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***PSEUDOMONAS AERUGINOSA* - *CANDIDA ALBICANS* INTERACTIONS FROM ECOLOGICAL AND MOLECULAR PERSPECTIVES**

by

FATHIMA FARAZ RINZAN

Under the Direction of Sidney A. Crow, Jr.

ABSTRACT

Pseudomonas aeruginosa and *Candida albicans* have shown antagonistic relationships, both *in vivo* and *in vitro*. The interaction between *P. aeruginosa* and *C. albicans* is one of the many prokaryotic-eukaryotic interactions existing in nature and needs more research due to their complex pathogenicity in humans. In this work, we have studied growth dynamics of *Candida* in a mixed population of *Pseudomonas* and *Candida*, and their receptor and ligand specificities, both from an ecological and a molecular point of view. Initially, growth, viability, and morphogenesis of *Candida* were studied in the presence of *Pseudomonas* and the conditioned medium of *Pseudomonas* using two *Candida* strains, namely CAF2 and *tup1* mutant. The killing effect of *Pseudomonas* was more pronounced on the hyphal form of *Candida*. However, growth of *Candida* was inhibited by *Pseudomonas* irrespective of its morphological form. The conditioned medium had no effect on the growth rate of *Candida*. Nevertheless, it completely inhibited the germination of *Candida*.

The attachment of *Pseudomonas* to *Candida* was studied using different strains of both, and with *Pseudomonas* cells harvested at different stages of its growth. The percent attachment varied with the age of the *Pseudomonas* culture. A *lecB* mutant of *Pseudomonas* showed a two–fold reduction in attachment compared to the wild type PAK strain. Carbohydrate inhibition studies confirmed that LecB is not directly involved in the attachment mechanism, but indirectly involves through regulating the expression of other proteins required for attachment.

A genomic DNA library of *Pseudomonas* PAO1 was screened for clones that had acquired the ability to attach to *C. albicans*. A clone was isolated with a small increase in attachment and was subjected to genetic analysis. It contained nucleotides 458565 to 475917 of the *Pseudomonas* genome including some genes of the *Pil-Chp* gene cluster. Seven transposon mutants that represent mutations in *ChpA,B,C,D,E* operon and three other ORFs were selected, and their attachment ability was tested. All seven mutants showed a reduction in attachment indicating that this was a non specific effect, which could be attributed to the *in vitro* manipulation of the bacteria. We conclude that the attachment of *Pseudomonas* to *Candida* is multi-factorial.

INDEX WORDS: *Pseudomonas aeruginosa*, *Candida albicans*, Attachment, Morphogenesis, Adhesins, LecB, Carbohydrate inhibition, Primary attachment, PAO1 genomic library

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FATHIMA FARAZ RINZAN

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Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

2009

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by

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May 2009

To my husband Rinzan,

children Sarah and Rayhan,

Mom and Dad,

and Grandma

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CHAPTER 1: INTRODUCTION

Inter–kingdom or interspecies interactions are ubiquitous in nature. These interactions are important for survival of species and ecological balance. Some of these interactions are beneficial and some are detrimental from a human standpoint. The interaction between *Pseudomonas* and *Candida* is an example of many prokaryotic-eukaryotic interactions, which needs more research due to the pathogenic nature of both organisms in humans. Acquiring an in-depth understanding of these interactions can be exploited for the benefit of mankind. Although phylogenetically, *Pseudomonas aeruginosa* and *Candida albicans* belong to two different domains of life, viz. prokaryotes and eukaryotes, they share many functional characters. A few traits shared among them are; being opportunistic pathogens in immunocompromised patients, ability to form biofilms on prosthetic devices, and increased drug resistance (Aaron et al., 2002; Chandra et al., 2001; Douglas, 2003; Ramage et al., 2001). These two organisms have shown antagonistic relationships both *in vivo* (Kerr, 1994) and *in vitro* (El Azizi et al., 2004; Hogan and Kolter, 2002; Hogan et al., 2004; Kerr, 1994; Kerr et al., 1999). Observation of an antagonistic relationship between microorganisms led to the discovery of antibiotics. As science and technology advanced, many such interactions have been recognized. It is now known that antagonistic interactions between microorganisms are not limited to antibiotics, but to a vast amount of cellular and extracellular factors produced by these organisms, that are either constitutively or conditionally regulated. The focus of this project is to study one such antagonistic interaction between *P. aeruginosa* and *C. albicans* both from an ecological and a molecular point of view.

1.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative aerobic rod. The planktonic state is motile by a single polar flagellum and it also has the ability to exist in sessile state as a biofilms. In the biofilm mode, the cells are attached by an alginate exopolysachcharide matrix to the surface (Wixon, 2000). *P. aeruginosa* is environmentally versatile and adapts and thrives in many ecological niches such as soil, water, plant and animal tissue. The exceptional ability to colonize ecological niches where nutrients are limited is attributed to its capability to utilize a variety of organic compounds as food sources (Stover et al., 2000).

