INVESTIGATION OF ANTIFUNGAL RESISTANCE EXHIBITED BY *CANDIDA ALBICANS*

by

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A dissertation submitted to the faculty of
The University of Utah
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Chemical Engineering

The University of Utah

August 2010

The University of Utah Graduate School

STATEMENT OF DISSERTATION APPROVAL

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ABSTRACT

The resistance exhibited by *C. albicans* biofilm, a common medical complication, has become a clinically important problem. Our lab was the first to separate and identify a small subpopulation of biofilm persister/ basal blastospores that are highly resistant to amphotericin B (AmB). An important aspect of the persister cells is that they are tightly adhered to the surface; the larger portion of the biofilm is easily sloughed off. While the mechanisms involved in formation of persister cell subpopulation are poorly understood, resistance could be due a number of factors. This study focuses on three possible mechanisms: 1) A reduction of ergosterol in the plasma membrane, 2) Increased production or cross linking of β-glucans in the cell wall and 3) Adhesion to surfaces.

Ergosterol is the primary sterol in *C. albicans*. AmB acts by binding to ergosterol and creating channels in the membrane, which then causes unregulated leakage of ions, damages the cell and eventually causes its death. Ergosterol and intermediates were measured in the persister cells and compared to other biofilm fraction and planktonic. Results show a decrease in ergosterol and formation of intermediates and side products only in persister cells. Though the ergosterol reduction in persister cells is consistent with their resistant

behavior; it still does not explain the magnitude of resistance exhibited by these persister cells.

The cell wall is physically and physiologically different from the plasma membrane. Direct analysis of the cell wall is scientifically challenging; however, one can analyze signaling pathways involved in cell wall synthesis. One such pathway involves the phosphorylation of MAP-kinase (MAPK). Results show that Mkc1 and Cek1 MAPK were activated in only the persister cells.

The most striking feature of the persister cell population was their increased adherence to the tubing. Farnesol is a known quorum sensing agent for *C. albicans*. Results show that farnesol had drastically reduced the attachment of cells to the tubing surface. This is an important finding, as it gives us a new avenue to impede the process of persister cell formation.

In summary, this work helped in gaining further insight into the formation of the persister cells.

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CHAPTER 1

INTRODUCTION

1.1 Clinical relevance of *Candida albicans* infections

Candida albicans is a commensal fungus that can be a component of the microbial floras of oral cavity, gastrointestinal tract or vagina (Odds 1988). Multiplication of organism is normally kept in check through physical barriers such as the skin, by competition with the endogenous microflora, and through host defense mechanisms. Under conditions of host immune suppression, or when natural barriers to infection are degraded, colonizing *C. albicans* can give rise to opportunistic infection (Cannon 1995 & 1999). Devices such as stents, shunts, prostheses, implants, endotracheal tubes, pacemakers, and various types of catheters, to name a few, have all been shown to support colonization and biofilm formation by *C. albicans* (Hawser 1994). Table 1.1 stresses the importance of tackling the *C. albicans* infections. In the current climate, with growing numbers of individuals affected by immune dysfunction, *C. albicans* poses an ever increasing threat and is one of the four most common causes of bloodstream infections (Douglas 2002). *Candida* species are also the third leading cause of catheter related infections, with the second highest colonization to infection rate and highest mortality rate (Ramage 2001, Ramage 2002, and Douglas 2002).

Table 1.1: Impact of *Candida* infections of medical devices

Device	Annual use in the United States	Overall rate of infection (%)	Proportion of infections due to *Candida* (%)	Mortality due to *Candida* infections (%)	Most common *Candida* species	Removal needed to achieve cure
Vascular catheters	5×10^6	3-8	10	26-38	*albicans, glabrata*	Yes
Joint prostheses	6×10^5	1-3	<1	NK	*albicans parapsilosis*	Yes
Dialysis access Hemodialysis fistulas	2.4×10^5	1-4	<<1	25-50	*albicans*	Yes
Hemodialysis grafts		10-35	<<1	25-50	*albicans*	Yes
Peritoneal dialysis catheters		23	2.4-7	5-25	*albicans, parapsilosis*	Yes
Cardiac devices						
Prosthetic valves	8.5×10^4	2.9	2-10 (45,	33g (112)	*albicans, glabrata parapsilosis*	Yes
Pacemakers	4×10^5	0.5-7.0 (36, 50, 120)	4.5 (6, 50, 120)	NK	*glabrata, albicans*	Yes
ICDs		2.2-7.2	<1	NK	*albicans*	Yes
VADs	700	28-66	25-39	100	*albicans, tropicalis*	Yes
Central nervous system devices						
VPSs	4×10^4	6-15	1	9-30	*albicans tropicalis*	Yes
Urinary catheters	3×10^7	10-30	21	19.8-39	*albicans, glabrata*	Yes
Penile implants	$1.5 - 2 \times 10^4$	1-9	5-9.2	NK	*albicans*	Yes

Modern technology has allowed the use of a wider and newer variety of medical devices. The combination of an increasingly aging population and consistently growing number of inserted devices is likely to escalate the occurrence of infectious complications related to medical devices (Kojic 2004). Clinically important antifungal agents - amphotericin B, fluconazole, flucytosine, itraconazole and ketoconazole - showed much less activity against *C. albicans* biofilms as compared to planktonic cells (Costerton 1999, Baillie 1999, Ramage 2001). Thus acquired resistance of *C. albicans* has become an important issue that needs to be addressed.

1.2 *Candida albicans* and biofilms

C. albicans are dimorphic fungi that can grow in variety of morphological forms, ranging from round budding yeast to psuedohyphae and true tubular hyphae. The budding yeast form is round in nature with a diameter of about 5 microns. In planktonic culture, they grow exponentially for about 6-8 hrs and reach stationary phase within 15 hrs. Because *C. albicans* is part of normal human flora, it represents an opportunistic infection. While *C. albicans* is the focus of intense research, knowledge of its biology is hampered by the fact that it is an obligate diploid lacking a sexual cycle (Larriba 2000). Infection of mucosal epithelium of human tissues by *C. albicans* as well as disease progression seems to require a reversible conversion of yeast from to a hyphal morphology. Hence, a large number of studies on *C. albicans* have focused on genes related to filamentation, which are linked to virulence (Larriba 2000).

A biofilm is a three-dimensional community of microorganisms embedded in an exopolymeric matrix and attached to a surface. Figure 1.1 shows a cartoon representing a heterogenous structure. From a human perspective, biofilms are important because they form on implanted medical devices and result in infections that are unusually refractory to antimicrobial therapy (Kumamoto 2005). Studies have shown that *C. albicans* biofilms are formed in several stages (Chandra 2001, Douglas 2003).

First, cells, typically yeast form, attach to a surface. Second, cells proliferate on the surface, forming microcolonies. Final stage includes growth of cells, production of hyphae and secretion of exopolymeric matrix result. The exopolymeric matrix is composed of carbohydrates, proteins and other unidentified components (Hawser 1998, Baillie 2000).

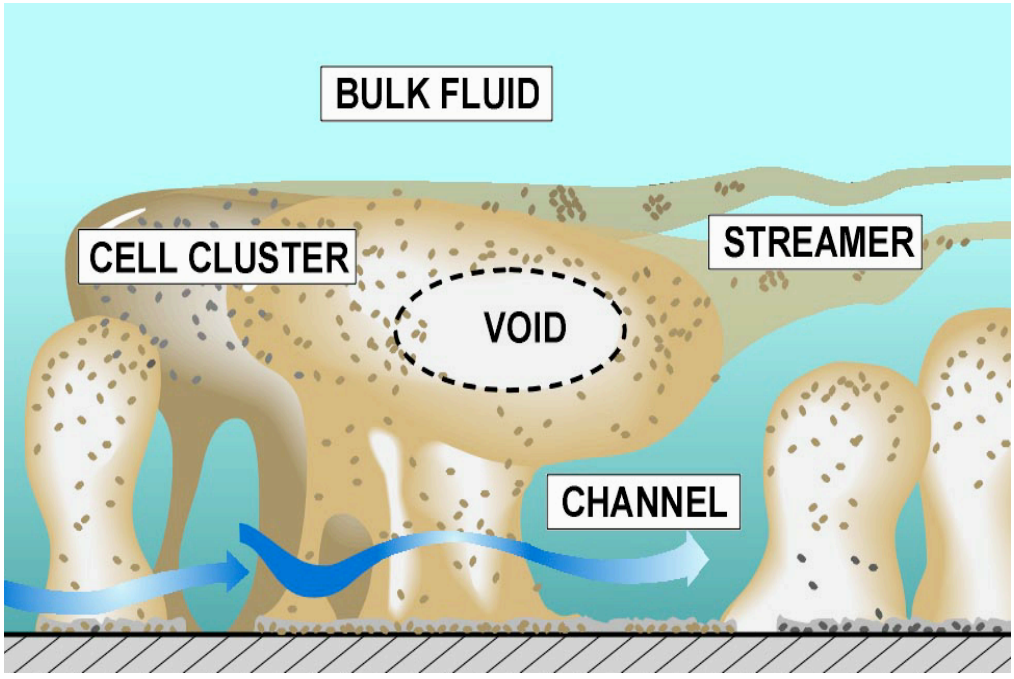

Figure 1.1: Cartoon of complex biofilm microflora

The structure of biofilm is strongly influenced by parameters such as media composition, temperature and nature of substratum (Kumamoto 2002). Mutants that are unable to form hyphae or yeast cells can nevertheless produce biofilms, demonstrating that a specific morphology is not strictly essential for biofilm formation (Baillie 1999). One of the biggest problems with biofilms is their high level resistance to antimicrobial drugs (Donlan 2001, Douglas 2003, and Kumamoto 2002). Recent studies have shown that basal blastospores have shown much higher level resistance to amphotericin B than rest of the biofilms (Khot 2006). These cells generally account for less than 2% of the biofilm. Another study has also shown that biofilms can produce antifungal-tolerant persister cells (Lafleur 2006). The existence of these persister cells has become the primary concern of our research as well as the fungal biofilm community. The genetic expression changes are quite different in this subpopulation as compared with the rest of the biofilm. Most of the current literature deals with biofilm as a whole. The molecular information from the smaller, highly resistant persister subpopulation is lost in this process.

In early stage biofilms, over expression efflux pump encoding genes, like CDR1, CDR2, and MDR1, have been shown to correlate with high resistance of biofilms (Ramage 2002). However this correlation has been proven otherwise for mature biofilms (Mukherjee 2003 and Ramage 2002). Decreased membrane ergosterol content (Mukherjee 2003) and altered expression of ergosterol biosynthetic genes (Garcia-Sanchez 2004 and Khot 2006) have been correlated to high drug resistance. Also b-1, 6 glucan pathway (Khot 2006, Nett 2007) and

surface adhesion related ALS family genes (Chandra 2001a) have been linked with resistant strains.

In summary, multiple mechanisms contribute to the increased drug resistance exhibited by biofilms. Hence it becomes critical to further investigate the molecular level changes in subpopulations of biofilm to understand the complex interaction and biology of *C. albicans* biofilm.

1.3 Biofilm subpopulation

There has been evidence of extremely resistant / persister/ basal blastospore cells found within the biofilm. However, only in the last half decade have researchers been able to gather more evidence for this idea. LaFleur et al. identified persister cells in a biofilm mode of growth. In a recent study, Khot et al. identified a subpopulation within a *C. albicans* biofilm that is highly resistant to amphotericin B. Using a continuous flow reactor, *C. albicans* biofilms were grown for 36 hrs and then divided, based on observation, into two fractions: A loose shear removed fraction (Figure 1.2) comprising the bulk of the biofilm and a much smaller basal blastopores fraction (Figure 1.3). The shear removed biofilm fraction constituted more than 98% of the biofilm and showed typical biofilm morphologies with dense colonies of mixed cell forms. Although this fraction has the morphological features typically associated with biofilm cells, these cells showed only a minimal increase in their resistance to amphotericin B (Figure.1.4). Hence, the separation of biofilms is critical to understanding the resistant behavior.

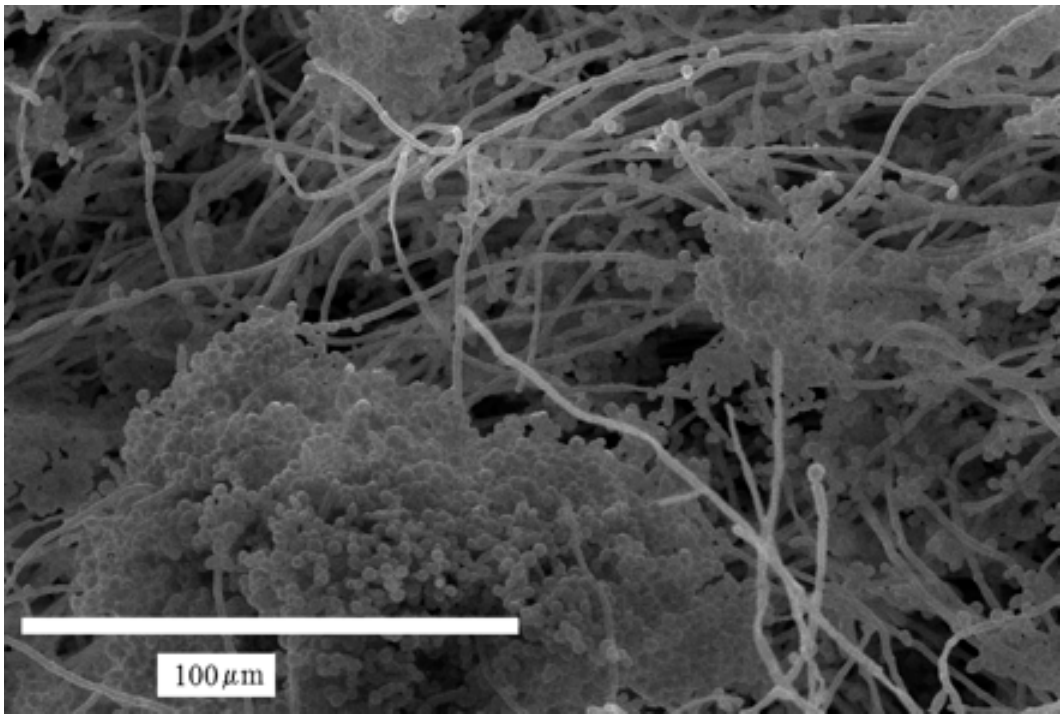

Figure 1.2: Scanning electron microscopy image of the shear-removed *C. albicans* biofilm

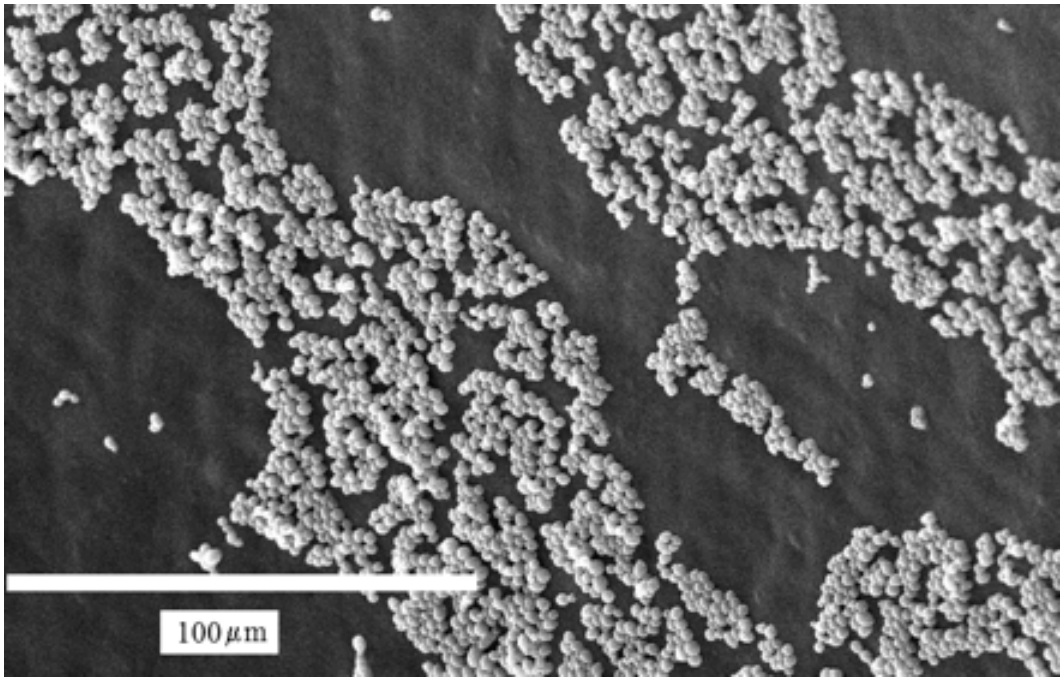

Figure 1.3: Scanning electron microscopy image of the *C. albicans* basal blastospore subpopulation

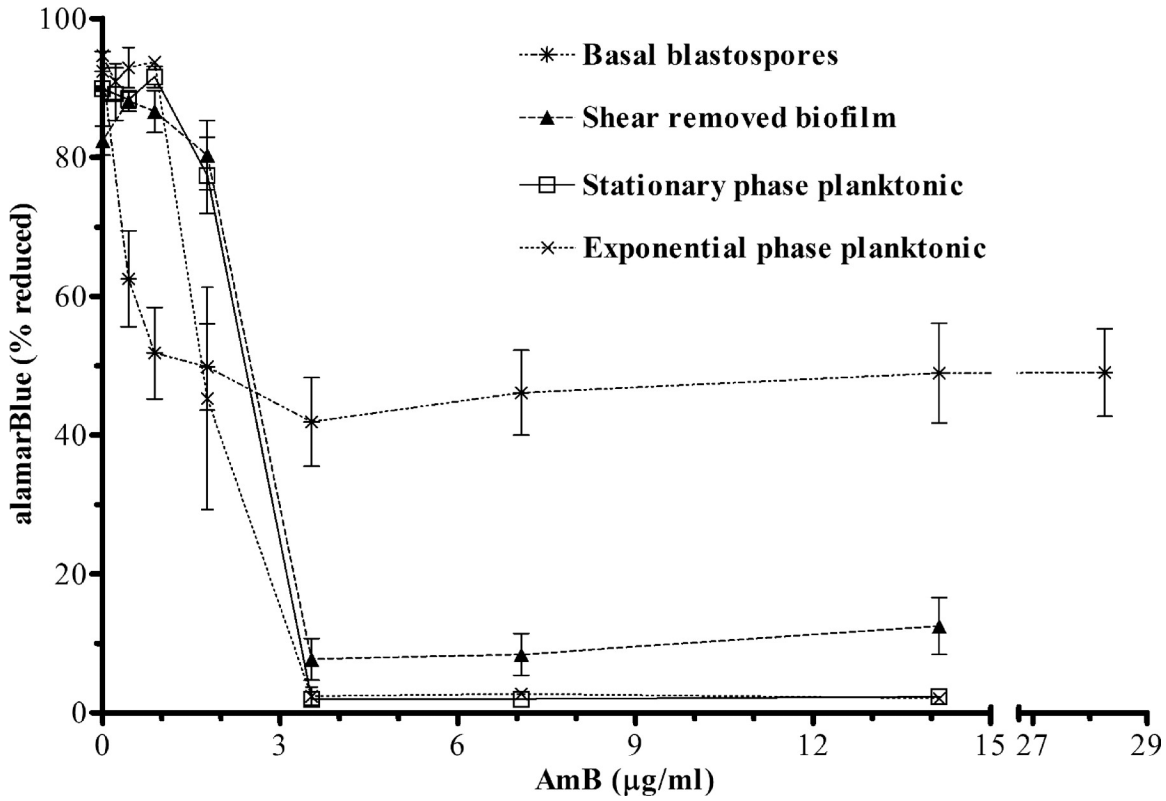

Figure 1.4: Amphotericin B dose-response curves for *C. albicans* biofilm and planktonic populations assessed by an alamarBlue metabolic assay

In contrast, the basal blastospores fraction constituted a submonolayer of budding yeast cells that exhibited extraordinary resistance to amphotericin B (Figure 1.4). The blastospores cells were resistant to around 10 times the amphotericin B. dose required to eliminate the other planktonic fractions. Although several researchers have published studies of differential gene expression in *C. albicans* biofilms, all these studies analyzed the whole biofilm. Interpretation of such results does not give a true picture because changes in gene expression within the very small subpopulation that is highly resistant are easily masked by the lesser-resistant majority cells within the biofilm. The

discovery of this resistant blastospore subpopulation represented a significant advance in the understanding of drug resistance in *C. albicans* biofilms. It was seen that the resistance to amphotericin B could possibly be due to a synergistic effect of changes in gene expression in two pathways - the ergosterol and the *β*-glucan pathways.

