spectrum of fungal targets and mechanism of action. I found that sEntV68 significantly reduced biofilm formation of clinical isolates, including azole resistant strains (Fig. 5.8A) (395, 412). Furthermore, sEntV68 was also active against biofilms formed by other C. glabrata, C. tropicalis, and C. parapsilosis (Fig..
Figure 5.6: Deletion of ZCF21 decreased *C. albicans* sensitivity to *E. faecalis* supernatant and sEntV\textsuperscript{68}.

*C. albicans* biofilms grown for 24 hours in YNBAS at 37°C for 24 hours with (A) sterile medium (blue) or supernatant (orange) and (B) 0.01% DMSO (blue) or 100 nM sEntV (orange). Biofilm biomass was quantified using the resazurin redox assay. Statistically significant differences relative to treated and control biofilms were calculated using a Student's t-test, and are indicated by asterisks (**, p<0.007).
Figure 5.7: Localization patterns of sEntV$^{68}$ on the surface of *C. albicans*.

Planktonic cultures of *C. albicans* expressing constitutive mCherry were incubated in YNBAS with 100nM sEntV$^{68}$-FITC at (A) 30°C for hour, (B) 37°C for 1 hour, or (C) 37°C for 1 hour without sEntV$^{68}$-FITC to allow for germ-tube formation followed by 1 hour incubation with sEntV$^{68}$-FITC. Localization of sEntV$^{68}$-FITC was observed by fluorescence microscopy (Olympus IX81) using slidebook 6 (3i, Intelligent Imaging Innovations). (D) Biofilms were grown with increasing concentrations of sEntV$^{68}$ or sEntV$^{68}$-FITC and the IC$_{50}$ calculated at 0.3 nM and 0.5 nM, respectively.
Figure 5.8: sEntV<sup>68</sup> target spectrum.

(A) sEntV<sup>68</sup> inhibitory activity against clinical isolates of <i>C. albicans</i>. Stain names in red are resistant clinical isolates that resistant to azoles. <i>C. albicans</i> biofilms grown for 24 hours in YNBAS at 37°C for 24 hours with 0.01% DMSO (blue) or 100 nM sEntV (orange). (B) sEntV<sup>68</sup> inhibitory activity against different Candida species. <i>C. albicans</i>, <i>C. tropicalis</i>, <i>C. parapsilosis</i> and <i>C. glabrata</i> biofilms grown for 24 hours in RPMI at 37°C for 24 hours with 0.01% DMSO (blue) or 100 nM sEntV (orange). Biofilm density was quantified by measuring resazurin fluorescence for three separate experiments and statically significant differences were calculated using a Student’s t-test (*, p<0.05; **, p<0.01).
Taken together these results suggest that EntV\textsuperscript{68} is a specific inhibitor of hyphal and biofilm growth with a broad spectrum of activity against \textit{C. albicans} and \textit{Candida spp}.

**Discussion:**

The mechanism by which EntV inhibits \textit{C. albicans} hyphal growth and biofilm formation is unresolved. However, homozygous deletion mutants resistant to \textit{E. faecalis} supernatant revealed that hyphal morphogenesis and surface stress response functions are important for the mechanism of action. Localization of the fluorescent peptide suggests a cell surface (wall or membrane) localization, and I have no evidence that it exerts any cytotoxic or lytic stress against \textit{C. albicans}. Quite the contrary: while sub-nanomolar concentrations of EntV are sufficient to inhibit biofilms, concentrations 10,000-fold higher do not reduce fungal growth, even in the presence of concurrent stresses that damage the cell wall or membrane.

Plausible mechanisms include EntV interfering with a cell surface receptor or a hyphal inducing factor or blocking cell-cell or cell-substrate binding mediated by one or more of the \textit{C. albicans} adhesins. Several of the homozygous deletion mutants, including \textit{ZCF21}, disrupt the cell wall composition that could lead to masking of a binding target protein or component of the cell wall or membrane. Disruption of the cell wall composition and structure can also impair biofilm development and is often mediated by cell stress response (20). Genetic and biochemical studies will be necessary to elucidate the mechanism of action.

Biofilms provide protection to microbes by production of the ECM that encases the microbes protecting them from the immune response and antibiotics (39). Thus, biofilm related infections present many clinical challenges by complicating treatment strategies (80). With the limited antifungals available fungal infections are difficult to treat. Furthermore, resistance to traditional antifungals is an increasing concern. Traditional antifungals interfere with essential
biological processes to kill or inhibit fungal growth and can have off target effects that can be toxic to the patient (91, 413). To overcome these challenges targeting virulence traits rather than essential fungal processes has recently been proposed to alleviate the selection pressure for resistance (410).