1.4 Specific research aims

1. **Optimize the Tubular Biofilm Flowcell to grow biofilms:** I have developed a Tubular Biofilm Flowcell to grow *C. albicans* biofilms. Optimal growth-media concentration, inoculum size, inoculum retention time, shear rate, tube dimensions and time of biofilm growth have been identified to yield biofilm samples to be used for further analysis. I have used growth kinetics curve and Colony Forming Unit protocol to quantify cells.

2. **Optimize the sterol extraction protocol and characterize these sterols using GC/MS:** Ergosterol is target for amphotericin B. In basal blastospore subpopulation, there is increased resistance towards amphotericin B. The gene expression studies have shown that there is change in the regulation of important genes in this pathway. I hypothesize that there is reduction in ergosterol levels, which leads to resistant behavior of basal blastospores.

3. **Impact of adhesion on MAP kinase pathway:** Two MAP Kinases Cek1 and Mkc1 in *C. albicans* have been identified to play a critical role in cell wall biogenesis and repair in cases of contact activated, antifungal agent and cell wall degrading enzyme induced stress. Gene expression studies have shown

that basal blastospores have over expression of β-glucan pathway genes. Hence I hypothesize that surface contact leads to activation of these Cek1 and Mkc1 MAP kinases in the basal blastospores. This could have been the possible trigger for up-regulation of β-glucan pathway genes.

4. **Effect of farnesol on biofilm formation:** Farnesol is a known quorum sensing agent for *C. albicans.* Quorum sensing has been observed to cause disruption of biofilm in bacterial models. A recent study has shown that farnesol has an effect on the architecture of biofilm in case of *C. albicans.* The critical step in transition of normal fungal cells to resistant phenotypes is attachment to surfaces. I hypothesize that by incorporating the farnesol into the tubing material; there will be reduction in the cells attaching to the surfaces. Therefore by reducing the number of cells attaching, there will be reduction in the resistant phenotype formation.

CHAPTER 2

MEMBRANE STEROL CHARACTERIZATION

2.1 Abstract

The plasma membrane consists of a lipid bilayer with sterols embedded in the inner hydrophobic core. Ergosterol is the primary sterol in C. *albicans*; it is the product of the ERG pathway, which starts with squalene and ends in production of ergosterol through a series of nine intermediate steps. Ergosterol modulates membrane fluidity. Amphotericin B (AmB), an antifungal drug, acts by binding to ergosterol and creating channels in the membrane, which then causes unregulated leakage of ions, damages the cell and eventually causes its death. Therefore, ergosterol mediated incorporation of AmB is critical to this process. Khot et al. showed that expression of key genes in the ERG pathway, ERG1 and ERG25, were altered, suggesting a reduction in ergosterol and the formation of intermediates. I hypothesized that in persister cells there will be a reduction of ergosterol and increased formation of intermediates.

Ergosterol and intermediates were measured in the persister cells and compared to other biofilm fraction and planktonic. Results show a decrease in ergosterol and formation of intermediates and side products only in persister cells. Consistent with my hypothesis and the gene expression analysis, I have been able to show that the ergosterol pathway is altered in C. *albicans* and could

play a role in resistance of persister cells. Other studies also show that strains of C. *albicans* that are resistant to AmB have drastically reduced ergosterol levels. Though the ergosterol reduction in persister cells is consistent with their resistant behavior; it still does not explain the magnitude of resistance exhibited by these persister cells. The following is a detailed description of the background, experiments, results and discussion. In conclusion, ergosterol is reduced in the membranes of the persister cells, explaining their resistance towards amphotericin B.

2.2 Introduction

2.2.1 Role of ergosterol

Sterols are an important component of the cell membrane. The cell membrane consists of a thin layer of amphipathic phospholipids which spontaneously arrange so that the hydrophobic "tail" regions are shielded from the surrounding polar fluid, causing the more hydrophilic "head" regions to associate with the cytosolic and extracellular faces of the resulting bilayer. This forms a continuous, spherical lipid bilayer.

Sterols are the other important component of cell membranes embedded in the hydrophobic areas of the inner (tail-tail) region. Figure 2.1 shows the structure of a typical fungal cell with its cell membrane. Cell membrane (plasma membrane) acts as the interface between cytoplasm and cell wall. Ergosterol is the primary sterol found in *C. albicans* membrane. The primary sterol in human cells is cholesterol. This chemical difference in fungal cells and human cells has made ergosterol pathway the prime target for antifungal drug design. One of the

main functions of ergosterol is to maintain the membrane fluidity in response to environmental changes. Ergosterol is responsible for membrane rigidity, stability and resistance to physical stresses. In addition, ergosterol modulates membrane fluidity, permeability and the activities of membrane-bound enzymes (Lees 1995, Parks 1995). They are also required in cell respiration and in altering growth rate of cells (White 1998).

2.2.2 Mechanism of amphotericin B resistance/action

Sterols are an important component of the cell membrane. Figure 2.1 shows the structure of a typical fungal cell with its cell membrane. The figure also shows the target for important classes of antifungal drugs that are in clinical use.

One such antifungal drug is a polyene called amphotericin B (Brajtburg 1990). Amphotericin B is widely used for the treatment of systemic fungal infections despite the severe side effects that it produces (Hartsel 1996, Brajtburg 1990). It is amphipathic, having both hydrophobic and hydrophilic sides and is amphoteric, having characteristics of an acid and a base. Amphotericin B preferentially binds to ergosterol and forms hydrophobic complexes. The seven conjugated double bonds of amphotericin B and the whole sterol molecule are involved in the formation of a complex through non specific Van der Waals interaction (Brajtburg 1990). This complex produces a permeability change in the membrane of the fungal cell, presumably caused by pores. This leads to leakage of ions from the cellular body. This leakage eventually results in cell death.

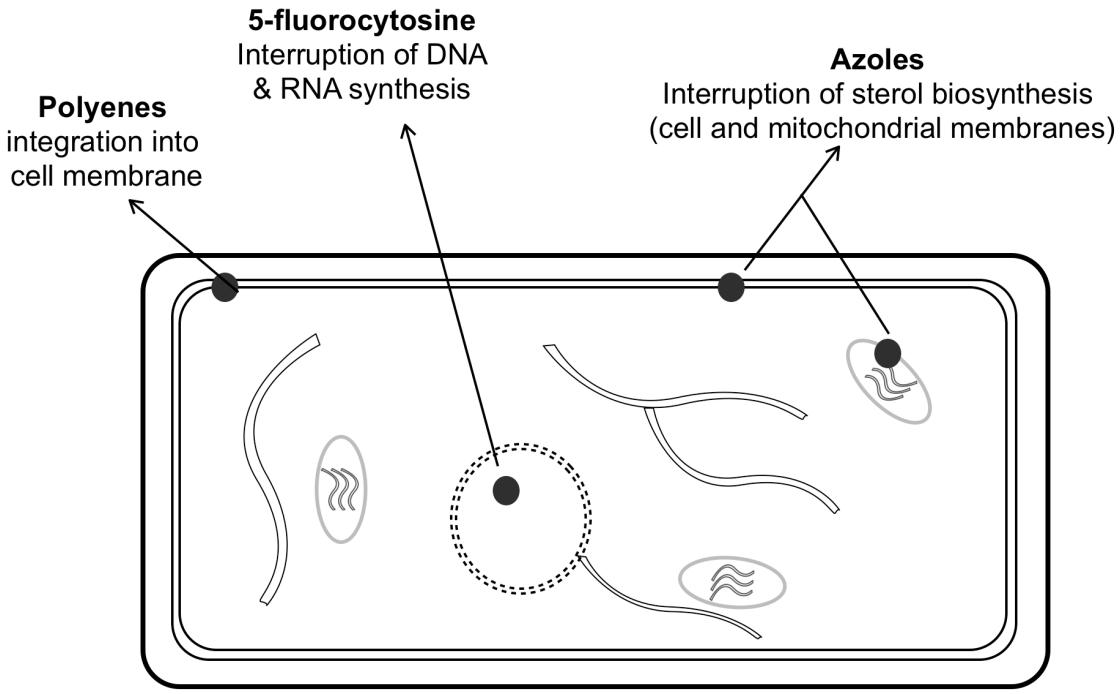

Figure 2.1: Structure of a fungal cell and the various drugs that target the cell membrane.

Amphotericin B binds to ergosterol molecules to form a barrel-like structure whose interior has a hydrophilic environment that allows for the passage of ions and other small molecules like urea and glucose (Gale 1986, White 1998, Ramage 2002, Loeffler 2003, Hartsel 1996). This unregulated leakage of metabolites damages the cell and eventually provokes its death. Thus the critical step in action of this drug is its binding to ergosterol. Once the complexes are formed, they diffuse towards each other to form pores, as shown in Figure 2.2. Reduction in ergosterol levels would lead to formation of fewer complexes (amphotericin B- ergosterol). With fewer complexes formed, the pores would be incomplete, hence ineffective. Therefore, reduction in levels of ergosterol could lead to ineffectiveness of amphotericin B.

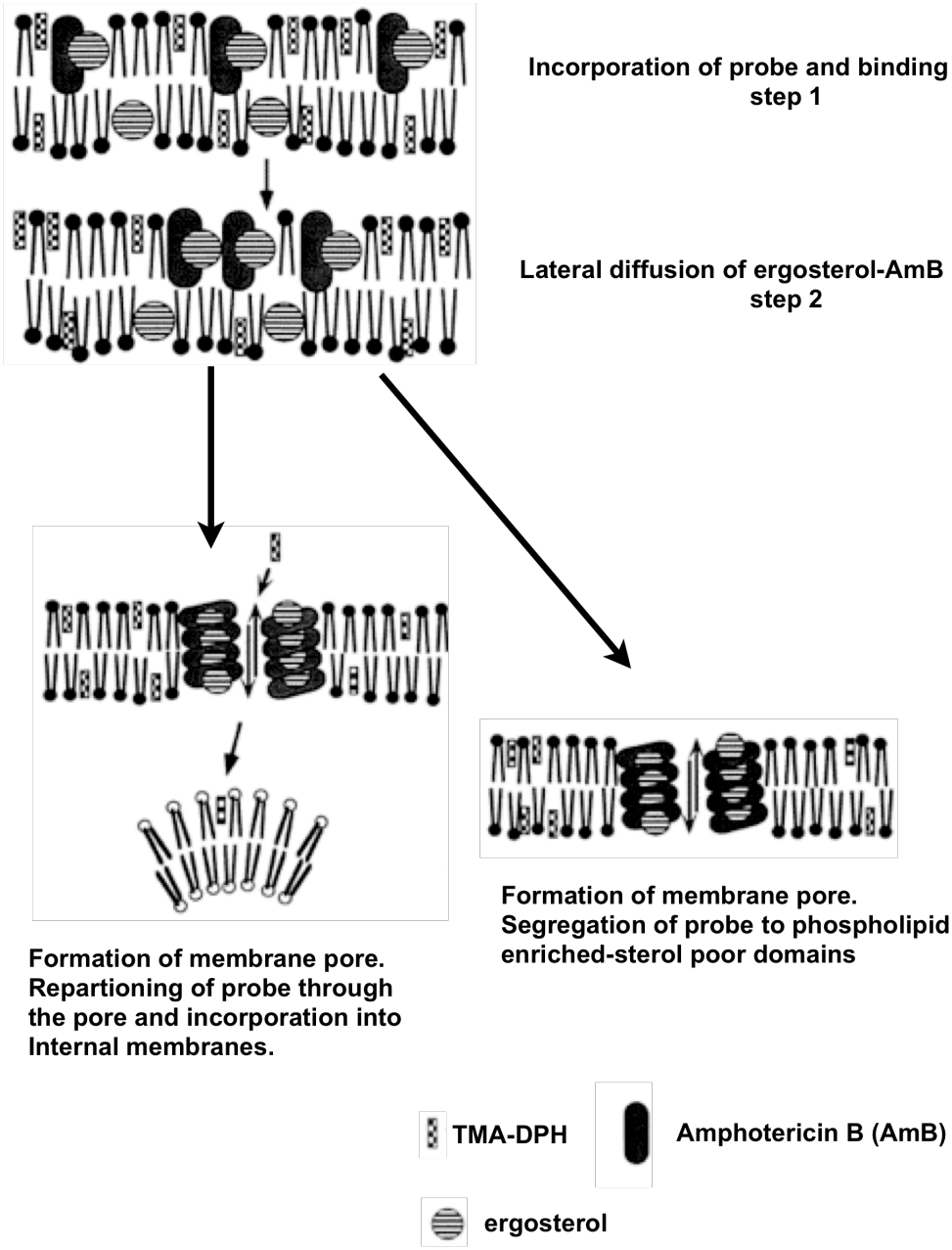

Figure 2.2: Mechanism of action of amphotericin B on fungal cell membrane.

2.2.3 Ergosterol levels change with resistant behavior

Among various classes of lipids in *C. albicans*, membrane ergosterol is an important target of common antifungal drugs like polyenes and azoles. Figure 2.3 shows the ergosterol biosynthesis pathway for *C. albicans.* Interestingly, the action of antifungal drugs is affected by the changes in membrane lipid composition in general and by ergosterol in particular. Because of the relationship between drug resistance and membrane ergosterol composition, many genes of the ergosterol biosynthetic pathway of *C. albicans* have been studied (Aaron 2001, Barker 2004, Khot 2006, Parks 1995a, Smriti 1999, Sanglard 2003).

Analyses of the gene expression of the ergosterol pathway genes, which are exclusively dedicated to the synthesis of ergosterol, have provided insights into the transcriptional mechanisms involved in regulating ergosterol levels (Barker 2004, Daum 1998). The first three steps of the pathway leading to formation of lanosterol from squalene are essential since no sterol molecule is synthesized until this point. These steps could be critical in regulation of this pathway. The remaining steps of the pathway form sterol intermediates, which may or may not substitute for ergosterol functionally (Daum 1998). The reduction of ergosterol in the plasma membrane of resistant cells leads to a lower affinity for amphotericin B, probably due to the lack of a binding site, thus increasing the resistance (Gale 1986, Loeffler 2003). Another study quantified the sterol distribution of the ergosterol pathway in *C. albicans* biofilms.

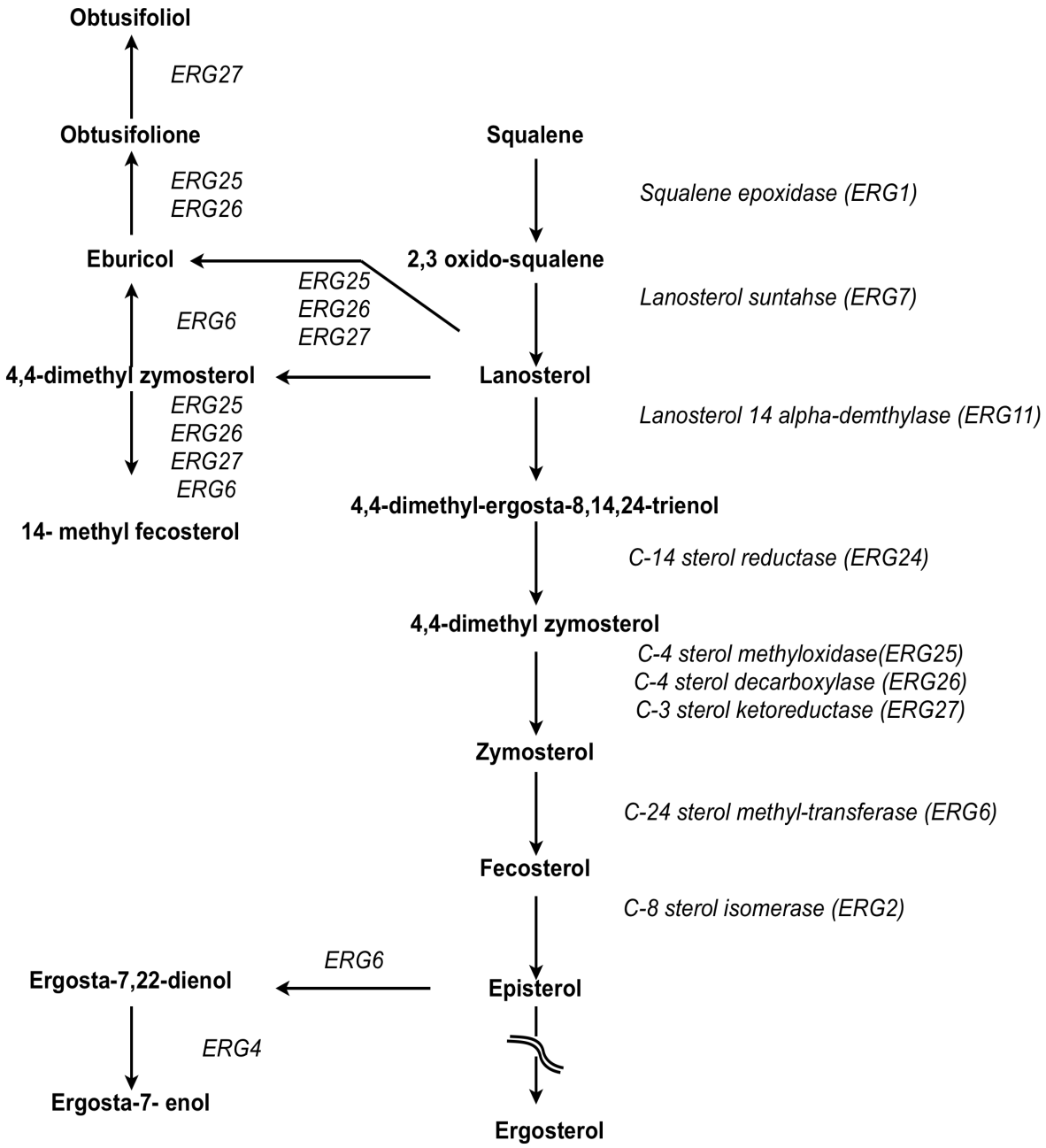

Figure 2.3: Ergosterol biosynthesis pathway

Sterol analysis data in Table 2.1 affirm the finding that *C. albicans* strains susceptible to amphotericin B are known to possess significantly higher ergosterol content than strains that are resistant (Barker 2004). Several other studies have supported this association (Dick 1980, Loeffler 2003, Peyron 2002, Sokol-Anderson 1988, Vanden 1994). These trends of significant reduction in ergosterol content were observed in resistant strains when compared to wild-type strains for *Candida krusei*, *Candida parakrusei* and *Candida tropicalis* which are closely related to *Candida albicans* (Safe 1977).

Table 2.1: Sterol analysis by GC/MS in strains SC5314-AR (strain resistant to amphotericin B and SC5314 (wild-type strain))

	Percentage content	
Sterols present	SC5314	SC5314-AR
Squalene	3.03	0.80
Zymosterol	3.46	0.00
Ergosterol	75.50	trace
Fecosterol	7.10	0.00
Episterol	4.30	0.00
Lanosterol	1.65	14.03
4,4-Dimethylzymosterol	3.10	0.00
14-Methylfecosterol	0.00	1.62
Obtusifoliol	0.00	7.86
24-Methylene Lanosterol (eburicol)	0.00	75.00

Although their study focused on fluconazole resistance; sterol data were generated from biofilms that were treated with fluconazole (Mukherjee 2003). This study showed that ergosterol levels have been modulated in both biofilms as well as planktonic cultures. The biofilm samples were not separated into two fractions. Considering that a small fraction of biofilm (Tightly adherent blastospores) showed high level of resistance to amphotericin B, it will be important to carry out these studies on separate biofilm fractions. These data show it is critical to study the potential relationship between ergosterol levels in biofilm subpopulations and its effects on amphotericin B resistance.

C. albicans biofilms cultured under our experimental conditions harbored a subpopulation of basal blastospores that were exceptionally resistant to amphotericin B. The molecular mechanisms underlying the resistance of the persister cell subpopulation were probed by performing a comparative analysis of transcript abundance for key genes in the ergosterol and β-1, 6-glucan pathways (Khot 2006). These results show that at this level of genetic regulation the basal blastospores subpopulation was distinct both from the bulk of the biofilm and from exponential- and stationary-phase planktonic populations. This suggests possible involvement of these pathways in conferring the resistant phenotype. Studies treating biofilm as two fractions would lead to better understanding of the resistant behavior. It will also lead to deciphering the various pathways involved in formation of resistant species. This will aid in development of newer antifungal drugs.