Moreover, targeting virulence factors will keep opportunistic pathogens in check rather than disrupting the entire mycoflora with antifungal treatment. One of the most abundant fungal species found in humans, *C. albicans* does not have any known environmental reservoir (410). This suggests that *C. albicans* has a profound association with humans and has coevolved considerably with the human host. Additionally, the majority of healthy individuals are colonized with *C. albicans* as part of the normal GI microflora (414, 415). That said, the beneficial effects of *C. albicans* colonization cannot be excluded, and might provide another rationale for why the use of an alternative antifungal could be advantageous. Collectively, our work suggests that EntV is a promising alternative therapeutic that targets hyphal morphogenesis and biofilm development with a spectrum of activity against clinical isolates and other pathogenic *Candida spp.*
Chapter 6:

Discussion and Perspectives
Summary:

*E. faecalis* and *C. albicans* occupy overlapping niches as ubiquitous parts of the gastrointestinal and oral microbiome. As opportunistic pathogens these species are also amongst the most important and problematic nosocomial pathogens. I have observed antagonistic interactions between these two species, where virulence of both microbes is attenuated in the *C. elegans* infection model. Inhibition of *C. albicans* hyphal morphogenesis was a striking phenotype observed in this infection model. Inhibition of hyphal morphogenesis was also obtained by addition of *E. faecalis* spent supernatant in the *C. elegans* infection model and in vitro biofilm model (115).

I have identified the inhibitory factor as an *E. faecalis* bacteriocin, EntV, which was necessary and sufficient for the reduction of *C. albicans* virulence and biofilm formation via its inhibition of hyphal morphogenesis. As illustrated in Figure 6.1, I have demonstrated that the EntV propeptide is post-translational modified for maximum activity. The synthetic version of the mature 68-amino acid peptide inhibited biofilm development in multiple media conditions and disrupted pre-formed biofilms. sEntV^{68} was protective in different fungal infection models at nanomolar or lower concentrations.

First, nematodes treated with the peptide at 0.1 nM are completely resistant to killing by *C. albicans* (data not shown see (303)). sEntV^{68} was protective in the macrophage infection model and works in synergy with the macrophages increasing their antifungal activity. Finally, EntV^{68} reduced epithelial invasion, inflammation, and fungal burden in a murine model of oropharyngeal candidiasis. Hyphal morphogenesis was inhibited in all three models. However, EntV^{68} had no effect on *C. albicans* viability, even in the presence of significant host-mimicking stresses. These findings demonstrate the therapeutic potential of EntV as a novel antifungal agent that targets virulence rather than viability, which is important considering the increase in resistance to traditional antifungals.
Figure 6.1: Summary of EntV processing and activity.

The inhibitory activity produced by *E. faecalis* was first characterized using an in vitro biofilm. EntV was identified as the inhibitory factor produced by *E. faecalis* and is proteolytically cleaved produce the active peptide that inhibits morphogenesis in the biofilm assay, murine macrophage infection model, and murine OPC model. Inhibition of hyphal morphogenesis reduced biofilm formation, and attenuated virulence of *C. albicans* during infection.
Furthermore, I demonstrated the physiochemical properties of EntV and the post-translational modifications that are necessary for activity. Upon assessment of the other closely related class IIIb bacteriocins from different bacteria, I observed prominent similarities in possible post-translational modifications and predicted secondary structures. This preliminary characterization of the class IIIb bacteriocins provides useful insight for the future design of EntV-based therapeutic strategies and demonstrates the importance of the class IIIb bacteriocins for these Gram-positive bacteria and their conceivable role in fungal-bacterial interactions.

To understand how *E. faecalis* inhibits *C. albicans* biofilms and hyphal morphogenesis, I identified *C. albicans* genes that confer decreased sensitivity to *E. faecalis* inhibitory activity during biofilm development. Screens of homozygous deletion mutants revealed that deletion of genes involved in regulation of hyphal morphogenesis, cell wall stress, and cell wall architecture decreased sensitivity to *E. faecalis* supernatant. This suggests that EntV may target processes involved in these functions, since perturbation decreases efficacy of *E. faecalis* inhibitory activity. Furthermore, sEntV\(^{68}\) localized to the cell surface of both yeast and hyphal cells. As illustrated in Figure 6.2, I have demonstrated that modification of *C. albicans* cell wall components or hyperfilamentation produces decreased sensitivity to *E. faecalis* mediated inhibition of biofilm development. Thus, I hypothesize that EntV targets the cell surface, which disrupts cell surface function(s) that are necessary for hyphal morphogenesis and biofilm development, most likely a function that is linked between these important virulence determinants. I also examined the target spectrum of sEntV\(^{68}\) and found that clinical isolates of *C. albicans*, including azole restraint strains, and other pathogenic *Candida spp.* are sensitive to sEntV\(^{68}\). This suggests that the mechanism of action targets a common function amongst these different strains and species. In total, these data demonstrate the therapeutic potential of sEntV\(^{68}\) or similar compounds for development of therapeutic strategies that target hyphal morphogenesis and biofilm development.
Figure 6.2: Summary of homozygous deletion mutant functions.