2.3 Materials and method

2.3.1 C. albicans strain and medium

C. albicans CA-1 is a clinical isolate obtained from the culture collection of Diane Brawner (Microbiology Department, Montana State University). The strain was stored at -80^{o}C. Planktonic cells were cultured in 2% YEPD medium (2% glucose, 1% Bacto yeast extract, and 2% Bacto peptone), and biofilms were cultured in a six fold dilution of this medium.

2.3.2 Planktonic cultures

Cultures were grown aerobically in 250-ml Erlenmeyer flasks containing 100 ml growth medium. The flasks were placed in a shaker incubator at 37^{o}C and 160 rpm for the desired period of growth. *C. albicans* grew as budding yeast under these conditions. Exponential-phase cells were harvested after a 6 hrs growth period and stationary-phase cells at 24 hrs.

2.3.3 Biofilm cultures

Biofilms were grown in a tubular biofilm flow cell (TBF). A schematic of the TBF is shown in Figure. 2.4. Silicone tubing (Cole-Parmer; catalog no. EW-95802-08) with an inner diameter of 4.78 mm, an outer diameter of 6.35 mm, a wall of 0.79 mm, and a length of 60 cm constituted the biofilm-growing region of the TBF. The source of growth medium for the TBF was a 2-liter Erlenmeyer flask. A bubble trap was placed between the Erlenmeyer flask and the biofilm growing region to prevent passage of air bubbles during biofilm growth. For a fixed tube diameter, the shear rate is proportional to the volumetric flow rate of

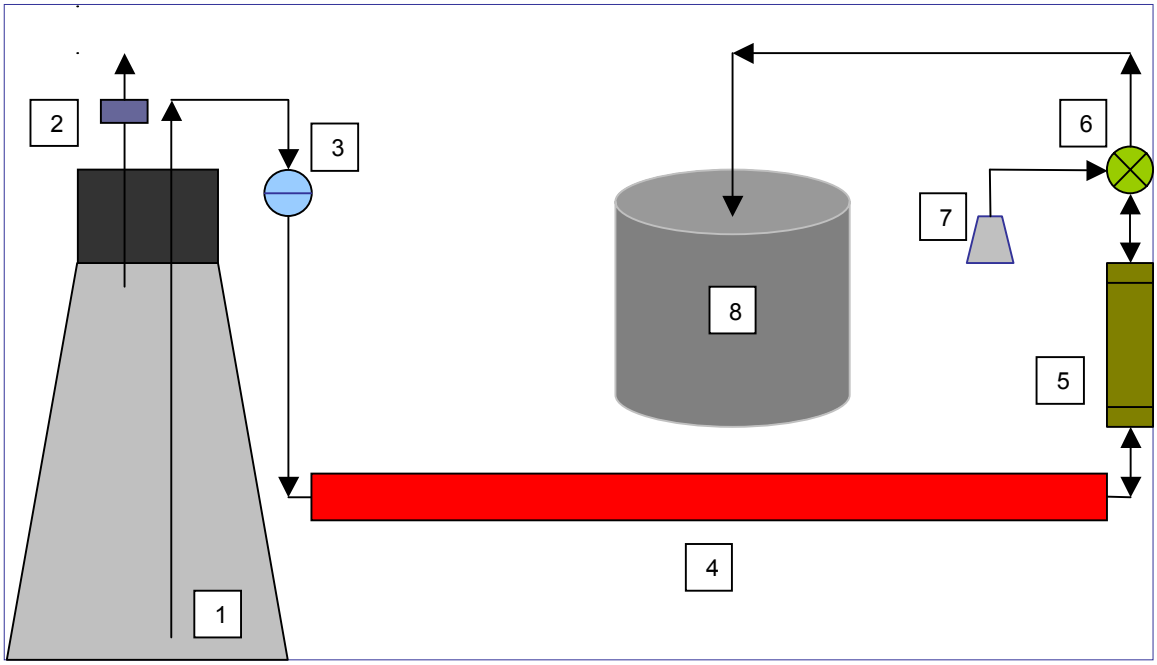

Figure 2.4: Schematic of tubular Biofilm flowcell (TBF) set-up. [1] 2-L Erlenmeyer flask containing sterile growth media. [2] 0.2-micron air filter. [3] Bubble trap. [4] Biofilm growing section of the TF tubing. [5] Peristaltic pump. [6] 3-way junction. [7] 250-ml Erlenmeyer flask with inoculum. [8] Waste container.

growth medium. Using peristaltic pump, flow rates of 1.17 ml/min (shear rate, 109.5 s^{-1}) were maintained. The residence time for the volume of liquid contained in the tubular reactor portion of the flow system (20-cm length of tubing) was 3 mins. This condition ensured that the contribution to the cell population in the TF from cells in the planktonic mode of growth (doubling time, approximately 80 min) was negligible.

After sterilization by autoclaving, the entire setup was placed horizontally on a grilled shelf in an incubator at 37^{o}C. The TF was filled with growth medium before being inoculated with cells. The inoculum was prepared from a 24 hrs

planktonic culture with a concentration of 10^8 cells/ml in 0.1 M phosphate-buffered saline (PBS, pH 7) buffer. It was fed into the TBF from the effluent end by reversing the direction of flow. Flow was then discontinued for 1 hr. After the 1 hr inoculation period, flow was resumed for 24 hrs. At the end of this culture period, the section of tubing in which the biofilm grew was clamped at both ends and removed by cutting the tubing with a sterile blade. The liquid column was drained into a petri dish by moving the tubing to a vertical position and releasing the clamps. The tubing was then rinsed with PBS buffer equivalent to one tube volume. The fraction of biofilm collected in the petri dish by this procedure is referred to as the shear removed biofilm (easily removed fraction). The cells that remained adhering to the walls of the tube are referred to as the basal blastospores or the basal blastospore subpopulation (tightly adherent fraction).

2.3.4 Extraction of sterol

Sterols are extracted from *C. albicans* using alcoholic KOH method. About 10^8 to 10^9 cells from either planktonic culture or biofilms are centrifuged at 300g to remove the supernatant media. To the cell pellet, 3ml of methanol, 2ml of 0.5w/v% pyrogallol in methanol and 2ml of 60 w/v% of KOH in water are added. The reaction is carried out in glass centrifuge tubes rated for 500g speed. The reaction tube is closed tightly and placed in a water bath at 90^oC for 2 hrs. The reaction mixture is allowed to cool to room temperature. The non-sapfonified sterols are extracted using 3x5ml n-heptane, with centrifuging at 500g for 5 min to facilitate phase separation.

2.3.5 Derivatization of sterol

In order to analyze sterols using GC/MS, the molecules need to be derivatized to achieve proper volatilization of sterols. Many compounds do not produce a useable chromatograph (i.e., multiple peaks, or one big blob), or the sample of interest goes undetected. As a result it is necessary to derivatize the compound before GC analysis is done. Silylation is the most commonly used derivatization procedure for sterols.

Silylation produces silyl derivatives, which are more volatile, less stable, and more thermally stable.

- Replaces active hydrogen with a TMS (trimethylsilyl group).
- Silylation occurs through nucleophilic attack (SN2). The better the leaving group, the better the silylation.
- Silylation reagents will react with water and alcohols first. Care must be taken to ensure that both sample and solvents are dry.
- Solvents should be as pure as possible. This will eliminate excessive peaks. Try using as little solvent as possible as this will prevent a large solvent peak.
- BSTFA (Bistrimethylsilyltrifluoroacetamide) is the silylation used for derivatization of sterols.

In order to achieve complete dryness before derivatization, samples were dried using a rotary evaporator. The sterol solution with n-heptane was run in rotary evaporator until the solution is almost dry. It was further dried under vacuum for 5 mins. Two ml. of benzene are added and dried using rotary

evaporator. Once dried, sterol samples are dissolved in 250 µl of 100% toluene. Under nitrogen environment, 250 µl of BSTFA were added to this mixture. The reaction was carried out at 80 ^{o}C for 45 mins. The derivatized samples were analyzed using GC/MS. The column used for separation was DB-5ms. Sample was introduced at 300^{o}C using splitless injection mode. Temperature of the oven was increased using two ramps: from 180 ^{o}C to 300^{o}C at 25 ^{o}C /min and from 300 ^{o}C to 325 ^{o}C at 5 ^{o}C/min. It was held at 325 ^{o}C for 15 mins (total run time of 24.8 mins).

2.3.6 Spike study

In order to study the extraction efficiency of the sterol extraction techniques, spike study was done. The spike study was done with stationary phase cells. Ten ml of cell culture solution were taken and centrifuged. The supernatant was discarded and wet weight of cells was noted. To this 10µg of ergosterol was added. The extraction of sterols was carried using above-mentioned technique. Once derivatized, the samples were analyzed using GC/MS. The study was done in triplicate. Known quantity of ergosterol was taken and dried using the above mentioned technique and derivatized. Calibration curve was generated using area under the curve versus ergosterol concentration.

2.4 Results

The first task accomplished was to check for the efficiency of the sterol extraction protocol. Calibration chart was generated using known quantities of ergosterol. The calibration chart used for quantification is shown in Figure 2.5.

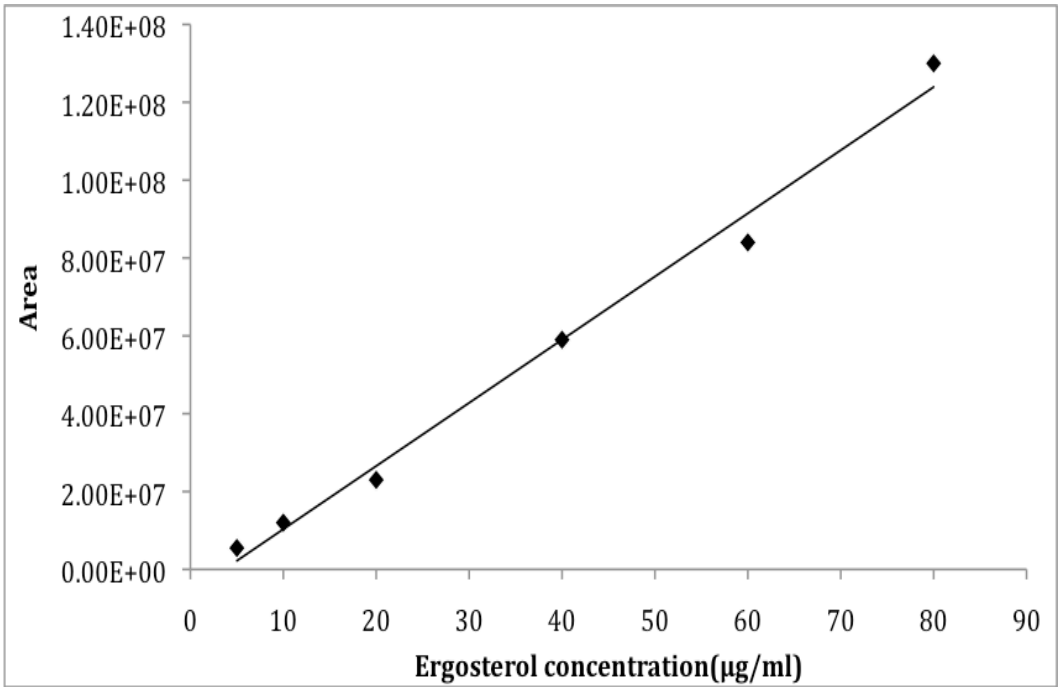

Figure 2.5: Calibration curve for ergosterol

The extraction efficiency was suffering due to the derivatization step of the process. I was able to achieve only about 65-78% extraction efficiency due to the moisture content in the samples or in the reagents used. Using rotary evaporator and freshly distilled 100% solvents, this problem was tackled.

For spike study, 10µg of ergosterol was added to each sample. The area under the curve was used to quantify the spiked levels of ergosterol. These runs were done in triplicate. Based on data from Table 2.2, the extraction efficiency was relatively good. The ergosterol difference in the last column was calculated using the difference in the spiked area and the average area from the stationary phase cells. The difference in area was converted to quantity of ergosterol using the calibration equation.

The sterol characterization of the planktonic phases gave almost same results. The total ion chromatogram showed just one peak corresponding to ergosterol. Figure 2.6 shows the chromatogram for the planktonic phases. The total ion chromatogram for sheared removed fraction showed a similar looking chromatogram.

There was change in the chromatogram for adherent cell population. Other than the ergosterol peak, there were four additional peaks after the ergosterol peak (Figure 2.7). The peaks in Figure 2.7 correspond to 1- ergosterol 2- lanosterol, 3 - eburicol and 4 - zymesterol. There was formation of intermediates of the ergosterol pathway. On quantifying the ergosterol levels in the different cell populations, there was an interesting trend that appeared. The samples were run in triplicate. For each sample, the GC/MS analysis was carried out twice.

For all subpopulation the primary sterol was still ergosterol. The quantity of ergosterol dropped in the case of biofilm populations. The ergosterol levels were lowest in the case persister cells as shown in Table 2.3 and Figure 2.8. These changes in the sterol correlate well with gene expression experiments done in our lab. The changes in ergosterol concentration highlight the fact that the persister subpopulations are changing their membrane dynamics and fluidity, thus restricting the access of antifungal drugs to their targets. This is the first study done on characterization membrane sterols of persisters cells in the biofilm.

Table 2.2: Extraction efficiency study

	Stationary (area)	Spiked sample (area)	Difference in area	Ergosterol difference (µg)
Run 1	34615778	46115778	10881447	10.88
Run2	35475310	44677890	9134283	9.13
Run3	35611904	46395281	10783377	10.78

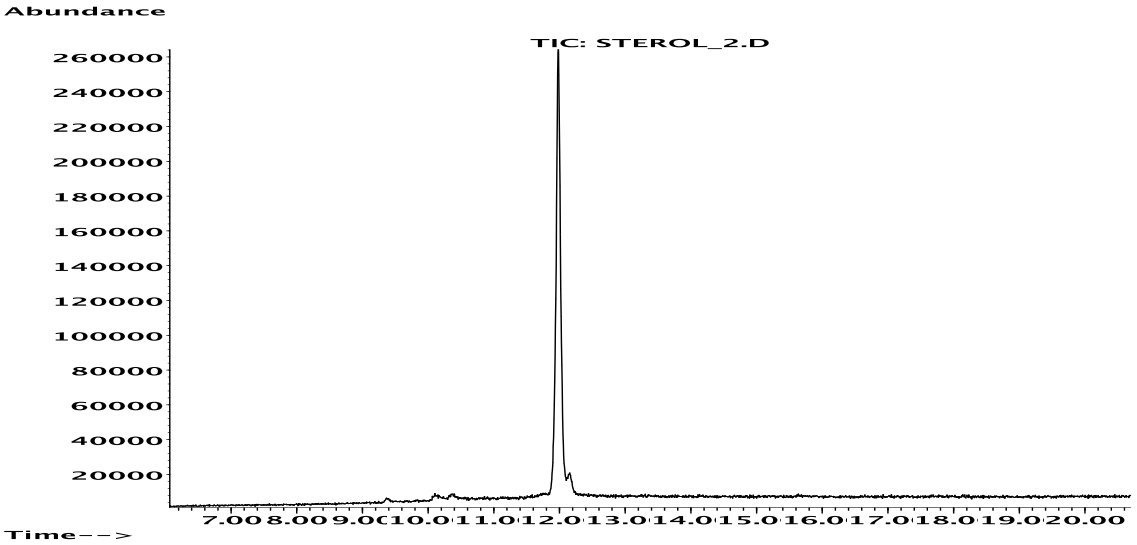

Figure 2.6: Chromatogram for planktonic culture.

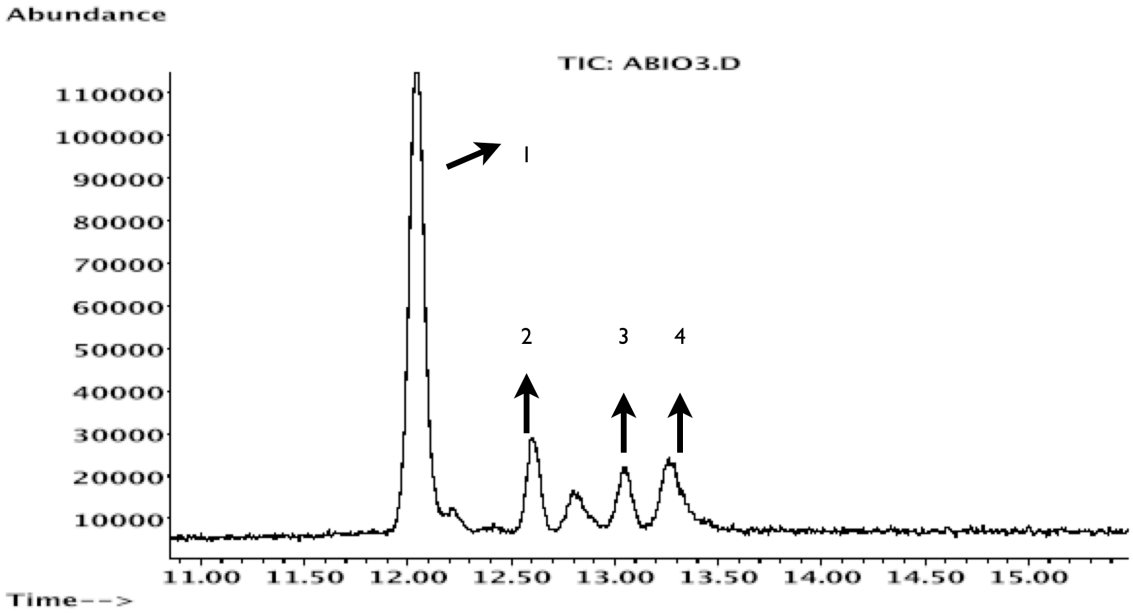

Figure 2.7: Chromatogram of persister cells from biofilm. 1- ergosterol 2- lanosterol, 3 - eburicol and 4 - zymesterol

Table 2.3: Ergosterol concentration in µg/mg of wet cell mass.

	Exponential	Stationary	Shear removed	Persister
Run1	15.52	15.31	13.58	10.96
Run2	16.17	14.23	12.29	9.60
Run3	14.67	14.45	13.66	10.50
Mean	15.46	14.66	13.18	10.36
Standard Deviation	0.75	0.57	0.77	0.69

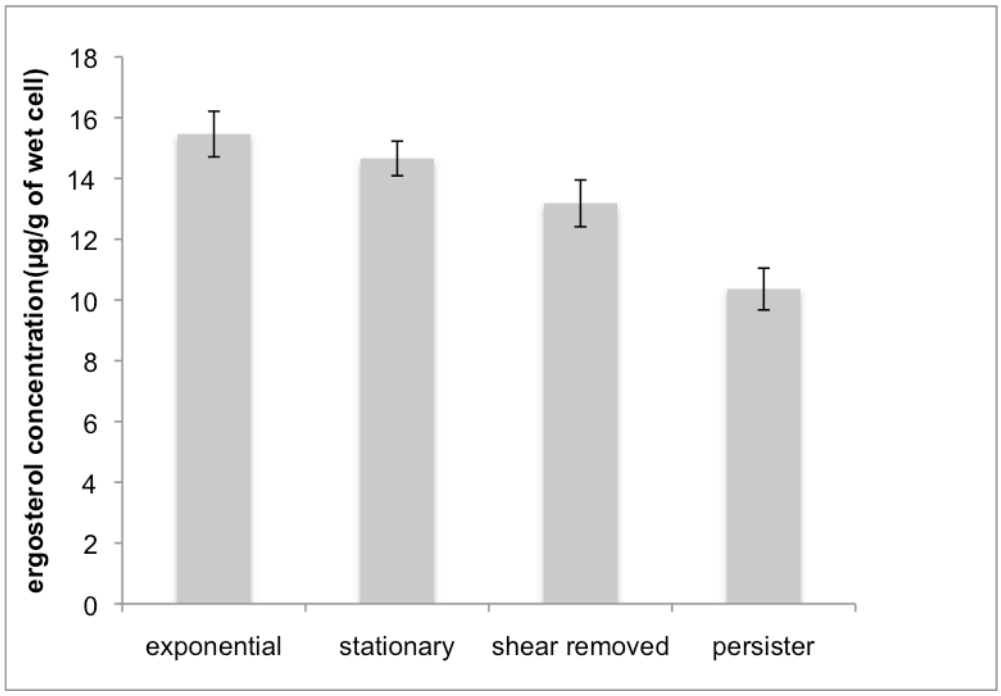

Figure 2.8: Bar chart of average ergosterol levels in different cell populations.