Of the 263 mutant screened 39 were less sensitive to *E. faecalis* supernatant. Collectively, most of these mutants have functions associated with cell wall composition and/or hyphal morphogenesis. Many of the cell wall mutants have functions associated with cell wall stress and are important for maintenance of cell wall integrity and architecture and deletion of these genes leads to cell surface remodeling. Of the 24 transcription factors, many have functions associated with regulating hyphal morphogenesis and many were hyperfilamentous as result of upregulation of hyphal specific genes.
C. albicans-E. faecalis interactions during commensalism and pathogenesis

Given the epidemiology of E. faecalis and C. albicans infections, their overlapping niches, and our current findings, it is plausible that these species have synergism during commensal colonization. As I observed previously in the nematode infection model, virulence of both microbes was attenuated, suggesting that their interactions promote non-pathogenic association with the host (115). Previous studies also demonstrate synergism during commensal colonization. Mason et al. (416) demonstrated that E. faecalis becomes more abundant after antibiotic treatment in mice colonized with C. albicans in both the stomach and the cecum. Considering C. albicans does not naturally colonize the GI tract of mice and antibiotic treatment is necessary to establish C. albicans colonization (417); it is plausible that the increase in E. faecalis also promotes commensal colonization of C. albicans (418).

On the other hand, during infection C. albicans and E. faecalis are commonly co-isolated in a variety of different types and severity of infections (109, 157, 159, 182). One common theme among these co-infections is the status of the host changes due to immune dysfunction and/or disruption of anatomical barriers, suggesting that a complex relation exists between E. faecalis and C. albicans that is dependent on the host. Consistent with clinical reports, Carlson et al. (151) found that co-infection with E. faecalis and C. albicans enhanced dissemination of E. faecalis pathogenesis in a murine intraperitoneal infection model. Considering these results and the clinical indices of E. faecalis and C. albicans co-infections, it is plausible that when immune dysfunction, loss of anatomical barriers, and/or disruption of the microflora occurs E. faecalis is able to hitch a ride, so to speak, with C. albicans during disseminated infection. A similar occurrence has been demonstrated in C. albicans and P. aeruginosa, where in vitro antagonism is observed and clinical co-infections are more severe (Chapter 1).

In view of our results, current literature, and clinical reports, Figure 6.3 illustrates our predicted model of the complex relationship that exists between E. faecalis and C. albicans. In
the presence of a healthy host both microbes are able to colonize as commensal microflora. However, with a change in the host status both microbes can become pathogenic (8, 366). During antibiotic treatment, \textit{E. faecalis} is capable of surviving because of their genomic plasticity and ability to take up resistance genes and virulence traits via conjugation [436]. Thus, \textit{E. faecalis} is capable of surviving with antibiotic treatment along with \textit{C. albicans}. However, with antibiotic treatment the population of \textit{E. faecalis} is likely reduced, repressing Fsr and in turn EntV production (356). Under these conditions, \textit{C. albicans} is able to grow, proliferate, and form hyphae. If the host has a compromised immune response, \textit{C. albicans} is able to disseminate and survive and possibly allow the remaining \textit{E. faecalis} a mode of dissemination (8). In the case of disrupted anatomical barriers, such as seen in intraperitoneal infections, both \textit{E. faecalis} and \textit{C. albicans} are able to survive in the hostile environment of the intraperitoneal cavity allowing further dissemination of both microbes (183, 184). To substantiate this model many more questions must be addressed, which will be discussed subsequently.

**Role of \textit{E. faecalis} bacteriocins in the host environment**

Many of the bacteriocins produced by \textit{E. faecalis} are found on plasmids and vary from strain to strain. Different bacteriocins-producing \textit{E. faecalis} strains have been isolated from a broad range of sources, from commensal strains found in food and feces to pathogenic strains found in different types of infections. Interestingly, some bacteriocins produced by \textit{E. faecium} are also produced by \textit{E. faecalis} commensal and pathogenic strains (419). Although the majority of bacteriocins produced by \textit{E. faecalis} target closely related Gram-positive bacteria and are thought to be important for competition with other bacteria, cytolysin is the exception because it targets other bacteria and eukaryotic cells. Cytolysin production was first observed in clinical isolates and conferred increased hemolysis (420). Furthermore, many \textit{E. faecalis}
strains produce multiple bacteriocins and many of the same bacteriocins are produced in strains isolated from extremely different environments, indicating that these bacteriocins are not

**Figure 6.3: Model of *C. albicans*-*E. Faecalis* interactions in the GI tract.**

In the healthy host *C. albicans* and *E. faecalis* have commensal like association with the host. There are abundant populations of *E. faecalis*. Thus, the Fsr system has been stimulated by accumulation of the QS molecule GABP and active EntV is being produced in sufficient concentrations to inhibit hyphal morphogenesis. In the unhealthy host who has compromised immune function, disrupted microflora, and/or loss of anatomical barriers *C. albicans* is able to penetrate the epithelial layer allowing *E. faecalis* to escape the intestinal lumen. As the status of the host deteriorates both *C. albicans* and *E. faecalis* become opportunistic pathogens.
niche specific and rather have a general advantage in any niche [440].

**Role of EntV in the host environment**

EntV is found in all sequenced *E. faecalis* strains, indicating that entV function is important for *E. faecalis* survival and is not niche specific. As mentioned previously, entV is regulated by the Fsr two-component system. The Fsr system is regulated by accumulation of the QS molecule GABP, suggesting that production and function of EntV is important when *E. faecalis* populations are at higher concentrations. In an environment such as the human GI tract *E. faecalis* populations can be $10^7$ CFU/μg. In this environment, competition with neighboring bacterial species that are closely related and likely require similar nutrients is important [441]. Production of a bacteriocin such as EntV could be advantageous.

Moreover, production of EntV in the GI tract could also be beneficial during commensal colonization with *C. albicans*. As demonstrated in our current and previous findings, EntV promotes commensal-like association with the host in *C. albicans* by inhibition of hyphal morphogenesis (115, 303). Although hyphal cells are considered the invasive and more pathogenic form of *C. albicans*, there is much debate on this topic (47). Some literature suggests that the yeast form is the commensal, noninvasive morphology and the hyphal form is the invasive pathogenic morphology. This is an over simplification. During invasive infection both morphologies are necessary and the dominance of one morphology over the other depends on the infected organ (421). Moreover, it is unclear what morphology predominates during commensal colonization. Kumamoto *et al.* (422, 423), reported the expression of hyphal-associated genes during commensal colonization in a murine GI colonization model, despite the fact that *C. albicans* was found predominately in the yeast form. That said, *C. albicans* mutant strains locked in either the yeast form or hyphal form have attenuated virulence, suggesting the transition between the two states is dynamic and necessary for maximum virulence (361, 424, 425). Thus, it is plausible that production of EntV during commensal
colonization with *C. albicans* is capable of inhibiting hyphal morphogenesis and promoting commensalism. Furthermore, yeast cells are far less immunogenic and are not recognized by phagocytes, while hyphal cell are much more immunostimulatory (426-428). Thus, controlling hyphal morphogenesis could benefit the microflora including *E. faecalis* by keeping the immune response restrained.

The class IIIb bacteriocins, including EntV, are unusual compared to other classes of bacteriocins. The majority of propeptides are small whereas the class IIIb bacteriocins are large and only about half of the propeptide is necessary for activity (Chapter 4). Most bacteriocins have an immunity protein that is encoded on a neighboring gene or with the same operon. This is the case with the class IIIb bacteriocins, except for EntV where the immunity protein has not been identified. Dundar *et al.* (245) attempted to identify the immunity protein to no avail. A translationally coupled gene was identified by bioinformatics as a candidate immunity factor (*ef1097b*). However, deletion of *ef1097b* had no effect on *E. faecalis* sensitivity to EntV. I considered whether the N-terminal portion of the propeptide might be the immunity factor. Nevertheless, Δ*entV* is not sensitive to sEntV68 (data not shown), suggesting that intrinsic immunity to sEntV68 occurs by another mechanism or gene and remains to be elucidated.