2.5 Discussion

Our lab demonstarted *C. albicans* biofilms cultured in our in vitro system harbored a subpopulation of persister cells that were substantially more resistant to AmB than both planktonic cells and the bulk of the biofilm that had been displaced from the substratum. Although several researchers have published studies of looking at different pathways affected in *C. albicans* biofilms, all these studies analyzed the whole biofilm. Such interpretation easily masks the information from persister cells, as they are only less than 1% of the total biofilm. The discovery of this resistant blastospore subpopulation represented a significant advance in the understanding of drug resistance in *C. albicans* biofilms.

The key result from the sterol study was reduction of ergosterol in persister cell subpopulation. Also there was accumulation of intermediates, instead of pushing the pathway all the way to production of ergosterol. These results corroborate the findings from Khot et al. The transcript analysis done by Khot et al. on these persister cell subpopulations indicated changes in the regulation of genes involved with both the ergosterol and *β*-1,6-glucan synthetic pathways. The results indicated that the persister cell subpopulation was a distinct population at this level of molecular characterization. Thus, the ergosterol level reduction and formation of intermediates could possibly be the reason for this highly resistant behavior exhibited by these persister cells.

The reduction in ergosterol levels in both biofilm fractions as compared with the exponential phase planktonic cells. The ergosterol reduction was the highest in case of persister cell subpopulation (Table 2.4). On carrying out the t-test at 99% confidence interval, the difference in the ergosterol levels between persister cell layer and exponential phase was statistically significant. Also, the ergosterol levels of persister cells were found to be statistically different when compared with shear removed and stationary phase cells. This result further validates our reasoning for treating the persister cell subpopulation differently from rest of the biofilm. By doing so, we can better understand the molecular mechanisms involved in development of highly resistant biofilm species. It is noteworthy that many features of fungal biofilms are shared by the better-studied bacterial biofilms. In both cases, biofilms are often formed by merging of initial founder microcolonies, are protected from the immune system by exopolymers,

Table 2.4: t-test on ergosterol levels with exponential phase as the reference state.

Sample 1	Sample 2	P value	Statistically Significant
Basal Blastospore	Exponential	0.0091	Yes
Shear Removed	Exponential	0.0215	Yes
Stationary	Exponential	0.2209	No

are composed of slowly growing cells, and exhibit multidrug tolerance (Kumamoto 2005, Lewis 2006).

Bacterial biofilms produce dormant persister cells that are largely responsible for multidrug tolerance (Brooun 2000, Keren 2004, Spoering 2001). Persisters are able to survive despite the presence of antibiotics at concentrations well above the MIC and are phenotypic variants of the wild type, rather than mutants (Keren 2004). Persisters are formed by all bacterial species studied and are present at 0.1 to 1% in the biofilms of *Pseudomonas aeruginosa*, *Escherichia coli*, and *Staphylococcus aureus* (Keren 2004). The discovery of persisters in bacterial biofilms helped explain the puzzling resistance of biofilms. A similar paradox exists in the field of fungal biofilms: planktonic cells may be highly susceptible to a range of antimicrobials, while mature biofilms appear to be resistant to killing by “everything” compared to planktonic populations (Keren

2004). Research done by our group (Khot et al.) has already identified persister cell population using alamarBlue metabolic assay. The persister cells were resistant to around 10 times the amphotericin B dose required to eliminate the other fractions as shown in dose response curve (Figure 2.9). Possibly triggers for formation of this highly resistant persister cell subpopulation could be due to nutrient depletion or attachment to surface.

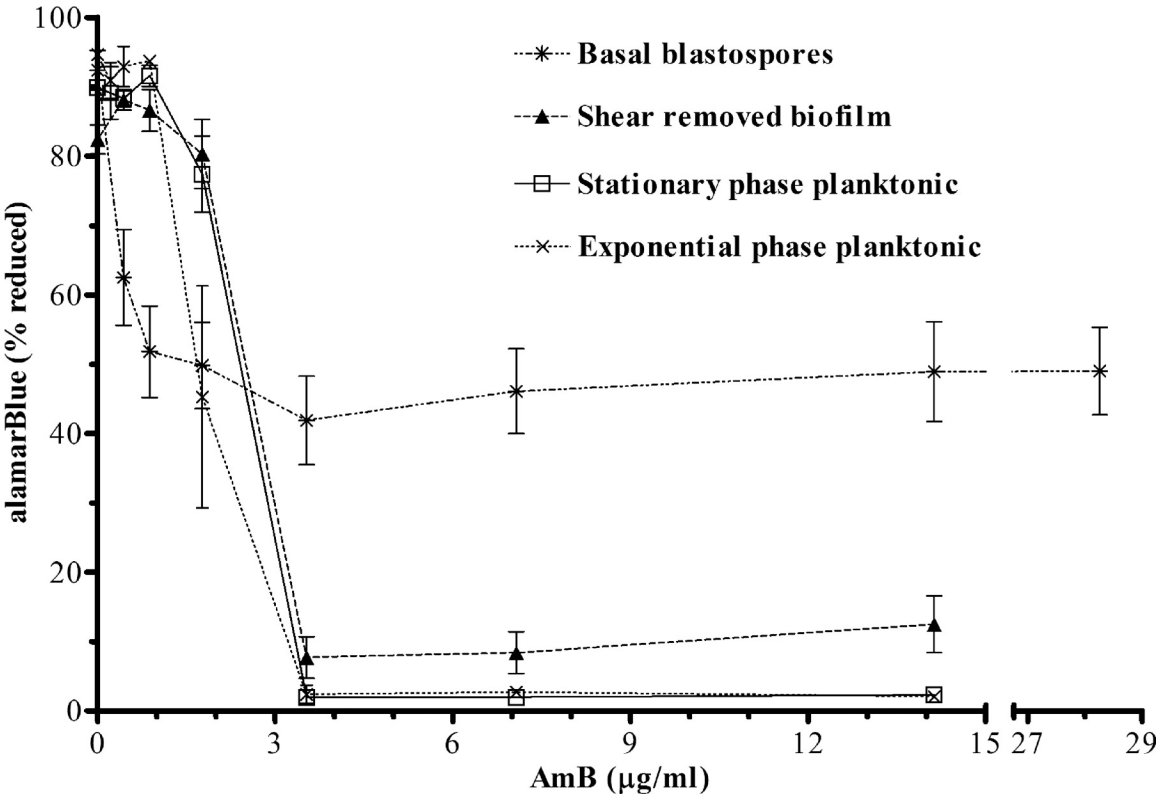

Figure 2.9: Amphotericin B dose-response curves for *C. albicans* biofilm and planktonic populations assessed by an alamarBlue metabolic assay.

In late stationary phase of planktonic growth, cells showed some resistance, but not to the extent of persister cells. The ergosterol level of stationary phase was not statistically significant from the planktonic phase. This possibly rules out nutrient depletion as the only cause for this phenotypic change to highly resistant behavior among persister cells. The other possible trigger for resistant species formation needs to be analyzed.

Transcript analysis done by Khot et al. on the persister cell subpopulation showed that abundance of the *ERG1* gene was significantly reduced in the resistant persister cell subpopulation (Table 2.5). The *ERG1* gene product catalyzes epoxidation of squalene to 2,3-oxidosqualene, a step that occurs early in the ergosterol synthetic pathway, just after commitment to sterol synthesis, and thus could serve as a point of down-regulation of ergosterol synthesis. In a *C. albicans* strain in which both copies of *ERG1* were disrupted, ergosterol was absent from the membrane and was replaced primarily by squalene (Groll 2004). This correlates well with the finding of this work that ergosterol levels have reduced in this basal blastospore biofilm subpopulation. Reduction of the ergosterol content of the plasma membrane is the most common mechanism for *C. albicans* to acquire resistance to amphotericin B (Ghannoum 1999). The ergosterol level reduction and increased resistance exhibited by persister cells can be positively linked. Based on the genes shown in Table 2.5, the other gene that changed in expression level was ERG25. Upregulation of ERG2 gene by four folds in the basal blastospores was reported. This is a possible explanation for the intermediates that showed up in the chromatogram of basal blastospores.

Table 2.5: Relative transcript abundance for selected genes of the ergosterol pathway in *C. albicans*. The gene expression fold change values were estimated relative to the exponential phase planktonic population (calibrator state) (Khot 2006)

Gene	Fold changes in Basal Blastospores
ACT1	0.9 ± 0.04
EFB1	1.11 ± 0.11
ERG1	-11.93 ± 4.69
ERG3	0.91 ± 0.22
ERG5	1.48 ± 0.27
ERG6	1.32 ± 0.55
ERG11	0.88 ± 0.27
ERG25	3.7 ± 0.73

A Study carried out by Barker et al. on wild type strain and resistant strain has shown over-expression of ERG25. They showed that pathway had completely shifted towards production of lanosterol and eburicol at the expense of conversion to 4,4-dimethyl-ergostera-8,14,24-trienol. By altering the pathway at this particular point, the cell would lose susceptibility to the effects of both fluconazole and amphotericin B(Barker 2004). This is a possible explanation to the up-regulation of the *ERG25* gene, in order to shuttle nonergosterol intermediates into the membrane. This behavior is seen among the persister cells subpopulation, as there is formation of intermediates like eburicol and lanosterol.

Though the resistance in persister cells and reduction ergosterol levels can be positively correlated, it does not explain the entire resistance. The

resistant strain study done by Barker et al. showed that ergosterol pathway had stalled in the early stages and shifted totally towards production of eburicol. Hence reduction in ergosterol level tells us one of the reasons why these cells are getting resistant; it does not explain the entire picture. The resistance to amphotericin B could possibly be due to a synergistic effect of changes in gene expression in more than one pathway. One such pathway that came into immediate considerations is the *ß* -glucan pathway. Transcripts encoded by two genes involved in *ß*-1, 6-glucan synthesis (*SKN1* and *KRE1*) were clearly more abundant in the persister cell subpopulation than in either the shear-removed biofilm or planktonic populations (Khot 2006)

One possibility is that these highly resistant persister cells regulated their genes in direct response to their relatively intimate association with the surface. This in turn leads changes in enzymes and final products of genetic expression, like sterols. A similar mechanism was proposed to explain AmB resistance of *C. albicans* biofilms grown on PVC catheters (Baillie 2000). Bacterial biofilms have been shown to alter genetic programs in response to surface association (Davies 1995, Kilic 2004, Moorthy 2004, Prigent-Combaret 1999), and even early-stage (monolayer) biofilms can become more resistant than their planktonic counterparts (Cochran 2000, Das 1998). It is not unreasonable to expect that the resistant persister cells used some of the same mechanisms previously identified in *C. albicans* for sensing surface contact (Watts 1998).

In conclusion, the persister cell subpopulation show changes in their molecular framework that is different from the rest of the biofilm. This is validated

by the reduction in the actual ergosterol levels and formation of intermediates observed in this study. The existing literature on *C.albicans* biofilm studies the biofilm as a whole, hence missing out on the changes taking place in less than 1% of the biofilm. This less than 1% of the biofilm holds the most critical information for really understanding the mechanisms behind development of resistant phenotypes like persister cells.

CHAPTER 3

MAP KINASE PATHWAY

3.1 Abstract

The cell wall is physically and physiologically different from the plasma membrane. It consists of glucans, chitin and manno-proteins and is thought to act as a physical barrier to the extracellular environment, i.e., providing increased strength and/or decreased permeability to toxins. Khot et al. showed that the genes of β-1-6-glucan pathway in the persister cells were up-regulated. For example, SKN1 and KRE1 were increased approximately 30 fold. This might result in the cell wall becoming thicker and/or increasing the number of crosslinks in the glucans.

Direct analysis of the cell wall is scientifically challenging; however, one can analyze signaling pathways involved in cell wall synthesis. One such pathway involves the phosphorylation of mitogen-activated protein-kinase (MAPK) (Kumamoto 2005a, Navarro-García 2005), i.e., Mkc1 and Cek1. Therefore, I hypothesized that Cek1 and Mkc1 are activated in persister cells. Results show that Mkc1 and Cek1 MAPK were activated in only the persister cells. While these results are consistent with my hypothesis further studies are necessary to determine if this pathway is critical in resistant behavior and/or if the cell wall plays a role in resistance. The latter could be determined by measuring

AmB resistance in presence and absence of zymolyase, an enzyme that degrades the cell wall.

3.2 Introduction

3.2.1 Cell wall

The cell wall was considered an almost inert structure that supplies rigidity and protection to the protoplast. Approximately 70 to 90% of the cell wall of *C. albicans* is carbohydrate. Three basic constituents represent the major polysaccharides of the cell wall: (i) branched polymers of glucose containing β-1,3 and β-1,6 linkages (β-glucans); (ii) unbranched polymers of *N*-acetyl-D-glucosamine (GlcNAc) containing β-1,4 bonds (chitin); and (iii) polymers of mannose (mannan) covalently associated with proteins (glyco[manno]proteins). In addition, cell walls contain proteins (6 to 25%) and minor amounts of lipid (1 to 7%) (Calderone 1991, Cassone 1989)

In current literature there is consensus on the role played by the cell wall as being essential to almost every aspect of the biology and pathogenicity of *C. albicans* (Calderone 1991, Calderone 1993, Cassone 1989, Chaffin 1998, Fukazawa 1997). The cell wall acts as a permeability barrier and is the structure that maintains the characteristic shape of the fungus (Calderone 1993). Also, as the most external part of the cell, the wall mediates the initial physical interaction between the microorganism and the environment, including the host (Cassone 1989, Chaffin 1998). For these reasons, the cell wall of *C. albicans* is the focus of study by numerous research groups. Their objectives are the elucidation of both

basic biological processes and functional mechanisms regulating the synthesis, organization, and environmental interactions of this complex macromolecular structure. Proteins have been implicated in most of the cell wall functions.

3.2.2 Persister cells

C. albicans biofilms cultured under our experimental conditions harbored a subpopulation of persister cells that were exceptionally resistant to amphotericin B. The sterol study showed that these cells were different from the rest of the biofilms with respect to their sterol composition. The sterol composition could not explain the level of resistance shown by these cells. Hence there is more than one factor contributing to the resistant behavior of these cells. A study done by Khot et al. on these persister cells using comparative analysis of transcript abundance for key genes in the β-1, 6-glucan pathways had shown that genetic regulation of this subpopulation was distinct from bulk biofilm and from exponential- and stationary-phase planktonic populations (Khot 2006). Khot et al. showed that the genes of β-1-6-glucan pathway in the resistant persister cells showed a high degree of differential up regulation of the *SKN1* (+30.7fold change) and *KRE1* (+29.86 fold change) genes. This result is suggestive of a bulking of the cell wall or higher level of crosslinking of the glucans in the cell wall. Both of these will increase the resistance of the *C. albicans*, as they will reduce the permeability of the antifungal drugs. Direct analysis of the cell wall is a challenge, as there are no good tools to study the carbohydrate moieties and crosslinking of carbohydrates involved in the cell wall matrix. However, one can analyze signaling pathways involved in cell wall synthesis.

3.2.3 General role of MAP kinases

MAP kinases signal transduction pathways are widespread mechanisms present in eukaryotic cells to couple environmental responses to transcriptional regulation. They comprise a conserved module of three kinases: the MAP kinase (MAPK), the MAP kinase kinase (MAPKK) and the MAP kinase kinase kinase (MAPKKK). When upstream signals are fed into the MAPKKK (by a variety of signaling mechanisms), the MAPKKK becomes phoshorylated and in turn phosphorylates the MAPKK that, in turn, does so to the MAPK. Once phosphorylated, MAP kinase is translocated into the nucleus. It in turn phosphorylates transcription factor. This leads to activation of the target gene and leads to an adaptive response to the original stimuli. The cascading signal of MAP kinase pathway is shown in Figure 3.1.

For mammalian cells, contact-dependent regulatory controls are crucially important for controlling cellular proliferation and preventing diseases such as cancer. *C. albicans*, an opportunistic fungal pathogen that normally resides within a mammalian host, also exhibits contact-dependent cellular behaviors such as invasive hyphal growth and biofilm development (Brown 1999, Ostrosky-Zeichner 2003). This signaling pathway could lead to finding the potential trigger for the transition of *C. albicans* from biofilm cells to persister cells. Understanding this pathway will be critical step in finding a real solution to tackle the resistant biofilm problem.

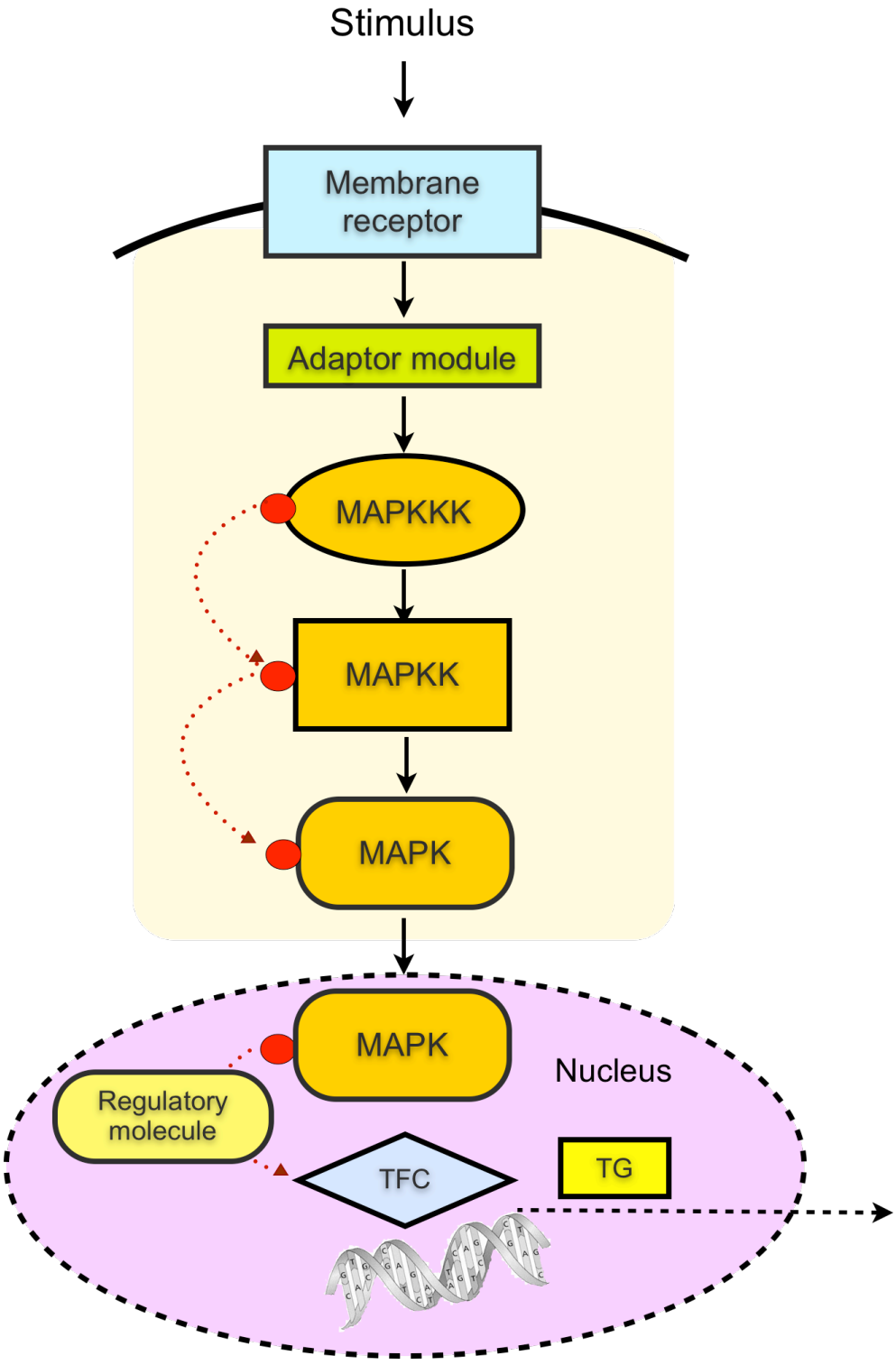

Figure 3.1: Typical signal cascading in MAP kinase pathway.

Candida albicans have shown adaptability to sustain at various environments. One of the strategies they have employed to survive is to continuously monitor the changes taking place in the surrounding environment. In eukaryotes, these external stimuli are usually processed through MAP kinase pathways, in which proteins are sequentially phosphorylated to respond appropriately to external changes (Navarro-Garcia 2005). There are several differences between higher mammalian cells and lower eukaryotic cells regarding the complexity and functionality of the elements involved. However, the core structure of the MAP kinase cascade is highly conserved.

These pathways have been extensively characterized in the non-pathogenic model yeast species *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Cid 1995, Fox 1999, Mosch 1999). However, despite the evidence that functionality of the signaling through these pathways can be essential for virulence, the knowledge of their relevance in pathogenic fungi is far from complete.

3.2.4 MAP Kinases in *C. albicans*

Genetic studies of pathogenic fungi have revealed that MAP kinases are also important virulence factors because mutants defective in them display a reduced virulence in certain animal models of infection (Kumamoto 2005a, Navarro-Garcia 1995 & 2005). The homologous relation of these pathways with those of other fungal models has helped in their identification of these kinases in *C. albicans* (Bardwell 1996, Csank 1998, Martín 2000, Whiteway 1992). Studies carried out to pinpoint the precise role of these kinases in *C. albicans* reveal

particular features in this dimorphic fungus that could reflect its adaptation to specific environments (Ibata-Ombetta 2001, Kumamoto 2005a, Lorenz 2004, Navarro-Garcia 2005, Phillips 2003). The central core gene of this pathway has been identified for most medically important fungi. Figure 3.2 lists the important genes in the MAP kinase pathways for *C. albicans* in pink (Roman 2007). The two MAP kinases, Mkc1 and Cek1, are of interest from the biofilm standpoint. These two kinases seem to play the major role in virulence, cell wall repair and cell wall biogenesis in *C. albicans*.

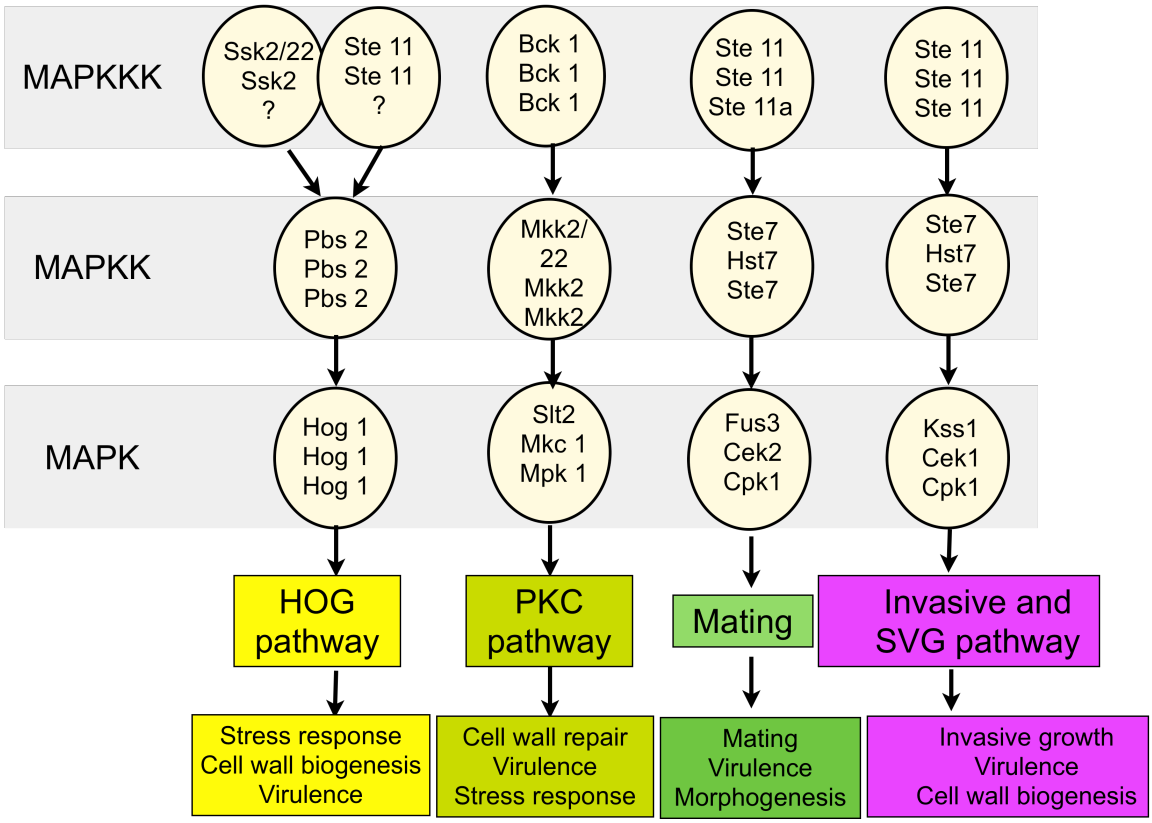

Figure 3.2: Map kinase pathways in important fungi. The genes in pink are the ones identified for *C. albicans*.

These pathways are critical in virulence exhibited by *C. albicans* cells. *C. albicans* with a deletion of the Cek1 gene are defective in shifting from a unicellular budding colonial growth mode to an agar-invasive hyphal growth mode when nutrients become limiting on solid medium with mannitol as a carbon source or on glucose when nitrogen is severely limited (Csank 1998). This study showed that Cek1p MAPK is part of the signal transducing machinery that coordinates hyphae formation in *C. albicans*. Their studies suggest that Cst20p, Hst7p, Cek1p, and Cph1p act sequentially in the order of a canonical MAPK cascade to induce the hyphae transition (Csank 1998). Cek2p together with Cek1p is shown to play a role in the mating process, only seen in rare strains *C. albicans*.

The key aspects of these kinases of interest are their role in cell wall repair and biogenesis. In *S. cerevisiae*, both pathways collaborate in the maintenance of cell wall integrity (Lee 1999). In *C. albicans,* Mkc1 and Cek1 MAPK mediate the cell integrity pathway (Kumamoto 2005a, Navarro-Garcia 1995 & 2005). It has also been shown that Mkc1 mutants are more sensitive to cell-wall-degrading enzymes and antifungals (Kumamoto 2005a, Navarro-Garcia 1995 & 2005). Navarro-Garcia et al. have shown that Mkc1p is activated by several kinds of stresses. On treatment with zymolyase, yeast cell wall degrading enzyme, Cek1 and Mkc1 were both activated (Navarro-Garcia 2005).

The unexpected phosphorylation of Mkc1 and Cek1 by various stimuli reinforces the importance of studying the activation of the *C. albicans* pathways in order to understand their function. It is very important to document the role of

these kinases from an ecological point of view. This helps in understanding the overall role of these kinases. *C. albicans* seems to have developed its own (and different) way to use the same set of tools that it shares with other yeasts to respond and adapt to the challenges in the human body. It will be critical to document these phosphorylation changes in these pathways in a biofilm model. It will important to understand whether this signal transduction pathway was phosphorylated when subjected to surfaces.

3.3 Materials and methods

3.3.1 *C. albicans* strain and medium

C. albicans CA-1 is a clinical isolate obtained from the culture collection of Diane Brawner (Microbiology Department, Montana State University). The strain was stored at -80^{o}C. Planktonic cells were cultured in 2% YEP medium (2% glucose, 1% Bacto yeast extract, and 2% Bacto peptone) and biofilm were cultured in a 6 fold dilution of this medium.

3.3.2 Planktonic cultures

Cultures were grown aerobically in 250-ml Erlenmeyer flasks containing 100 ml YEP growth medium. The flasks were placed in a shaker incubator at 37^{o}C and 160 rpm for the desired period of growth. *C. albicans* grew as budding yeast under these conditions. Exponential-phase cells were harvested after a 6 hrs growth period and stationary-phase cells at 24 hrs.

3.3.3 Biofilm cultures

Biofilms were grown in a tubular biofilm flow cell (TBF). Silicone tubing (Cole-Parmer; catalog no. EW-95802-08) with an inner diameter of 4.78 mm, an outer diameter of 6.35 mm, a wall of 0.79 mm, and a length of 60 cm constituted the biofilm-growing region of the TBF. The source of growth medium for the TBF was a 2-liter Erlenmeyer flask. A bubble trap was placed between the Erlenmeyer flask and the TF to prevent passage of air bubbles during biofilm growth. Peristaltic pump coupled at the effluent end of the TF helped maintain flow rates of 1.17 ml/min (shear rate, 109.5 s^{-1}). The residence time for the volume of liquid contained in the tubular reactor portion of the flow system (20-cm length of tubing) was 3 min. This condition ensured that the contribution to the cell population in the TF from cells in the planktonic mode of growth (doubling time, approximately 80 min) was negligible. After sterilization by autoclaving, the entire setup was placed horizontally on a grilled shelf in an incubator at 37^{o}C. The TF was filled with growth medium before being inoculated with cells. The inoculum was prepared from a 24 hrs planktonic culture with a concentration of 10^{8} cells/ml in 0.1 M phosphate-buffered saline (PBS, pH 7) buffer.

It was fed into the TF from the effluent end by reversing the direction of flow. Flow was then discontinued for 1 hr. After the 1 hr inoculation period, flow was resumed for 24 hrs. At the end of this culture period, the section of tubing in which the biofilm grew was clamped at both ends and removed by cutting the tubing with a sterile blade. The liquid column was drained into a petri dish by moving the tubing to a vertical position and releasing the clamps. The tubing was

then rinsed with PBS buffer equivalent to one tube volume. The fraction of biofilm collected in the petri dish by this procedure is referred to as the shear removed biofilm. The cells that remained adhering to the walls of the tube are referred to as the basal blastospores or the basal blastospore subpopulation.

3.3.4 Protein extraction

Total proteins from cells were extracted using a protein extraction protocol outlined by Diez Orejas et al. (1997), with modifications. Cells were pelleted by centrifugation and washed twice in ice-cold water. A protease inhibitor Tablet (Complete Mini, Roche) was dissolved in 1.5 ml Milli Q water and aliquots of 0.15 ml were stored at -20 ^{o}C. Cell pellet was suspended in 0.850 ml lysis buffer (50 mM Tris-HCl, pH 7.5, 1mM EDTA, 150 mM NaCl, 1mM DTT) with 0.15 ml of protease inhibitor solution. This cell suspension was added to a 1ml equivalent volume of glass beads (0.5 mm, Biospec) and beaten for 25s on a Mini Bead beater (Biospec) followed by cooling on ice for 1 min. This cycle of beating and cooling was repeated 25 times. The cell extract containing proteins was separated from the debris and the glass beads by centrifuging at 13000 rpm for 15 min at 4 ^{o}C and pipetting the supernatant. The supernatant was then centrifuged again to purify the protein. The protein solution was concentrated to about 0.2ml solution using Millipore centrifuge filters for sizes above 10kda. For concentration, solution was centrifuged at 5000rpm for 25 mins at 4 ^{o}C. The concentrated protein sample was stored in aliquots at -80 ^{o}C for further use.

3.3.5 Protein quantification

Protein quantification was performed using the BCA assay (Quantipro BCA assay kit, Sigma). The bicinchoninic acid (BCA) assay depends on the formation of a protein-*Cu*2+ complex followed by the reduction of *Cu*2+ to *Cu*+ by peptide bonds and amino acid residues such as tryptophan, tyrosine, and cysteine. BCA forms a purple complex with *Cu*+ in an alkaline environment. The reduction is dependent on the amount of protein present and can be monitored by measuring the absorbance at 562 nm. A standard curve was made using standard protein (BSA protein standard, Sigma) concentrations from 0 - 30 µg /ml, the linear range of the BCA assay and protein concentrations of unknown samples were found by diluting to within this range using 0.1N NaOH.

3.3.6 Running gels

Protein extracts were diluted to a concentration of 300 µg/ ml to make solution of 10µl. To this 10µl of laemmli buffer was added and heated at 95 ^{o}C for 5 mins. The electrophoresis was carried out using Bio-Rad Mini Protean gel electrophoresis system. The 20 µl samples were loaded onto precast minigels (Bio-Rad) onto each lane. Samples were run at 200 V for 1hr.

3.3.7 Western blotting

PDVF Membrane of required size was cut and activated for 10s by placing in methanol. Activated membrane was shaken in transfer buffer solution (Glycine 57.6 g, Tris Base 12.0 g, SDS 3.0 g, Methanol 800 ml and H2O to 4 liters) for 10 mins. The sponge for transfer was soaked in transfer buffer and placed on the

semidry transfer plate. Placing SDS-gel on top of membrane and sponges on top and bottom made a sandwich. Any bubbles that were formed in this sandwich were removed by rolling a pin along the sandwich. The semidry apparatus was closed and run at 14V for 20 mins. After disassembling the setup, the membrane was taken out and washed in TBST (Biorad) for 5 mins. The membrane was then blocked using 5% nonfat dry milk in TBST for 15 mins. The plots were then incubated with primary antibody at appropriate dilutions with TBST+5% nonfat dry milk overnight at 4^{o}C and continuously shaken. The membrane was then washed 3 times for 15 mins each in TBST. The membrane was then incubated with secondary antibody with appropriate dilutions in TBST+5% nonfat dry milk for 4 hrs at room temperature. The membrane was then washed 3 times for 15 mins each in TBST. The blot was placed with the protein side down on the ECL solution (Bio-Rad). It was incubated for 1 min and excess reagent was drained. The blot was then transferred to a plastic report cover. Film was exposed immediately for 40 mins. The primary antibody used for the studies were Rabbit p42-44 MAP kinase and Rabbit phospho p42-44 MAP kinase. Both of these were ordered through Cell Signaling Inc. Anti-Rabbit IgG, HRP-linked antibody was the secondary antibody used. The TEYVATRWYRAPE motif, which is characteristic of the VIII subdomain of the p42-44 MAP kinase family of protein kinases. The T and Y residues are phosphorylated in the activated form of the kinase. This sequence is present in both proteins of interest Mkc1p - 59 kDa and Cek1p - 48·7 kDa. Hence, the same primary antibody can be used to probe them. The

two proteins can be indentified based on their mass. They will form two distinct bands on the gel and can be matched with the size ladders.

3.4 Results

Protein quantification was performed using BCA assay. A standard curve was made using standard protein (BSA protein standard, Sigma) concentrations from 0 - 30 µg /ml. The standard/ calibration curve obtained in the assay is shown in Figure 3.3 along with a linear fit between 0 - 30 µg /ml.

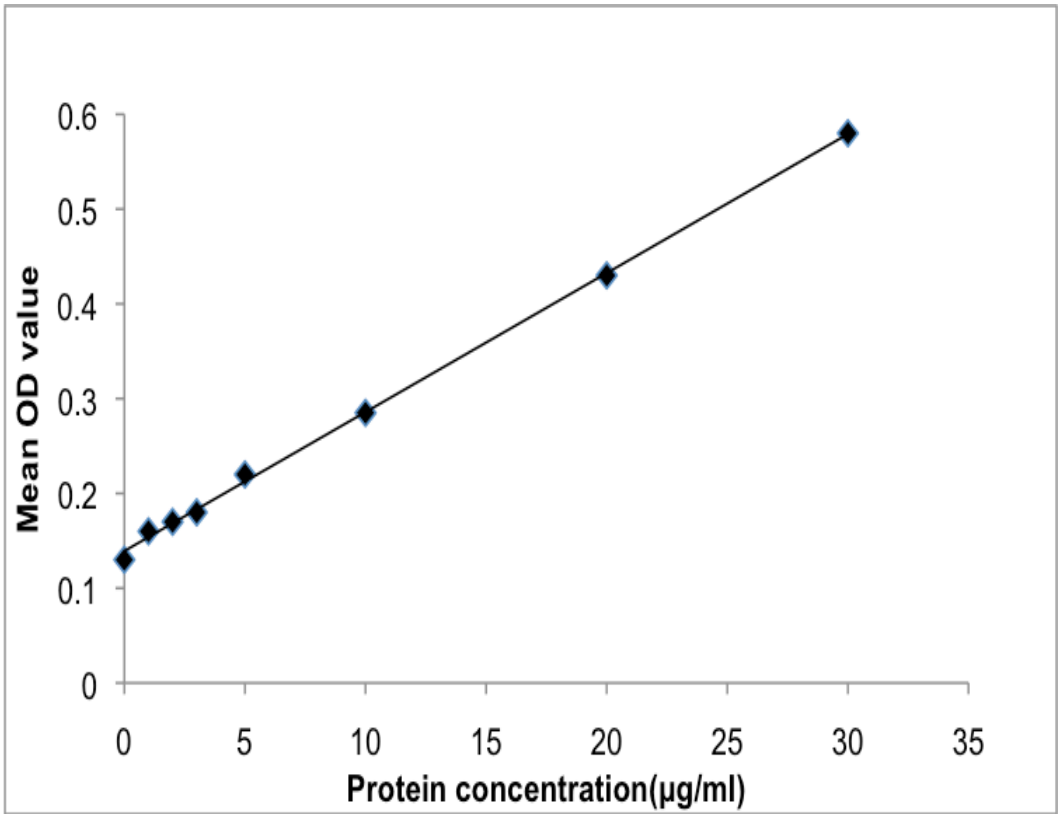

Figure 3.3: Protein calibration curve using BCA assay

For accurate quantification of protein, the unknown samples were diluted serially to have concentration within the linear range (0 – 30 µg) of this assay. The SDS-PAGE gel was used to test for the extraction efficiency and also protein stability of cell-lysis technique. The gel shown in Figure 3.4 shows that we can see bands across the gels and also there was equal loading of these gels. The protein bands were stained with Coumassie blue stain.

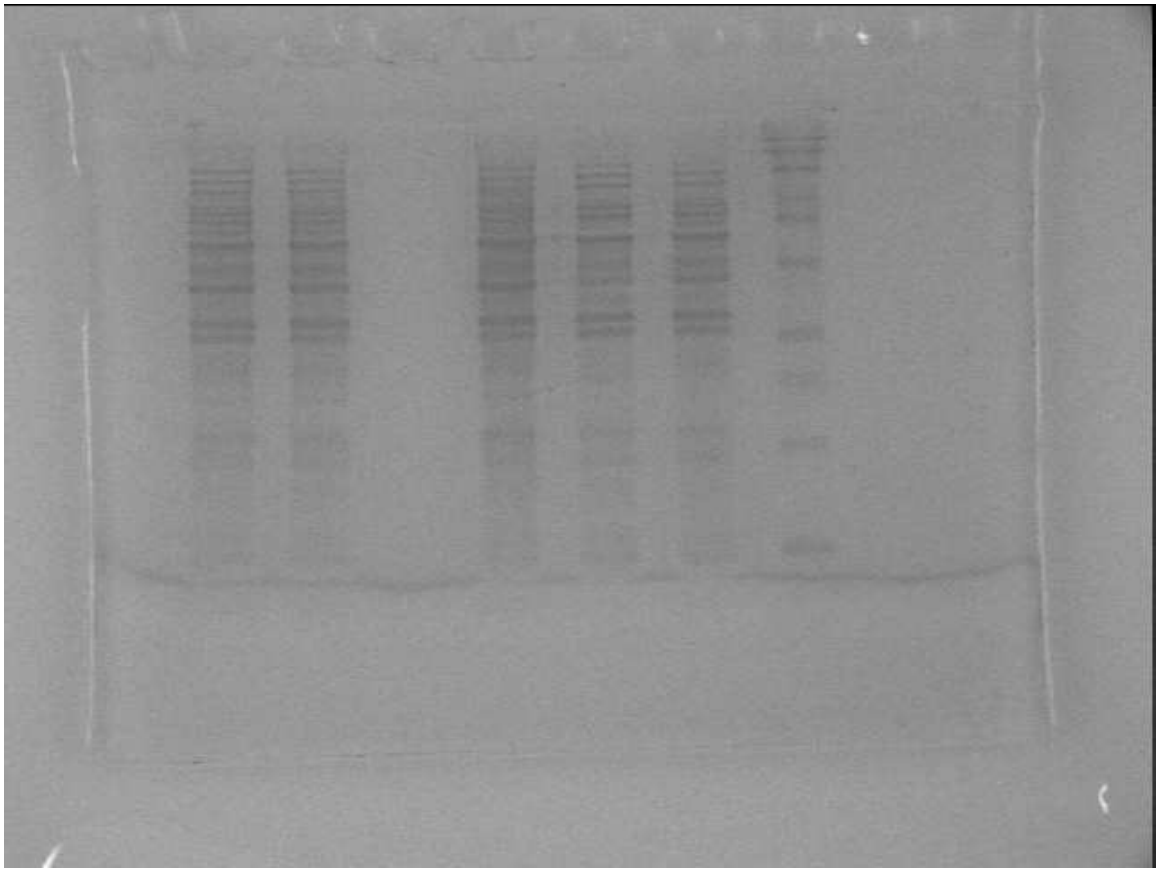

Figure 3.4: SDS-PAGE of total protein extracts.