Gene expression data demonstrated that *entV* expression is the same in the pathogenic strain *E. faecalis* V583 as the commensal strain *E. faecalis* OG1RF, suggesting that EntV is expressed in all *E. faecalis* strains in a similar manner (356, 357). Furthermore, Bourgogne *et al.* (356), identified a FsrA binding consensus sequence that is present in all strains of sequenced *E. faecalis*. In addition to the Fsr operons, *gelE* and *sprE*, and *entV*, *ef1352* that encodes a putative magnesium-translocating P-type ATPase was unregulated in response to GABP stimulation (Fsr activation) (357). Nonetheless, *ef1352* does not have an FsrA binding consensus sequence, suggesting that *ef1352* is not directly regulated by the Fsr system and perhaps is induced by EntV causing ion leakage. This remains to be elucidated and would be in stark contrast to the immunity factors of other class IIIb bacteriocins (243, 358, 429).
The EntV mechanism of action against bacteria has not been fully elucidated, aside from the non-lytic manner of bactericidal activity. However, other class IIIb bacteriocins have been studied in much more detail. DysA (dysgalacticin) is produced by Streptococcus dysgalactiae subsp. equisimilis and the mechanism of action for DysA has been investigated in some detail. Swe et al. (394, 429) found that DysA has a non-lytic mode of action that is bactericidal, inhibited glucose fermentation by targeting glucose/mannose-PTS, increased membrane permeability that mediated intracellular ion leakage. Given the similarity in secondary structure I hypothesis that EntV has a similar mode of action that is mediated by insertion into the membrane of target bacteria (Fig 6.4). AMPs with similar physiochemical properties as EntV can target both fungi and bacteria (295, 302, 430). Thus, it is conceivable that EntV also interacts with the membrane of C. albicans, albeit to a lesser degree given the complex structure of the cell wall of C. albicans and differences in lipid composition compared to bacteria (13, 431). Human AMPs that are active against C. albicans, Hst-5, LL-37, hBD-3, are also active against bacteria, and inhibit hyphal morphogenesis and biofilm adhesion at sub-lethal concentrations in C. albicans. Considering this it is plausible that only low concentrations of EntV are able to insert into the membrane of C. albicans causing membrane stress that mediates inhibition of hyphal morphogenesis and biofilm development. However, given the lack of synergism of EntV with known membrane and cell wall stressors, this may be an oversimplification of a very complex interaction.

Therapeutic potential of bacteriocins and future application

As previously mentioned, application of bacteriocins as therapeutics for human use has been limited to probiotics and application in food as preservatives (Introduction). Reports of in vivo use of bacteriocins have also focused on the use of probiotic bacteria that express and potentially secrete the bacteriocins in the gastrointestinal tract (432). Although the use of bacteriocin-producing probiotics may be as beneficial as prophylactics, application of purified
Figure 6.4: Predicted membrane topology of active EntV.

The two-amphipathic helices of EntV are predicted to insert into the cytoplasmic membrane of susceptible targets. The disulfide bridge is predicted to remain on the outer leaflet of the membrane. Membrane topology was predicted using PHDhtm transmembrane helices predictions (NPS), where the EntV amino acid sequence is compared and aligned to similar sequences to obtain a more accurate predicted membrane topology (310).
bacteriocins would be superior at countering established infections. Application of purified nisin has been successful, but has been limited to application in food (244). One of the greatest impediments in the therapeutic application of purified bacteriocins is the stability of the bacteriocin in the host environment (433). Engineering bacteriocins to increase stability and potency will overcome this problem. To design bacteriocins with increased stability and potency an understanding of bacteriocins that are effective in vivo is necessary. The stability and target spectrum of EntV have not been extensively studied, thus in the event that an enhanced version would be ideal strategies for modification of class II bacteriocins can be used for EntV. Another, important factor to consider is that the size of EntV is twice as large as the class II bacteriocins, which have been extensively used and tested and are more cost effective treatment strategies.

The class IIa bacteriocins structure-function relationship has been extensively studied and the in vivo efficacy has been examined. Dabour et al. (434) found that intra-gastric application of pediocin PA-1, produced by Pediococcus acidilactici, was efficacious in mice with GI infections of Listeria monocytogenes. Infected mice where given repeat doses (250 μg/ day) of purified pediocin PA-1 for three days, which resulted in up to 2-log reductions in L. monocytogenes in feces and translocation into the liver and spleen was slowed. Furthermore, L. monocytogenes completely disappeared in the liver and spleen within six days of treatment. Another class IIa bacteriocins that has been studied in vivo is piscicolin 126 from Carnobacterium piscicola. Ingham et al. (435) found that after tail vein injection into mice piscicolin 126 retain activity against L. monocytogenes. Mice were infected intravenously with L. monocytogenes followed by intravenous treatment with purified piscicolin 126, which significantly reduced L. monocytogenes in the liver and spleen reducing clinical signs of disease. Likewise, Rihakova et al. (436) demonstrated that the divercin RV41 bacteriocin produced by Carnobacterium divergens significantly reduced L. monocytogenes during invasive infection. The efficacy of structural variants with mutation in different amino acids was
also tested and while all retained activity against *L. monocytogenes* the level of potency varied, demonstrating that certain amino acids are required for activity.