The western blots that were developed with 10 seconds of exposure time did not yield any results. On changing the exposure time to about 40 mins the band appeared on the film. The western blots (Figure 3.5) developed with the Rabbit p42-44 MAP kinase antibody showed the same bands of equal intensity for all cell populations. This shows us that the genetic level expression was the same for all cases. An *in silico* analysis of the *C. albicans* genome using the BLAST algorithm (http://genolist.pasteur.fr/*Candida*DB/) and the TEYVATRWYRAPE motif, which is characteristic of the VIII subdomain of the p42-44 MAP kinase family of protein kinases. These hits match the sequence exactly in three cases (Mkc1p, 59 kDa; Cek1p, 48·7 kDa; and Cek2p, 43·3 kDa), and for this reason they can be considered p42-44 homologues.

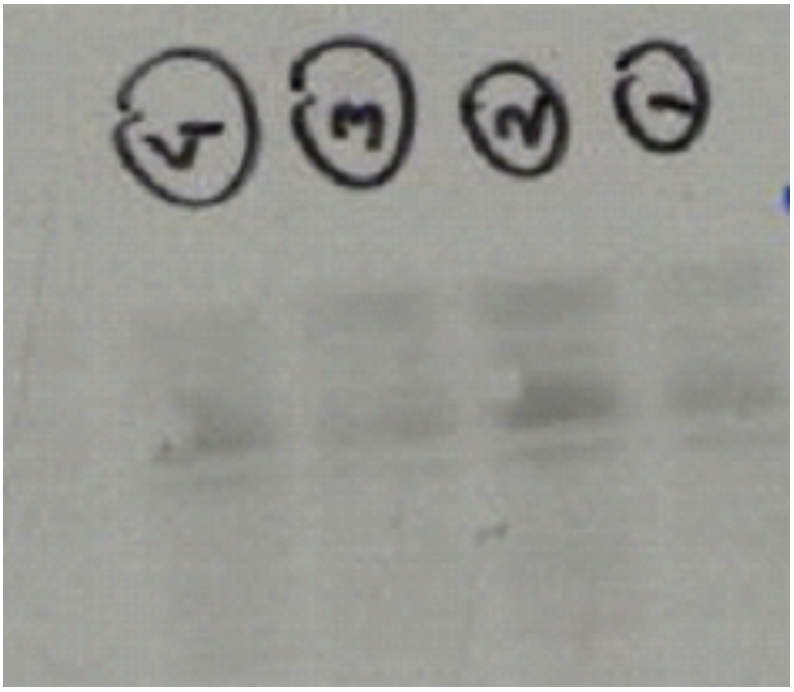

Figure 3.5: Western blot using Rabbit p42-44 MAP kinase as the primary antibody. Lanes: 1 – exponential, 2: stationary, 3 – shear removed, 4 – basal blastospores.

The western blots (Figure 3.6) developed with the Rabbit Phospho p42-44 MAP kinase antibody showed couple of distinct bands only for adherent cell subpopulation of the biofilm. The four independent samples that ran for this cell type showed the same bands. These bands were not visible in any other cell populations. Based on the size of these bands and comparing with other literature, the lower dark band corresponds to Cek1p Map kinase in *C. albicans*. The upper dark band corresponds to the Mkc1p MAP kinase in *C. albicans*. These data when clubbed with the data generated in our lab, show that the basal blastospores are distinctively different from other cell populations.

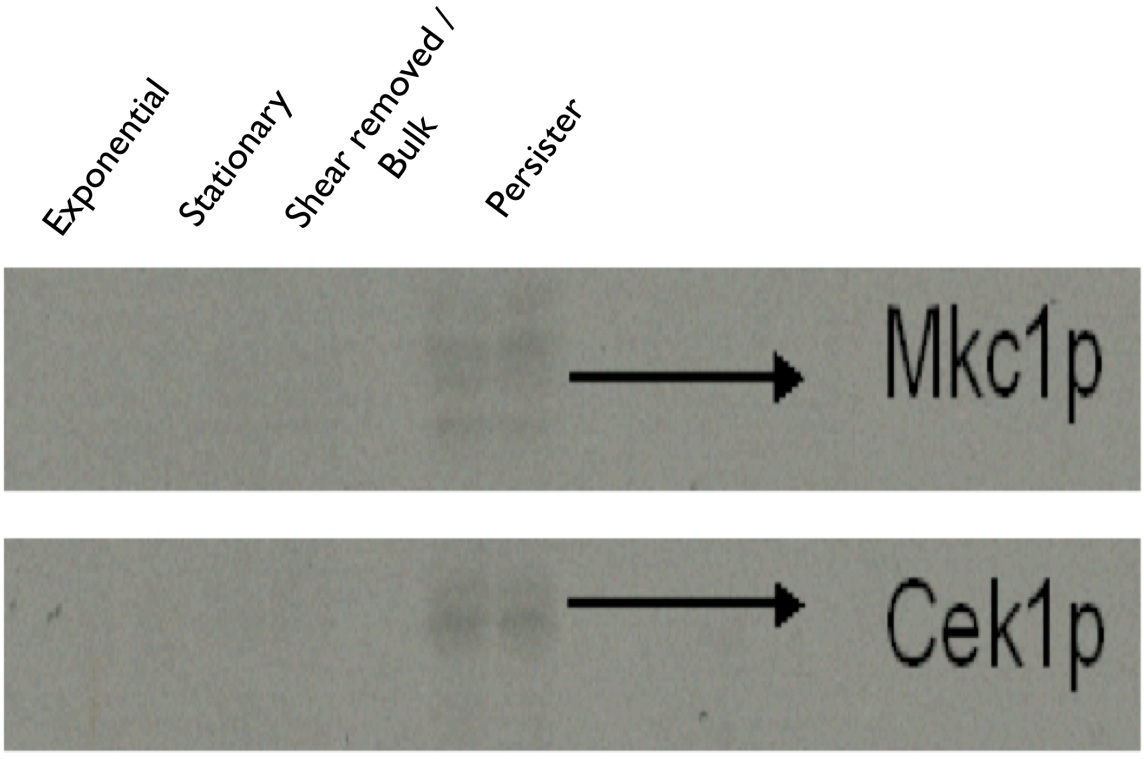

Figure 3.6: Western blot using Rabbit phospho p42-44 MAP kinase as the primary antibody.

The phosphorylation of Cek1 and Mkc1 MAP Kinases confirms the hypothesis . Also there were a couple of fainter bands that belong to the family of p42-44 MAP kinases that were activated in the basal blastospores.

3.5 Discussion

The conclusion from this study is that cell integrity MAP Kinase pathway was activated in the persister cell subpopulation. Both the important MAP kinases, Cek1 and Mkc1, were both activated only in the persister cell subpopulation. There was not any change in the genetic level expression of these MAP kinases as shown in my probing with p42-44 MAP kinase antibody. All the fractions showed similar levels of MAP kinases. The change was only in the phosphorylation of the above mentioned kinases. This is typically triggered by some stress signal, probably due to contact based stress. The network of MAPK signaling pathways represents an array of cascades that are essential for morphogenesis, cell growth, biogenesis of the cell wall and stress response, among others, in *C. albicans*.

According to previous work with the MAP kinases of different organisms, phosphorylation of the threonine and tyrosine residues in the TEY signature of domain VIII of the p42-44 MAP kinase family is necessary for phosphorylation activity. Only when both residues are dephosphorylated, p42-44 MAP kinases are unable to phosphorylate their substrates (Anderson 1990; Nishida 1993; Flury 1997). Accordingly, antibodies against the phosphorylated forms of p42-44 MAP kinases are used in many eukaryotic organisms (fungal, mammalian and plant) as indicators of the activation of these MAP kinases. This is the approach I used

to detect and determine the phosphorylation and thereby the activation of different p42-44 MAP kinases, mainly Mkc1 and Cek1, in *C. albicans*.

Mkc1 and Cek1 are elements of the cell integrity pathway, since they are necessary to appropriately respond to cell wall injuries. In *S. cerevisiae*, both pathways collaborate in the maintenance of cell wall integrity (Lee 1999). Navarro-Garcia et al. checked the phosphorylation state of Mkc1p after treatment with the cell-wall-disrupting agents like calcofluor white and Congo red. After 2 hrs, an increase in the phosphorylation of Mkc1p was detected (Navarro-Garcia 2005). On treatment with zymolyase, yeast cell wall degrading enzyme, Cek1 and Mkc1 were both activated (Navarro-Garcia 2005). This further confirms their role in cell wall repair. In *C. albicans*, Mkc1 mutants and Cek1 mutants are more sensitive to cell-wall-degrading enzymes and antifungals (Kumamoto 2005a,Navarro-Garcia 1995 & 2005). This clearly demonstrates that by the deletions of essential elements of these cascades there is a reduction in resistance shown by these mutants.

An important task for a pathogenic microorganism is to determine its location within the host. Experiments carried out by Kumamoto demonstrate that without Mkc1p, the response of cells to the presence of a surface is aberrant, and thus the cell integrity pathway is a source of important information that allows monitoring of the immediate environment (Kumamoto 2005a). A null mutant lacking Mkc1p exhibits reduced virulence and reduced contact with surfaces, suggesting that virulence of the organism is associated with the ability to initiate contact-dependent responses (Ramage 2002).

Khot et al. showed that the genes of β-1-6-glucan pathway in the resistant basal blastospores showed a high degree of differential up regulation of the *SKN1* (+30.7fold change) and *KRE1* (+29.86fold change) genes. The *KRE1 and SKN1* genes exhibited an interesting trend of increasing transcript abundance for populations exhibiting increased resistance at higher concentrations of amphotericin B. This suggests that there are changes taking place in the cell wall composition in these basal blastospores. Literature already shows that Mkc1 and Cek1 are both activated on contact activated stress. They are also activated when subjected to antifungal and cell wall degrading enzyme stresses. The fact that these kinases are activated in basal blastospores suggests that these kinases are activated due to some contact related stress. The ability of a cell to sense and respond to surface contact is wide-spread in biology. This activation could be possible signal that lead to the gene expression changes observed by Khot et al. This result along with the ergosterol reduction leads to the hypothesis that the resistance of basal blastospores to amphotericin B is possibly due to a synergistic effect of changes in two pathways - the ergosterol and the beta-glucan pathways.

This work has shown that growth of *C. albicans* in contact with a surface results in mechanical perturbation of the cell wall or plasma membrane. This perturbation is detected by *C. albicans* using a signal transduction pathway known as the cell integrity pathway, a MAP kinase pathway. Mkc1 and Cek1 MAP Kinases were found activated in persister cell subpopulation.

CHAPTER 4

QUORUM SENSING BY FARNESOL

4.1 Abstract

The most striking feature of the persister cell population was their increased adherence to the tubing, relative to the rest of the biofilm. Studies performed in our lab show that genes involved in adhesion were up-regulated in the persister cells (Srinath Rangarajan-unpublished work). Bacterial biofilm studies have shown that adherence to surfaces results in alteration of the cell wall or plasma membrane, which lead to increased resistance. Quorum sensing molecules have been shown to reduce adhesion of bacteria biofilms.

I hypothesized that farnesol, a quorum-sensing molecule in *C. albicans*, will reduce the attachment of *C. albicans*, thereby reducing persister cell formation. Results show that farnesol had drastically reduced the attachment of cells to the tubing surface. Traditional treatment of infectious diseases is based on compounds that aim to kill or inhibit microbial growth. A major concern with this approach is the frequently observed development of resistance to antimicrobial compounds. This makes the above mentioned finding important, as it gives us a new avenue to impede the process of persister cell formation.

4.2 Introduction

Two major aspects of the host-parasite interactions are the adhesion of *C. albicans* cells to host cells and tissues and the immuno-modulation of the host immune response. Adhesion is a prerequisite for colonization and an essential step in the establishment of infection. *C. albicans* adheres to epithelial cells, endothelial cells, soluble factors, extracellular matrix, and inert materials implanted in the body of the host. Multiple adherence mechanisms appear to be used by *C. albicans* cells (Calderone 1993 & 1991, Fukazawa 1997). Previously done work on *C. albicans* by Khot et al., and Rangarajan have shown that persister cell subpopulations have marked changes in their genetic expressions in various pathways as compared with the other fractions of biofilm. This was also shown with the ergosterol study and MAP kinase study done as part of this dissertation.

Previously done work on *C. albicans* reported by Khot et al. and Rangarajan have shown that persister cell subpopulations have marked changes in their genetic expressions in various pathways as compared with the other fractions of biofilm. The planktonic stationary phase was not associated with the same changes in gene expression. This indicates that resistance of *C. albicans* to AmB is due to changes in a number of pathways, not just the $\beta(1,6)$ glucans and ergosterol pathways, but that the cell wall subpopulation, indicating a correlation between adhesion and resistance.

4.2.1 Resistance towards antifungal agents

Several groups have demonstrated that the *Candida* biofilm lifestyle leads to dramatically increased levels of resistance to the most commonly used antifungal agents (Baille 1999, Chandra 2001, Hawser 1998 & 1994, Khot 2006, Kuhn 2002, Lamfon 2004, Lewis 2006, Ramage 2001). As yet, there appears to be no one specific resistance factor responsible for the increased recalcitrance to antimicrobial agents exhibited by biofilms. Instead, biofilm resistance is a complex multifactorial phenomenon, which still remains to be fully elucidated and understood. Bacterial biofilms of this type, including those produced by *Staphylococcus epidermidis* and *Pseudomonas aeruginosa*, are relatively insensitive to antibiotic treatment and eradication of implant-associated infections often involves removal of the infected prosthesis (Brown 1993, Costerton 1987). This holds true in case of even fungal biofilm centered infections.

The majority of implant infections attributable to fungi are caused by the pathogenic *Candida* species, especially *Candida albicans* (Hawser 1994). The usage of antifungal drugs induces resistance and thus development of persister cells is a growing concern with the medical community. As shown by Khot et al., the persister cell subpopulations are resistant at even high concentration of amphotericin B. The high concentration of amphotericin B is known to have severe side effects. Traditional treatment of infectious diseases is based on compounds that aim to kill or inhibit microbial growth is failing in case of biofilms. One of the critical steps in switching to the resistant phenotypes in case of

biofilms is attachment to surfaces. If this process of attachment could be inhibited, the switch to the resistant phenotype could be halted.

4.2.2 Adhesion of persister cells

An infectious pathogen such as *C. albicans* develops resistance using a multitude of pathways, making it much more complex, difficult to find suitable drug targets and to develop drugs. Various mechanisms have been suggested trying to explain the resistance shown by these cells. One critical factor differentiating these persister cells from the rest of the biofilm is that they are tightly adhering to the substratum. Rangarajan (PhD dissertation, 2008) reported few important genes such as MNT1, CSA1, ALS1 and YWP1 were differentially regulated in the blastospore subpopulation. This study linked the adhesion of cells as a trigger mechanism towards development of resistant phenotype.

Tranformation of planktonic population of cells to highly resistant persister subpopulation cells can be envisioned as concerted changes in the cell wall of the resistant persister cells leading to resistance, as a consequence of a trigger such as adhesion to an implant substrate. Starting as a response to adhesion, the adhesive proteins trigger signalling pathways (currently unknown) that reach the interior of the cell, the nucleus and initiate changes in gene transcription. Ultimately, the proteins affected by these gene expression changes in turn affect cell wall molecules such as the ß-glucans and the sterols in the plasma membrane. As a consequence, the cell responds by changing the structure of the cell wall and cell membrane. Pursuing this avenue of research is bound to yield better drug targets because all subsequent events are downstream. Currently

most pharmaceutical companies are developing drugs that act by targeting downstream events/pathways. These events are generally those that happen at a much later stage - after the organism has firmly established itself on the implant surface. The adhesins responsible for resistance or triggering resistance may be used as novel drug targets.

4.2.3 Quorum sensing

Quorum sensing refers to phenomenon whereby the accumulation of signaling molecules enables a single cell to sense the number of cells (cell density) around it. Quorum sensing research holds key to understanding the adaptability of cells to their surrounding. Production of farnesol by *Candida albicans* is the first quorum-sensing system discovered in a eukaryote (Hornby 2001).

Farnesol is a fungal quorum-sensing molecule with intriguing regulatory properties in *Candida albicans*. This compound is excreted continuously by *C. albicans*, and when it accumulates beyond a threshold level, it blocks the yeast-to-mycelium conversion (Hornby 2001). Farnesol blocks mycelial development initiated by any of three chemically distinct triggers for germ tube formation: L-proline, *N*-acetylglucosamine, and serum. Farnesol has been shown to affect the dimorphism in *C. albicans* (Hornby 2001). Thus farnesol's mode of action and the therapeutic potential of farnesol analogs are of interest to the research community.

Farnesol is a component of many perfumes, including Chanel No. 5, and its distinctive aroma was used initially in its purification from *C. albicans* (Hornby

2001). Farnesol can exist as four isomers, but only the *E*,*E* isomer has QSM activity (Shchepin 2003). Figure 4.1 shows the structure of all farnesol isomers. Recent studies have shown the linkage between farnesol production and sterol biosynthesis pathway as shown in Figure 4.2. Hornby et al. investigated the connection between sterol pathway and production of farnesol. They found increased production of farnesol with squalene synthase inhibition by zaragozic acid B. They have confirmed similar results indicating inhibition of lanosterol 14α-demethylase by four cytochrome P450-inhibitory azoles.

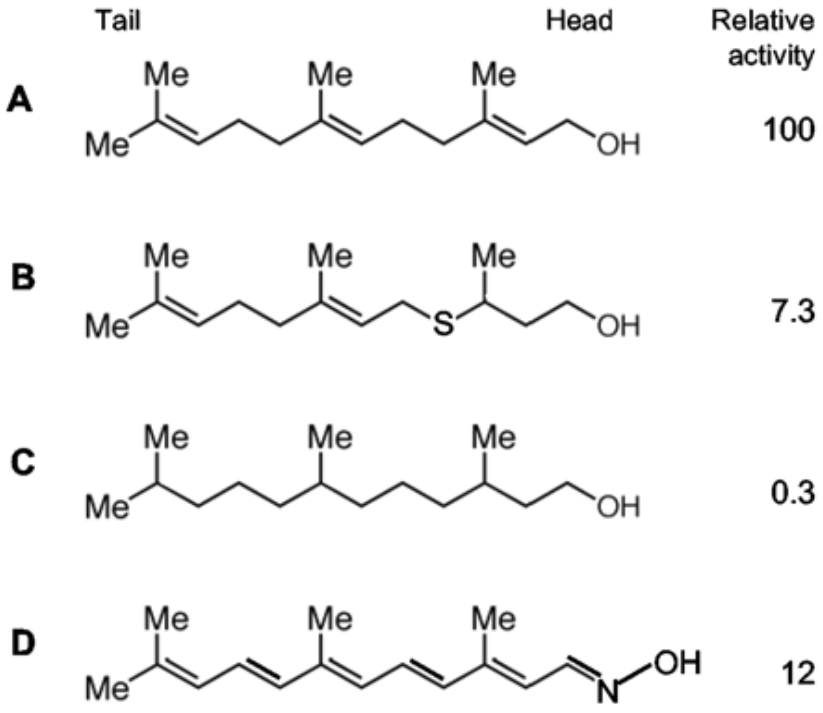

Figure 4.1: Farnesol isomers. A (E, E, farnesol) is the only one with quorum sensing effect on *C. albicans*

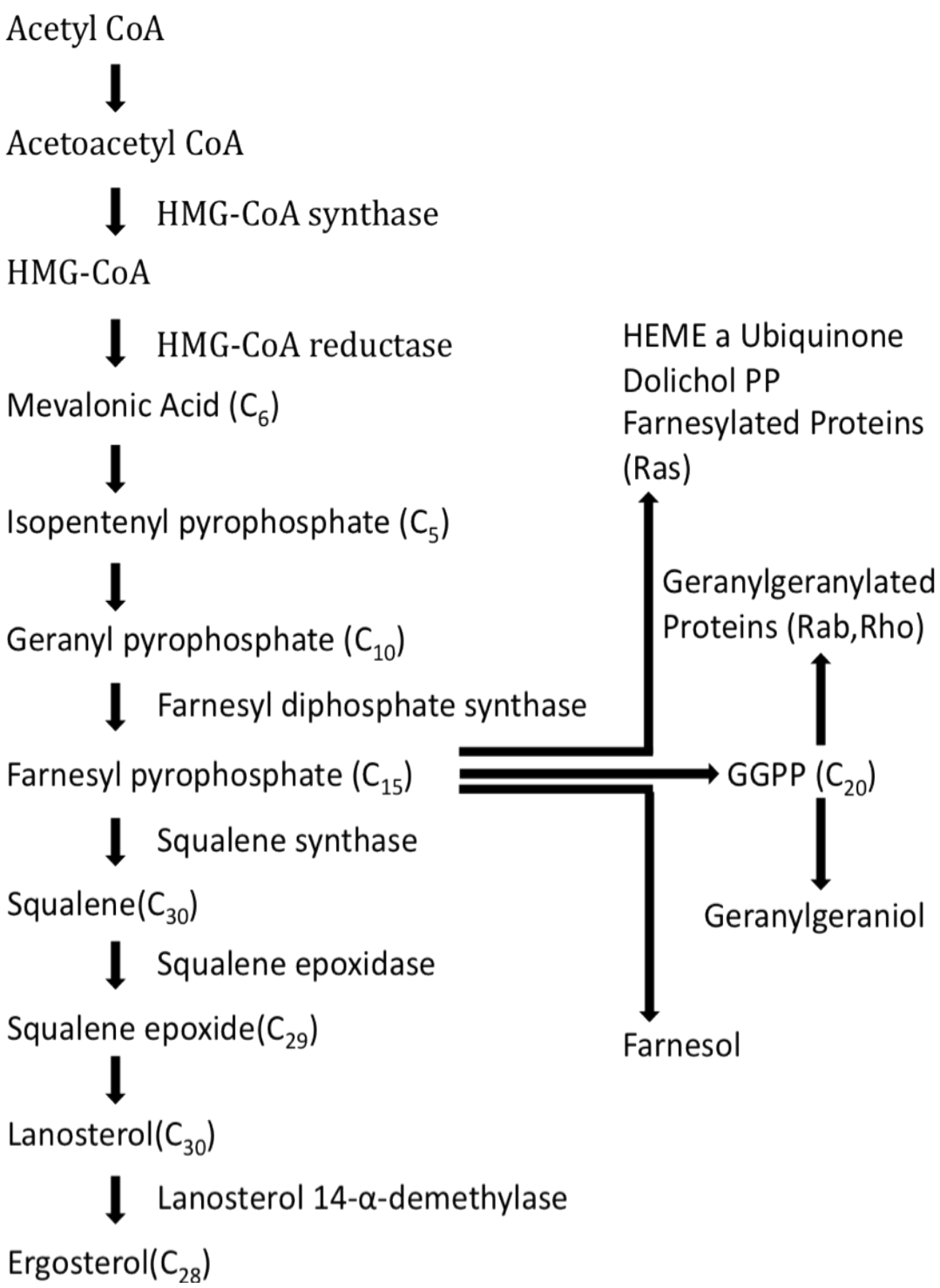

Figure 4.2: Farnesyl pyrophosphate as linkage between sterol synthesis and farnesol production

For most strains of *C. albicans*, any drug that inhibits a step in the sterol biosynthetic pathway, thus inhibiting carbon flow to ergosterol, may also increase farnesol production (Hornby 2004). This would lead to a higher level of communication between cells, and thus a mode for adaptation towards their changing environment.

4.2.4 Quorum sensing effect on biofilm formation

A typical biofilm formation in *C.albicans* is initiated by colonization of yeast cells is followed 3 to 6 hrs later by germ tube formation. The adhering yeast cells form a basal layer that firmly attaches the biofilm to the substrate, while subsequent germination generates the bulk of the biofilm. After 24 hrs, a mature biofilm typically contains yeasts, hyphae, and pseudohyphae. Ramage et al. have shown that the effect of farnesol on biofilm development was time dependent. Addition of 30 to 300 μM farnesol at time zero inhibited biofilm development, but addition 1 to 2 hrs later did not. That is, once hyphal formation had been initiated, it could no longer be inhibited by addition of farnesol. Interestingly, mature biofilms (24 hrs) became sensitive to farnesol (Ramage 2002a). The results reported provide the first example of a quorum-sensing event in fungal biofilms. Farnesol plays a crucial role in biofilm development and survival. Biomaterial infections are an increasingly alarming problem, and due to their intrinsic recalcitrance to conventional therapy new methods of dealing with these infections must be explored. These studies were carried out with farnesol being introduced at certain time points. It will be interesting to study the effect of continuous release of farnesol during biofilm formation.

4.3 Materials and methods

4.3.1 *C. albicans* strain and medium

C. albicans CA-1 is a clinical isolate obtained from the culture collection of Diane Brawner (Microbiology Department, Montana State University). The strain was stored at -80^{o}C. Planktonic cells were cultured in 2% YEPD medium (2% glucose, 1% bacto yeast extract, and 2% bacto peptone). The solid agar medium was 1% glucose, 0.5% bacto yeast extract, 2% bacto agar, 0.1% ammonium sulfate dissolved in 20% tap water and 80% D.I. water.

4.3.2 Planktonic cultures

Cultures were grown aerobically in 250-ml Erlenmeyer flasks containing 100 ml growth medium. The flasks were placed in a shaker incubator at 37^{o}C and 160 rpm for the desired period of growth. *C. albicans* grew as budding yeast under these conditions. Stationary phase cells were harvested after a 24 hrs growth period and washed using cold PBS buffer.

4.3.3 Biofilm cultures

Biofilms were grown in a tubular biofilm flow cell (TBF). A schematic of the TBF is shown in Figure. 4.3. Silicone tubing (Cole-Parmer; catalog no. EW-95802-08) with an inner diameter of 4.78 mm, an outer diameter of 6.35 mm, a wall of 0.79 mm, and a length of 20 cm constituted the biofilm-growing region of the TF. The source of growth medium for the TF was a 2-liter Erlenmeyer flask. A bubble trap was placed between the Erlenmeyer flask and the TF to prevent passage of air bubbles during biofilm growth.

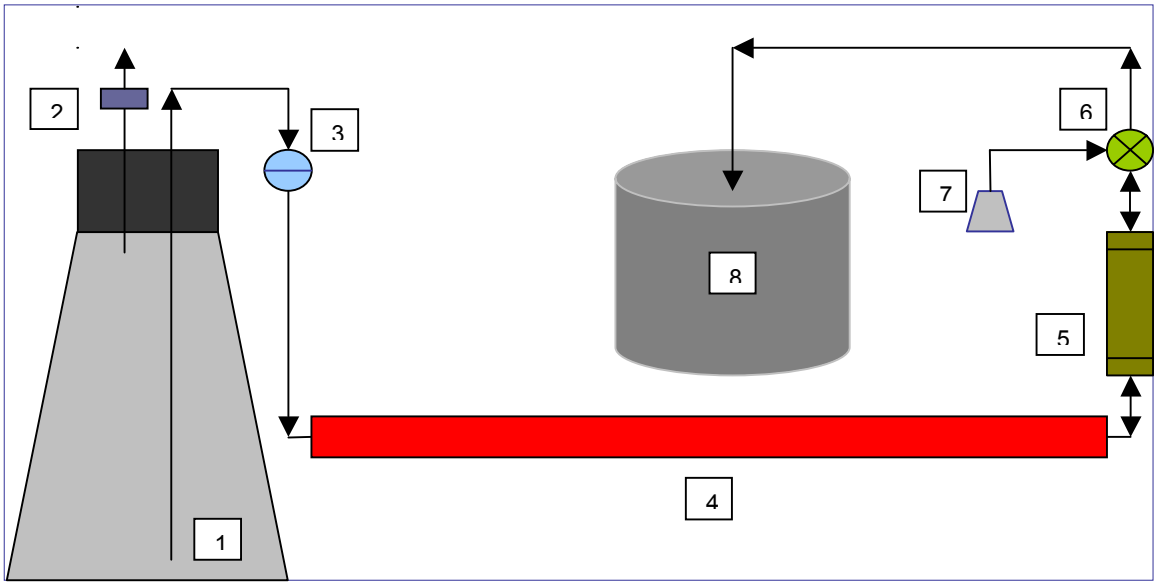

Figure 4.3: Schematic of tubular biofilm flowcell (TBF) set-up.1] 2-L Erlenmeyer flask containing sterile growth media. [2] 0.2-micron air filter. [3] Bubble trap. [4] Biofilm growing section of the TF tubing. [5] Peristaltic pump. [6] 3-way junction. [7] 250-ml Erlenmeyer flask with inoculum. [8] Waste container.

Peristaltic pump coupled at the effluent end of the TBF helped in maintaining a flow rate of 1.17 ml/min (shear rate, 109.5 s^{-1}). The residence time for the volume of liquid contained in the tubular reactor portion of the flow system (20-cm length of tubing) was 3 mins. This condition ensured that the contribution to the cell population in the TBF from cells in the planktonic mode of growth (doubling time, approximately 80 mins) was negligible. After sterilization by autoclaving, the entire setup was placed horizontally on a grilled shelf in an incubator at 30 ^{0}C. The TBF was filled with growth medium before being inoculated with cells. The inoculum was prepared from a 24 hrs planktonic culture with a concentration of 10^{8} cells/ml in 0.1 M phosphate-buffered saline (PBS, pH 7) buffer. It was fed into the TBF from the effluent end by reversing the direction of flow. Flow was then discontinued for 1 hr. After the 1 hr inoculation

period, flow was resumed for 24 hrs. At the end of this culture period, the section of tubing in which the biofilm grew was clamped at both ends and removed by cutting the tubing with a sterile blade. The liquid column was drained into a petri dish by moving the tubing to a vertical position and releasing the clamps. The tubing was then rinsed with PBS buffer equivalent to one tube volume. The cells that remained adhering to the walls of the tube are referred to as the persister subpopulation. The persister cells were removed using a sterile cell scrapper. The procedure was carried out under highly sterile conditions.

4.3.4 Farnesol incorporation

In order to study the effect of farnesol on biofilm, farnesol was incorporated into the silicone tubing. A 10%v/v solution of farnesol in methylene chloride was made. The desired length of silicone tubing was immersed in this solution for 30 mins. The tubing was left in a fume hood for 1 hr until the tube deswelled. This tube was washed with water and sterilized for use. Tubing was sterilized using either steam or ethylene oxide.

To test the concentration of farnesol in tubing, 1 cm of sterilized tubes were cut into small portions and placed in 1 ml of methylene chloride for 30 mins. The methylene chloride layer was then pipetted into a glass vial and analyzed using GC/MS. The column used for separation was DB-5ms. Sample was introduced at 300^{o}C using splitless injection mode. Temperature of the oven was increased using two ramps: from 50 ^{o}C to 225^{o}C at 25 ^{o}C / min and from 225 ^{o}C to 280 ^{o}C at 10^{o}C/min (total run time of 12.5 mins). The calibration curve was generated using known concentration of farnesol in methylene chloride.

4.3.5 Farnesol effects on biofilm study

Considering the heterogeneous nature of biofilms, these studies were carried out in tubes which had farnesol incorporated into only one half of the tube. This served the purpose of internal standard. The farnesol incorporated end served as either the top side (Top Farnesol, Figure 4.4) of the reactor or the bottom end (Bottom Farnesol, Figure 4.5) of the reactor.

4.3.6 Plate counts

Colony forming units (CFU) were used to estimate the numbers of cells attaching to silicone tubing (concentration of basal blastospores). After Biofilm ran for 24 hrs, the pump was stopped. The reactor section of the tubing was washed using PBS buffer (0.1M, pH 7.0). The tube was split into smaller section using sterile surgical blades. For each section a lengthwise slit was made about a 1ml of PBS was pipetted. The adherent cells were removed using a sterile scraper. The solution collected was serially diluted in 2 ml cuvettes. The serial dilution was 10 fold across each cuvette.

The required numbers of serial dilutions per sample were judged based on trial and error. A volume of 100 µl from each serially diluted cuvette was spread as a separate lane on an agar plate. Each agar plate had a maximum of 4 lanes. Plates were incubated at 37^{o}C for 24 hrs. CFU were estimated from lanes of serial dilutions whose numbers fell in the range of 10 to 100 colonies per lane. The appropriate dilution factor was multiplied to estimate the final viable cell concentration for every sample.

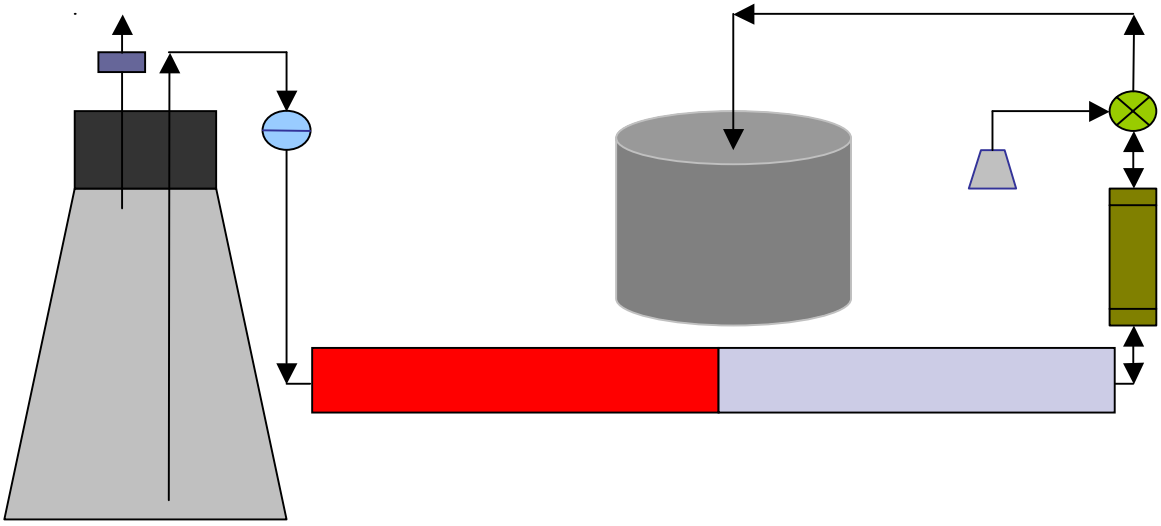

Figure 4.4: Top farnesol, where the upstream end of the tubing has farnesol incorporated (red section)

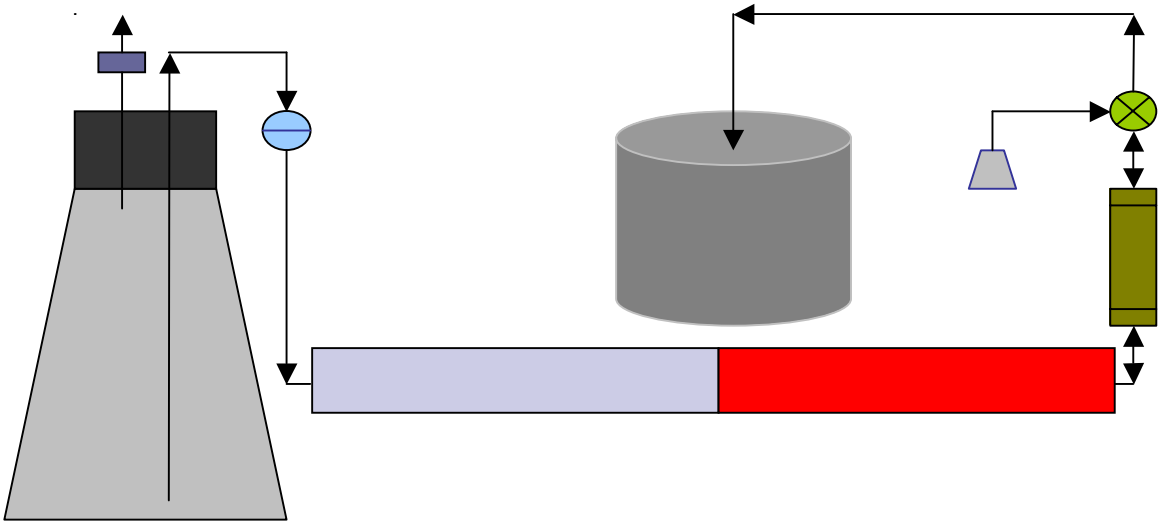

Figure 4.5: Bottom farnesol, where the downstream end of the tubing has farnesol incorporated (red section)

4.3.7 Farnesol release study

In order to study the release of farnesol from the tubing, same setup as a TF setup was used. Instead of the media, water was used. Water flowing out of the reactor was collected using glass centrifuge tubes at different time points. For every 15 ml of water collected, 1ml of methylene chloride was added. The solution was mixed gently for 15 mins. After 15 mins of mixing, tubes were centrifuged at 300g for 5 mins. The methylene chloride layer (bottom layer) was analyzed using GC/MS.

4.4 Results

In order to generate the calibration curve, a varying amount of known concentration of farnesol was used. The solution was made up to 1ml using methylene chloride. Figure 4.6 shows the calibration curve used for the farnesol experiments. Calibration curve was generated using the area under the curve and concentration of farnesol in methylene chloride.

Farnesol concentration in the tubing used was calculated using GC/MS. Figure 4.7 and 4.8 are the total ion chromatogram for farnesol peaks and mass spectra for farnesol molecule. The two peaks that show up in the chromatogram are due to the fact that farnesol is available commercially as a mixture of isomers. The concentration of farnesol in steam sterilized tubing was 4.08±0.25 mg/cm and concentration of farnesol in case of ethylene oxide sterilization was 12.01 ± 0.42 mg/cm.

Also to check for farnesol release form the tubing, an experiment was conducted to with water as the running media. The GC/MS of the samples

collected showed that farnesol could be detected for 11 hrs. The farnesol peaks could not be quantified as they were below the quantifiable limit of 2µl/ml.

The biofilms samples from farnesol incorporated tubing were collected and cell density was estimated using CFU counts. The data files shown below used three kinds of tubing. The top farnesol is the tubing in which top half of the tube was exposed to farnesol treatment. The bottom farnesol is the tubing in which bottom half of the tube was exposed to farnesol treatment.

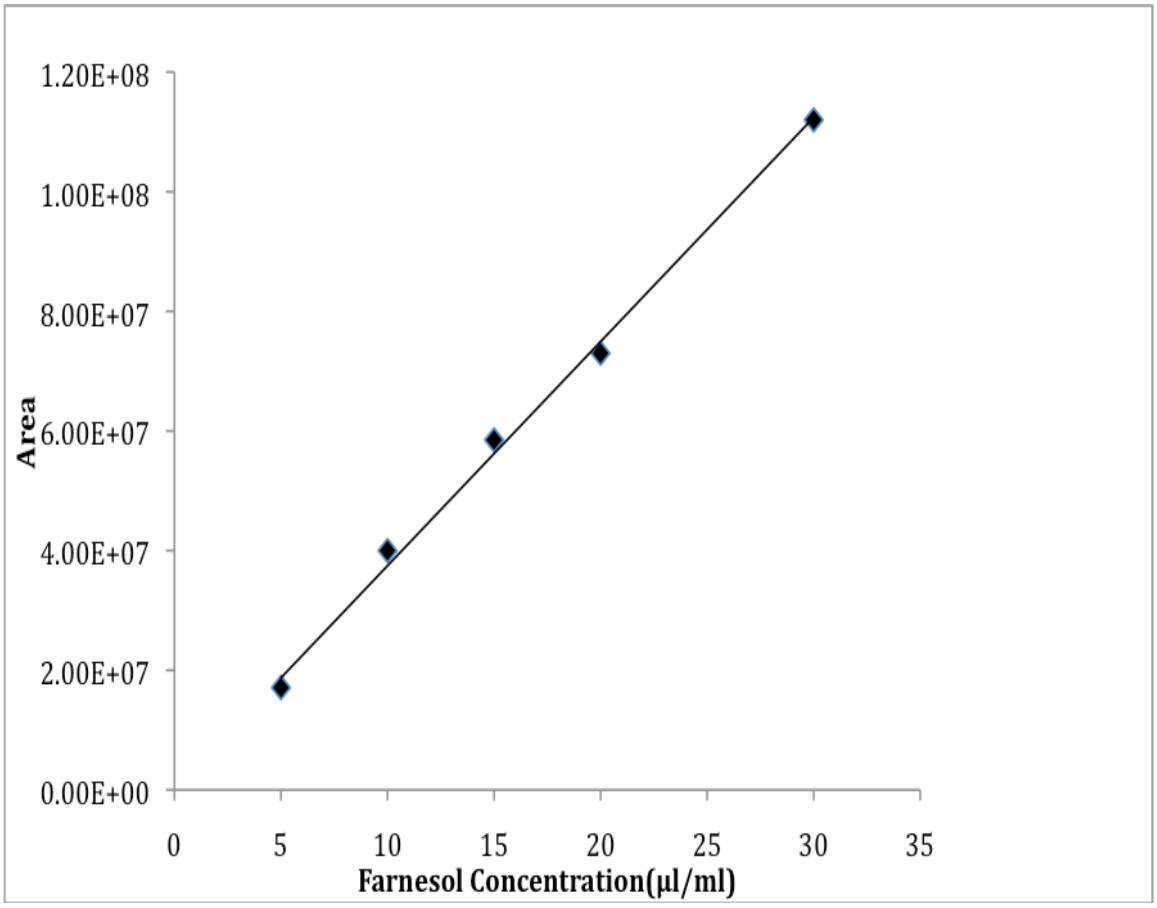

Figure 4.6: Calibration curve for farnesol concentration.