Unlike traditional antibiotics bacteriocins are confronted with challenges related to their structure as peptides. The structure-function relationships and in vivo efficacy observations can provide insight to determine engineering methods to enhance stability and potency of bacteriocins, such as EntV. Some commonly used methods to increase stability are: (1) introduction of additional disulfide bridges (437, 438), (2) amino acid substitutions including incorporation of D-amino acids (439, 440) (3) encapsulation of bacteriocins in liposomes (441-443). The class IIa bacteriocins have one or more disulfide bridges that are important for activity and target specificity (242). Fimland *et al.* (437) found that addition of a C-terminal disulfide bridge into sakacin P produced by *Lactobacillus sakei*, which only has an N-terminal disulfide bridge, broadened the target spectrum, increased potency and decreased temperature sensitivity. To reduce proteolytic cleavage of bacteriocins by proteases found in the gastrointestinal tract, O'Shea *et al.* (439) altered trypsin recognition sites in salivaricin P, a class IIb bacteriocin produced by *Lactobacillus salivarius*. Antimicrobial activity was retained with these alterations and salivaricin P variants were resistant to trypsin digestion. In the event that modification of the bacteriocin disrupts activity, encapsulation of the bacteriocin can be preserving. Were *et al.* (443) observed that encapsulation of nisin and lysozyme increased efficacy of nisin against *L. monocytogenes* by stabilizing the bacteriocin activity and structure.

To enhance the potency of bacteriocins the physiochemical properties that contribute to the mode of action must be considered. As demonstrated in Chapter 4, active EntV is amphipathic and has a net positive charge (~9.84). Proteolytic cleavage of the propeptide increased activity against *C. albicans* (Chapter 3). This corresponds with enhancement of the positive net charge that promotes the initial electrostatic interaction with the target cell. Likewise, Kazazic *et al.* (444) demonstrated that addition of positively charged amino acids at specific residues enhanced activity of sakacin P by increasing cell binding.
There are different methods that have been used previously with success to develop engineered bacteriocins (433). Rational substitution of amino acids is one such method that depends on the structure-function relationship of the bacteriocins. Use of error-prone PCR to randomly generate mutants is another approach. However, greater modifications are usually required to improve bacteriocin activity, as bacteriocins have evolved to be as effective as possible (242, 445). An alternative approach that allows for major modification is creating chimeras of with different bacteriocins from the same class to create a DNA-shuffling library to create hybrids with increased activity and broadened spectrum targets (445).

Traditional antimicrobial therapies often have broader-spectrum of activity than bacteriocins, thus having a greater impact on the commensal microflora. Often keeping opportunistic pathogens at bay, the commensal microflora acts a barrier to infection and disruption of the commensal microflora with antimicrobial treatment is a growing concern. An advantage over traditional antimicrobials, bacteriocins often have specific microbial targets, and due to their composition are less likely to be toxic because they are easily broken down into nontoxic amino acids. Correspondingly, our cytotoxicity assays (Chapter 3) demonstrated that sEntV$^{68}$ is not toxic to murine macrophages or Human cervical epithelial cells. Likewise, Jasniewski et al. (446) found that two class IIA bacteriocins, Cbn BM1 and Cbn B2 produced by *Carnobacterium maltaromaticum*, in combination or alone had no cytotoxic effects on human gastrointestinal cells (Caco-2) at concentration 100-folder than what was required for antimicrobial activity.

**Future perspectives and directions**

As mentioned previously, *C. albicans* biofilms are a growing concern in the clinical setting. Due to their complex structure, encasement in the ECM, and production of persister cells traditional antifungals are ineffective at eradicating *C. albicans* biofilms. In addition there are currently no biofilm-specific therapeutics used clinically further complicating treatment
strategies (39). Several chemicals have been identified that specifically target biofilm formation, while others target hyphal morphogenesis (92, 111, 447). These studies are insightful and show promise for the development of biofilm specific treatment strategies. Likewise, our findings demonstrate the possible use of a natural-based peptide for control and treatment of C. albicans biofilms that has many advantages over the use of other treatment.

Here, I have described very complex and ecologically important interactions between E. faecalis and C. albicans. As described in Chapter 3, I have identified a bacteriocin, EntV that has therapeutic potential in different in vitro and in vivo models. Furthermore, I have characterized EntV activity with respect to post-translational processing that results in production of a highly active version of EntV. Additionally, I have identified genes that when deleted decrease C. albicans sensitivity to E. faecalis supernatant and sEntV\textsuperscript{68}. Lastly, I examined the spectrum of target activity for sEntV\textsuperscript{68}. Although, I have addressed many of the questions I had when starting this project, there are still many more intriguing questions left to answer.

**Is sEntV\textsuperscript{68} efficacious in other mammalian infection models with C. albicans?**

C. albicans can cause a variety of different infectious, from superficial infections (OPC, vaginal candidiasis) to systemic infections (endocarditis, candidemia, intraperitoneal infections). Recall that during invasive infection the yeast-hyphal transition is necessary for maximum virulence. However, the dominance of one morphology over the other depends on the infected organ (421). Thus, it would be insightful to assess the efficacy of EntV an invasive infection model, such as the intravenous tail vein injection model. Considering that other bacteriocins are effective in invasive bacterial infections, EntV would be expected to decrease dissemination of C. albicans. Nevertheless, EntV does not have fungicidal activity during planktonic growth so the effects of EntV may be moderate.
Given our observation in the *C. elegans* infection model, where commensal-like association with *C. elegans* was observed, I speculate that these species have synergism during commensal colonization. This could be confirmed using the murine colonization model (422). Perez *et al.* (9) screened homozygous transcription factor deletion mutants in both the murine intestinal colonization and systemic infection models. Eight transcriptional regulators were identified that have roles in at least one of the models examined. 800 target genes were identified by genome-wide chromatin immunoprecipitation that are regulated by the eight transcriptional regulators. Interestingly, many pathogenesis associated genes were upregulated during commensal colonization suggesting that commensalism and pathogenicity are genetically intertwined.

Addition of EntV to either of these models could change the gene expression profiles or the specific genes identified.

**Is sEntV\(^{68}\) efficacious against other fungal pathogenes not closely related to *C. albicans*?**

sEntV\(^{68}\) was active against biofilms formed by other *C. glabrata*, *C. tropicalis*, and *C. parapsilosis*. Although all of these species belong to the *Candida* genus, only *C. tropicalis* and *C. parapsilosis* are closely related to *C. albicans* and are part of the CUG clade that translate the CUG codon as serine instead of leucine (448). *C. glabrata* is more closely related to *S. cerevisiae*. The *Candida* genus originally included yeast species that are able to form hyphae or psuedohyphae and have no observed sexual spores. Although *C. albicans* remains the leading of cause of candidiasis, isolation of non-albicans *Candida* (NAC) species is increasing in patients. *C. glabrata* followed by *C. parapsilosis* and *C. tropicalis* were the most common NAC species isolated worldwide in a 10 year retrospective analysis of NAC species infections (449). Interestingly, EntV has inhibitory activity against all of these species, suggesting the mechanism of action requires a common feature found in these different species.
C. albicans, Cryptococcus neoformans and Aspergillus fumigatus are among the most prevalent fungal pathogens. Resistance to traditional antifungals is also an increasing problem in C. neoformans and A. fumigatus infections (450). Thus, development of novel treatment strategies that do not promote resistance is also critical to treating these infections. Since C. neoformans and A. fumigatus are important pathogens and not closely related to C. albicans, examining the efficacy of EntV would provide useful insight into the mechanism of action and could be used as a novel treatment strategy.

**What is the role of the disulfide bridge and how is it formed?**

Recall that EntV contains two cysteines that form a putative disulfide bond that is necessary for bactericidal activity and inhibitory activity against C. albicans (Chapter 4) (358). Until recently disulfide bond formation in Gram-positive bacteria was not well understood and was not thought be important (381). However, recently thiol-disulfide oxidoreductases (DsbA-like proteins) have been characterized in Gram-positive actinobacteria (382). Furthermore, a homolog of dsbA has been identified in E. faecalis and disruption of dsbA by transposon mutagenesis resulted in reduced inhibitory activity suggesting that dsbA is required for producing active EntV. However, the role of the disulfide bond formation by DsbA remains to be fully elucidated. I am currently examining the role of dsbA in producing active EntV and other possible roles in E. faecalis (Armand Brown, Ph.D.). Currently, there are only limited reports on the dsbA homolog (EF1088) in E. faecalis thus this work will not only add to our understand of EntV, but the physiology of E. faecalis.

**What role if any does SprE play in post-translational processing of sEntV?**

I hypothesized that GelE and/or SprE proteolytically process EntV. However, I did not determine the role of SprE for EntV activity. To address this, we are currently working on examining the in vitro processing of EntV in deletion mutant of E. faecalis (ΔgelE, ΔsprE,
ΔgelEΔsprE) compared to WT. EntV will be purified from the supernatant and cell lysates of the
E. faecalis strains and subjected to SDS-PAGE and visualized by I stern blotting polyclonal
antibodies. Our previous attempts to observe in vivo cleavage of EntV did not work because we
was expressing EntV with a C-terminal Hexa-HIS tag expressed from the endogenous
promoter in the mutant strains and WT E. faecalis. However, I was not able to visualize the
EntV-6XHIS by western blot with HIS antibodies. The EntV-6XHIS construct fully
complemented the ΔentV mutant, suggesting EntV is being produced. This along with our in
silico analysis of proteolytic cleavage by the GelE homolog, thermolysin, suggests that the
Hexa-HIS tag is being cleaved in vivo by GelE.