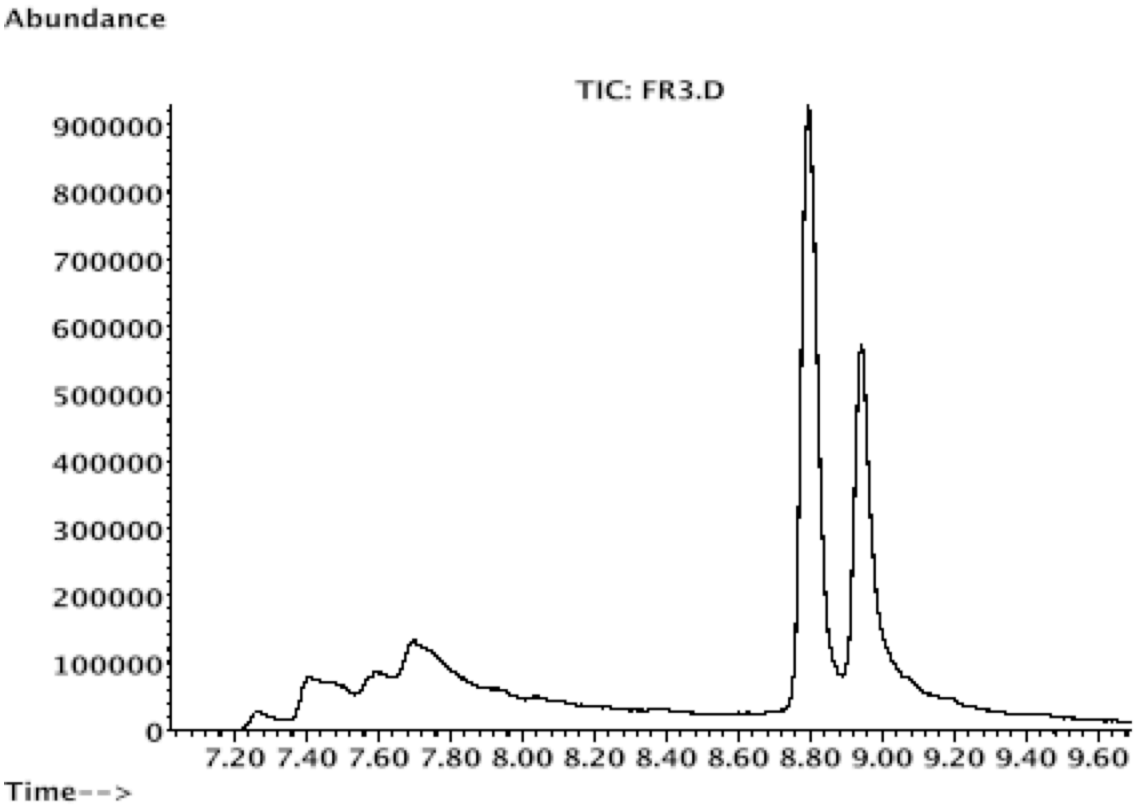

Figure 4.7: Farnesol extraction from tubes chromatogram.

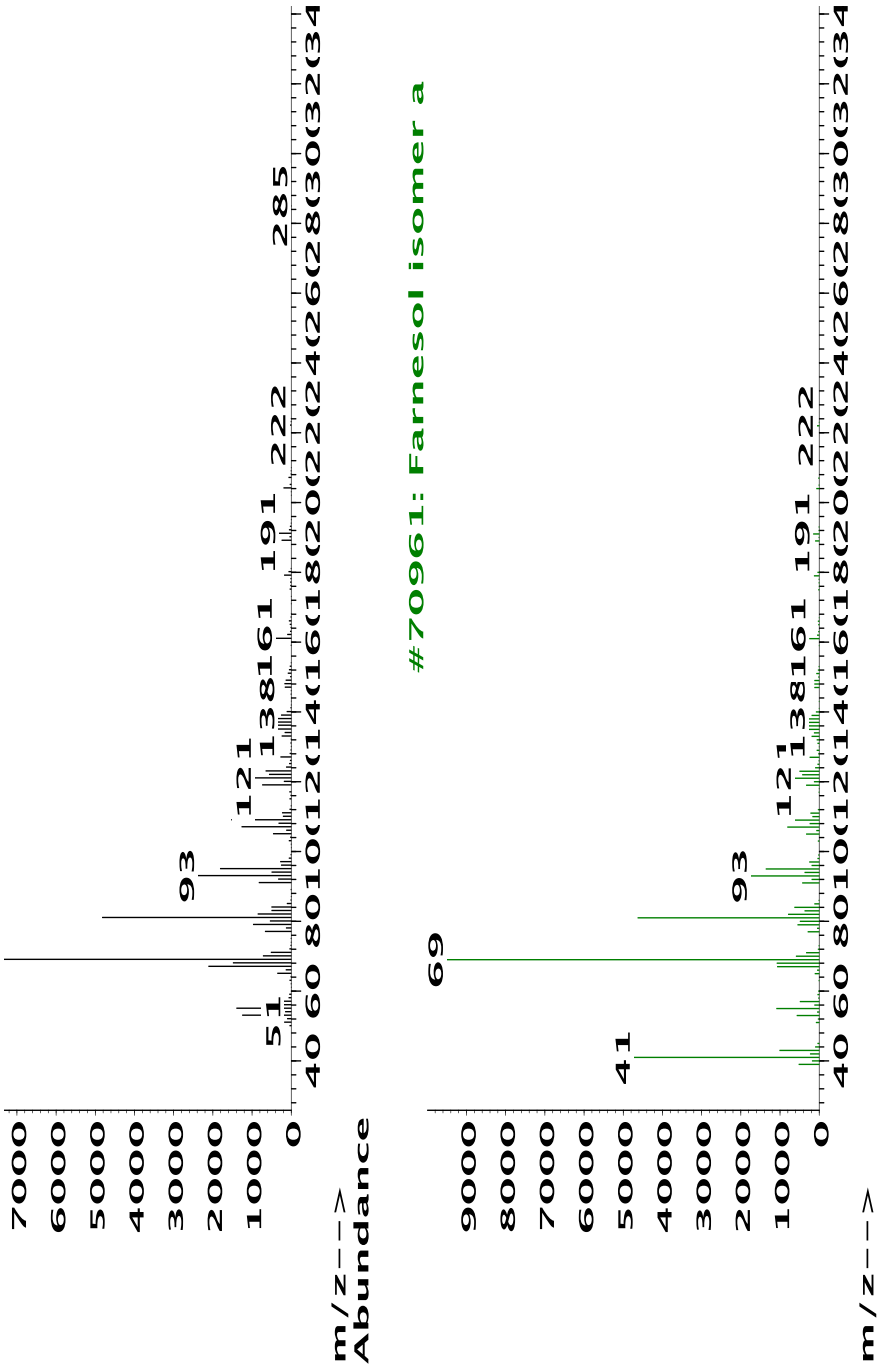

Figure 4.8: Mass spectroscopy of farnesol

The CFU counts are were taken in triplicates at each dilution and separately done to top half of the tubing and bottom half of the tubing. This tubing was sterilized using either the steam or ethylene oxide. The data from CFU counts from four experiments are tabulated in Tables 4.1, 4.2, 4.3 and 4.4. There were two controls used in all four runs. Control-1 was tubing subjected to methylene chloride treatment without farnesol. Control-2 was virgin tubing. There were interesting data that came out of these experiments. The drastic effect was seen in case of ethylene oxide sterilization.

The control-1 tubing used showed no affect due to the chemical process it was subjected too. The attachment of cells was very similar to that of virgin tubing (control-2). This can be seen in all four experiments done. Hence the methylene chloride treatment did not have any residual effect on the cell attaching in both cases of sterilization. Table 4.1 and 4.2 show data from the steam sterilization study. In case of Top-farnesol tubing, lesser cells have attached to farnesol treated surface as compared to the control tubing. The effect of farnesol was seen in the bottom section of the tubing too. This was probably due to release of farnesol from the top section. This corroborates well with the finding from the farnesol release study. Table 4.2 shows the CFU counts for cells in case of bottom half of the tubing exposed to farnesol treatment. The bottom farnesol tubing showed some difference in terms of cell attachment. This data was not as good as the top farnesol tubing. Also the top section of the tubing did not show much reduction in cells attached probably due to the fact that there was no farnesol present in this section of the tubing.

Table 4.1: Cell density of adherent fraction of biofilm in case of top farnesol tubing in case of steam sterilization

Section		Run 1	Run2	Run3	Run4	Run5	Run6
	Tubing	Top farnesol	Top farnesol	Top farnesol	Top farnesol	Control-1	Control-2
top		(cells/cm)	(cells/cm)	(cells/cm)	(cells/cm)	(cells/cm)	(cells/cm)
	count1	1.10E+03	9.12E+02	3.10E+03	1.21E+03	7.15E+05	4.20E+05
	count 2	2.45E+03	3.80E+04	1.12E+03	4.12E+03	2.87E+05	3.50E+06
	count3	1.54E+03	4.70E+03	2.15E+04	5.68E+03	5.60E+05	2.45E+05
	average	1.32E+03	1.45E+04	8.56E+03	3.67E+03	5.21E+05	1.39E+06
bottom							
	count1	3.10E+03	2.57E+03	5.68E+03	3.42E+03	4.20E+05	1.79E+06
	count2	5.41E+03	3.41E+04	1.41E+03	4.51E+04	3.50E+06	4.50E+06
	count3	4.31E+03	1.12E+03	3.68E+03	3.46E+04	2.45E+05	8.45E+05
	average	4.27E+03	1.26E+04	3.59E+03	2.77E+04	1.39E+06	2.38E+06

Table 4.2: Cell density of adherent fraction of biofilm in case of bottom farnesol tubing in case of steam sterilization

section		Run 1	Run2	Run3	Run4	Run5	Run 6
	Tubing	Bottom farnesol	Bottom farnesol	Bottom farnesol	Bottom farnesol	Control-1	Control-2
Top		(cell/cm)	(cell/cm)	(cell/cm)	(cell/cm)	(cell/cm)	(cell/cm)
	count1	2.90E+05	1.21E+06	6.20E+04	4.79E+05	3.47E+05	4.71E+04
	count 2	3.21E+05	4.50E+03	2.31E+03	2.31E+04	7.89E+05	2.36E+04
	count3	4.20E+03	3.10E+04	6.72E+04	3.57E+04	1.40E+06	8.19E+05
	average	2.05E+05	4.15E+05	4.38E+04	1.79E+05	8.45E+05	2.97E+05
Bottom							
	count1	2.31E+04	3.42E+03	1.25E+04	2.72E+04	4.68E+05	6.71E+04
	count2	4.58E+04	1.95E+04	3.57E+03	3.14E+03	3.92E+05	8.90E+05
	count3	3.92E+03	6.41E+03	7.14E+02	2.14E+03	1.25E+04	9.80E+04
	average	2.43E+04	9.78E+03	5.58E+03	1.08E+04	2.91E+05	3.52E+05

Table 4.3: Cell density of adherent fraction of biofilm in case of bottom farnesol tubing in case of ethylene oxide sterilization

Section		**Run 1**	**Run2**	**Run3**	**Run4**	**Run5**	**Run6**
	Tubing	**Top farnesol**	**Top farnesol**	**Top farnesol**	**Top farnesol**	**Control-1**	**Control-2**
top		**(cells/cm)**	**(cells/cm)**	**(cells/cm)**	**(cells/cm)**	**(cells/cm)**	**(cells/cm)**
	count1	1.10E+03	9.12E+02	3.10E+03	1.21E+03	7.15E+05	4.20E+05
	count 2	2.45E+03	3.80E+04	1.12E+03	4.12E+03	2.87E+05	3.50E+06
	count3	1.54E+03	4.70E+03	2.15E+04	5.68E+03	5.60E+05	2.45E+05
	average	1.32E+03	1.45E+04	8.56E+03	3.67E+03	5.21E+05	1.39E+06
bottom							
	count1	3.10E+03	2.57E+03	5.68E+03	3.42E+03	4.20E+05	1.79E+06
	count2	5.41E+03	3.41E+04	1.41E+03	4.51E+04	3.50E+06	4.50E+06
	count3	4.31E+03	1.12E+03	3.68E+03	3.46E+04	2.45E+05	8.45E+05
	average	4.27E+03	1.26E+04	3.59E+03	2.77E+04	1.39E+06	2.38E+06

Table 4.4: Cell density of adherent fraction of biofilm in case of bottom farnesol tubing in case of ethylene oxide sterilization

section		Run 1	Run2	Run3	Run4	Run5	Run 6
	Tubing	Bottom farnesol	Bottom farnesol	Bottom farnesol	Bottom farnesol	Control-1	Control-2
Top		(cell/cm)	(cell/cm)	(cell/cm)	(cell/cm)	(cell/cm)	(cell/cm)
	count1	1.50E+05	1.34E+05	9.20E+04	7.20E+04	1.65E+06	5.61E+05
	count 2	7.91E+04	4.50E+04	3.42E+04	4.51E+04	7.12E+05	2.36E+04
	count3	5.50E+03	1.11E+04	6.40E+03	9.80E+02	7.23E+06	5.41E+05
	average	7.82E+04	6.34E+04	4.42E+04	3.94E+04	3.20E+06	3.75E+05
Bottom							
	count1	1.30E+03	1.79E+03	2.18E+03	2.70E+02	3.45E+05	1.21E+04
	count2	4.52E+02	4.31E+02	1.89E+03	4.10E+02	9.23E+05	4.50E+05
	count3	2.10E+02	1.56E+02	1.12E+02	1.20E+02	4.25E+04	3.20E+04
	average	6.54E+02	7.94E+02	1.39E+03	2.67E+02	4.37E+05	1.65E+05

Data (Table 4.3 and 4.4) from ethylene oxide showed the most effect on cell attachment. As shown in Table 4.3, there was drastic reduction in the amounts of cell attaching to the tubing. The trend has been that top farnesol tubing has shown overall the best case. The top section of tubing had fewer cells as compared to any other region. The bottom section in this case also had lower amount of cells probably due to farnesol released from the top section of the tubing.

Table 4.4 shows that clearly farnesol is affecting the attachment of cells. There was drastic reduction in cells in the bottom section of the tubing. The effect was seen in the top section of this tubing as there was more farnesol in the general system. Since farnesol is semivolatile, some amount of farnesol was getting vaporized during steam sterilization. Hence, less farnesol was present in steam-sterilized tubing. This explains the difference seen in cell attachment in case of steam sterilization and ethylene oxide sterilization.

This work shows that amount of cells attaching to the tubing was drastically reduced by incorporating farnesol into the tubing. One of the facts established with researchers was that farnesol does not increase the resistance in *C.albicans*. Combined with this fact and that farnesol reduces attachment to surfaces, this strategy is a possible solution to the big problem of biofilm formation on surfaces.

4.5 Discussion

The result confirms the fact that farnesol affects the biofilm formation in C. *albicans*. Ethylene oxide sterilized tubing showed a drastic reduction in the

amount of cells attached to the tubing surface. Biofilms are highly organized communities of cells (Costerton 1995). Like the cells of a tissue that communicate via autocrine and paracrine stimulation, cells of microbial biofilms release chemical compounds that act in concert, reaching threshold densities that signal the initiation of coordinated cellular differentiation events (Davies 1998, Miller 2001, Parsek 1999, Singh 2000). In essence, biofilms may represent the foundation of multicellular life. Understanding the way in which these complex structures communicate provides insights into biofilm prevention strategies.

Most of the current literature on quorum sensing to tackle biofilm formation has been carried out on bacterial biofilms. Inhibition of quorum-sensing in *Staphylococcus* has been shown to enhance biofilm formation. Down-regulation or mutation of the *Staphylococcus* quorum-sensing system increases bacterial persistence in device-related infection, suggesting that interference with quorum-sensing would enhance rather than suppress this important type of staphylococcal disease (Otto 2004). In the case of *Pseudomonas aeruginosa* biofilms, the quorum sensing molecule did not affect initial attachment to the abiotic substratum. It does, however, affect the architecture of the biofilm and enhances the process of bacterial detachment, leading to a loss of bacterial biomass from the substratum (Hentzer 2002). It seems self-evident that quorum sensing may govern biofilm development, and indeed bacterial quorum-sensing molecules have been shown to govern both aggregation and dispersal of biofilm cells (Hammer 2003, Parsek 2005, Suntharalingam 2005).

This strategy of cell-cell communication benefits the biofilm community by preventing and controlling unnecessary overpopulation and competition for nutrients and has important implications for the infectious process, especially for dissemination and establishment at distal sites of infection (Ramage 2002). Exogenous farnesol inhibits biofilm formation (Ramage 2002) and, in so doing alters expression of 274 genes (Cao 2005).

Farnesol plays a crucial role in biofilm forming ability of *C. albicans* as proven by the farnesol incorporated tubing studies. This is the first study that employed continuous release of farnesol from tubing. The attachment of cells to the surface is proven to be the trigger for formation of persister cells. Hence by reducing the amount of cells attaching, there is a drastic reduction in this critical population. Biofilm formation is a common problem associated with implanted medical devices. Medical device centered infections are an increasingly alarming problem, and due to their intrinsic recalcitrance to conventional therapy, new methods of dealing with these infections are harder to develop. The only solution to this problem is physical removal of the medical device. This is an expensive medical procedure and also risky at times.

Hence by reducing the attachment of cells to the surface, the problem of biofilm formation can be tackled. Farnesol proves to be an interesting prospect as an anti-infective strategy in this setting. This finding provides evidence that there are alternatives to the traditional mode of Figurehting, hard to treat, fungal infections. Today, we find ourselves in urgent need for novel antimicrobial drugs, as many important human pathogens have acquired multiple antibiotic resistance

factors. Hence by designing an effective mixture of quorum sensing agents for different species that attach to medical devices, the problem of biofilm formation can be derailed.

CHAPTER 5

ORIGINAL CONTRIBUTIONS AND FUTURE DIRECTIONS

1. **Development of an in vitro model for biofilm:** The tubular biofilm flowcell is a better representation of the in vivo biofilm model as compared with the multiwell batch model used by current researchers. The advantages include well defined shear conditions, continuous replenishment of media and transport of waste. The most important aspect is the separation of biofilm into two subpopulations: shear removed fraction and basal blastospores / persister cell subpopulation. This step is critical as the more resistant, Basal blastospores are less than 2% of the biofilm. On treating them as a whole, the molecular information from these cells will be masked by the majority fraction.
2. **Characterized the sterols in the membrane for different subpopulations**: The persister cell subpopulations came out to be the most different cell populations as compared with the rest of the species. There was a decrease in ergosterol levels in these cells and also there was expression of different intermediates of the sterol cycle. These data correlate well with other studies done in our lab and stress the importance of looking at the persister cell subpopulation. This is consistent with the resistant behavior exhibited by persister cell subpopulation, yet does not explain the whole

picture. In future, the study of the cell wall components will elucidate the role of cell wall in resistance.

3. **Phosphorylation of p42-44 family MAP kinases:** It was detected only in the persister cell subpopulation. I identified Cek1 and Mkc1 phosphorylation in this subpopulation. These MAP kinases are typically associated with cell integrity and cell wall repair in *C. albicans.* The fact that these subpopulations have phosphorylation of these kinases indicates that they are responding to some stress signal that is associated with being attached to surfaces. Hence respond in a hunkered down version with lesser metabolic activity and also changes in their membranes. These results indicate that there are major changes in genetic level expression in these cells. It is not the effect of one pathway at a time. Rather there are changes in quite a few pathways. In future, a better understanding of what triggers these signals will help in understanding the stress that causes a switch to resistant phenotype. These data also prove that cell wall study gives more answers towards understanding the resistant behavior of basal blastospores. One of the interesting experiments will be to block the phosphorylation of these MAP kinases and see if these cells are still able to show the same amount of resistance. This will positively link these MAP kinases with the resistant phenotype switching.

4. **Role of farnesol in biofilm formation:** Farnesol was deterring the attachment of cells onto the surface. There was drastic reduction in the amount of cells that attached onto tubing surface. These molecules have

been shown not to induce any from of resistance buildup in *C .albicans*. With the failure of most available antifungal drugs to treat biofilm related infection, this experiment gives a new direction towards inhibiting cells from attaching to surface. These molecules, along with a mixture of other quorum sensing molecules could serve as a possible solution to development of better biomaterials that resist biofilm formation. In future, attempts could be made to immobilize farnesol molecules onto the tubing material and carry out attachment pattern studies.

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