What is the sEntV68 mode of action against C. albicans?

As previously mentioned, I speculate that EntV interacts with the membrane of C.
albicans at low concentration causing minimal perturbation of the membrane that in turn
disrupts biofilm formation and hyphal morphogenesis. This hypothesis also corresponds with
our mutant library screens, where deletion of genes that disrupt the cell wall components or
increase filamentation are less sensitive to E. faecalis supernatant. Thus, it is plausible that
disruption of the cell wall components masks or interferes with EntV targeting the membrane. If
indeed EntV68 is causing membrane stress by leakage of ions then there should be a change in
membrane potential and intracellular ATP, which can be measured and quantified using
fluorescence assays and bioluminescence assays, respectively (382).

Furthermore, treatment of C. albicans with human AMPs LL-37, β-defensin-3 hβD-3, or
Hst-5 causes vacuolar expansion, which is thought to occur as a result of osmotic stress from
membrane permeabilization and cytosolic ion leakage (280, 451). The vacuole can be
visualized by staining with FM4-64 (Invitrogen), a red fluorescent lipophilic stain that
intercalates into the membrane and then is transported to the vacuole, allowing assessment of
the physical characteristics of the vacuole (302, 452). This could determine if *E. faecalis* causes membrane perturbation resulting in an increase in vacuole size.

**Are the other class IIIb bacteriocins active against *C. albicans***?

Examining the efficacy of the other class IIIb bacteriocins against *C. albicans* could provide further insight into the mechanism of action and would also suggest that the interactions observed between *E. faecalis* and *C. albicans* are more widespread, which has ecological implications. Furthermore, understanding the structure differences and possible activity differences could contribute to engineering possibilities of EntV (51, 453). As mentioned previously, the potency and stability can be improved by enhancing the physiochemical properties that contribute to the mode of action. If these bacteriocins are active against *C. albicans* the construction of chimeric bacteriocins is possible using different portion of the class IIIb bacteriocins to create hybrids that may have increased activity and broadened target spectrums (433). This could also provide insight into what portions of the peptides are necessary for activity increasing our ability to develop EntV-based treatment strategies.

**How does *C. albicans* gene expression change in the presence of *E. faecalis***?

The EntV mechanism of action is one of the most important questions left to answer. Changes in *C. albicans* gene expression in the presence of *E. faecalis*, *E. faecalis* supernatant, and EntV can be used to elucidate the mechanism of action. The most comprehensive way to address this question would be by RNA sequencing, which would tell us the global changes in gene expression. This assay can be done in *C. albicans* grown in the in vitro biofilm assay grown in YNBAS for 24 hours. Previous studies have observed gene expression changes in *C. albicans* grown as monoculture and polymicrobial biofilms with different bacteria and growth conditions (445). While the transcription factor mutant library screen gives some insight into gene regulation in the presence *E. faecalis* supernatant only a select number of genes can be
assessed, which only include non-essential genes and only a small portion of the genes expressed by \textit{C. albicans}. RNA sequencing can also provide insight into gene expression in different infection models that have already been tested (nematode, macrophage, OPC) or new models to understand the effects of EntV during colonization and systemic infections. Not only will RNA sequencing provide insight into gene expression of \textit{C. albicans}, but also gene expression of \textit{E. faecalis}.

I also observed attenuation of \textit{E. faecalis} virulence in the \textit{C. elegans} infection model, where survival of the nematode was increased during co-infection with \textit{C. albicans} and \textit{E. faecalis} compared to nematodes infected with \textit{E. faecalis} only. The \textit{E. faecalis} mode of virulence is less striking than that of \textit{C. albicans}, where hyphal cells invade through the body of the nematode. Nematodes infected with \textit{E. faecalis} become impacted with the bacteria and are unable to expel them and eventually die (LD~10). Preliminary observation demonstrate that spent media from \textit{C. albicans-E. faecalis} biofilms inhibits biofilm formation of \textit{E. faecalis} (Melissa Cruz). Furthermore, alteration of bacterial gene expression by \textit{C. albicans} has been previously reported, such as what is seen in \textit{S. aureus} and \textit{C. albicans} biofilms (42, 454, 455).

This project has overall provided valuable insight into the growing knowledge of polymicrobial interactions. Moreover, I have demonstrated that a bacteriocin produced by \textit{E. faecalis} has therapeutic potential against \textit{C. albicans} in different in vitro and in vivo assays. The future directions of this study can lead to advancements in treatment of antifungal resistant \textit{C. albicans} by exploiting bacterial mediated mechanisms to treat \textit{C. albicans} infections.
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Vita

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