This organism has one of the largest bacterial genomes sequenced to date. The genome is 6.3-Mbp which contributes to its complexity compared to many other bacteria (Stover et al., 2000). Comparison of the distribution of genes amongst functional categories has shown that *P. aeruginosa* has the largest proportion of regulatory genes of all the sequenced bacterial genomes (Wixon, 2000).

Pathogenesis and Virulence factors

P. aeruginosa is an opportunistic pathogen that causes serious infections in immunocompromised patients (Van Delden and Iglewski, 1998). The three main diseases caused by *P. aeruginosa* include bacteremia in severe burn victims, chronic lung infections in cystic fibrosis patients, and ulcerative keratitis in extended wear soft contact lens users (Lyczak, 2000). In addition to these diseases, *P. aeruginosa* is also responsible for nosocomial infections (Avichezer et al., 1992). The virulence of this organism is attributed to a large number of cell-associated (flagellum, pilus, nonpilus adhesins, alginate or exopolysaccharide) and extracellular (proteases, hemolysins, exotoxin A, exoenzymes, pyocyanin) factors (Gilboa-Garber, 1988; Van Delden and Iglewski, 1998). The cell-associated factors aid in translocation and primary attachment of the organism to host. Cell-associated factors are prerequisites for infection whereas the extracellular factors aid in in-

vasion and progression of disease. For example, in burn wound infections pili and flagella render *P. aeruginosa* the ability to persist at the wound site and disseminate through the wound. Elastase degrades collagen and non-collagen proteins and disrupts the integrity of the host basement membrane, and promotes spread of infection. At present, chronic bacterial lung infection accounts for the majority of the morbidity and mortality in cystic fibrosis (CF) patients. Cystic fibrosis is an autosomal recessive disorder resulting from mutation of the cyclic AMP regulated chloride ion channel protein known as CF transmembrane conductance regulator. Once chronic infection is established, *P. aeruginosa* changes its phenotype from a non-mucoid to mucoid form that produces alginate or mucoidexopolysacchcharide. Alginates play an important role in adherence of bacteria to host cells and evasion of the host immune response. Ulcerative keratitis is a rapidly progressing inflammatory response to bacterial infection of the cornea, and has been called the most destructive bacterial disease of the human cornea. *P. aeruginosa* glycocalyx, endotoxin, exotoxin, protease, flagella, and pili are factors which affect the initiation and progression of corneal infection (Lyczak, 2000).

1.2 *Candida albicans*

Candida albicans is a polymorphic fungus with yeast, hyphal and pseudohyphal forms in its life cycle. It is a commensal organism in healthy individuals and an opportunistic pathogen in immunocompromised patients (Douglas, 2003). *C. albicans* is found as part of the normal flora of the oral cavity, gastrointestinal tract and female genital tract of humans (Fowler and Jones, 1992). Immunosuppresive therapy, antibiotic therapy, use of indwelling devices, HIV infection, diabetes and old age are some of the predisposing factors for *C. albicans* infection (Ramage et al., 2002).

Pathogenesis and virulence factors

C. albicans is the fourth most commonly isolated blood stream pathogen and the third leading cause of catheter related infections (Ramage et al., 2002). It causes a variety of superficial, mucosal, and systemic infections (Douglas, 2003). Candidiasis is found to be associated with biofilm formation on prosthetic medical devices such as catheters, heart valves, dental implants, vascular bypass grafts, ocular lenses, artificial joints, and central nervous system shunts (Chandra et al., 2001). A number of virulence factors have been proposed for this organism, including the production of hydrolytic enzymes such as aspartylproteases and phospholipases (Ibrahim et al., 1995), morphogenesis (Liu, 2001), phenotypic switching (Jin et al., 2005) and host recognition biomolecules or adhesins (Calderone and Fonzi, 2001).

Morphology and morphogenesis

C. albicans is a polymorphic fungus. Four distinct morphological forms have been identified in its life cycle: yeast, hyphae (germ tubes), pseudohyphae, and chlamydospores. Reproduction is by budding and formation of yeast cells which are also known as blastospores or blastoconidia. Hyphae are generated by budding cells by changing their growth mode to continuous apical extension followed by septation. Pseudohyphae are created by unipolar budding with the newly formed cells remaining attached to the mother cell. Both pseudohyphae and true hyphae produce chains of cells. True hyphae have no constrictions at their septa, and they have parallel cell walls with branches forming perpendicular to the walls. Also, *C. albicans* can form chlamydospores, which are round, refractile spores, larger than yeast cells, with a thick cell wall under certain non-optimal growth conditions (Cassone, 1989; Sudbery et al., 2004).

Morphological changes are often a survival strategy used by the fungus to adapt to new environmental conditions (Cassone, 1989; Sudbery et al., 2004). The yeast to hyphal morphogenesis

is thought to be important for tissue invasion and progression of infection (Gow et al., 2002). A correlation has been noticed between fungal adherence to human buccal epithelial cells and formation of germ tubes (Kimura and Pearshall, 1980). Some important environmental factors that affect the morphological changes and germ tube formation in *C. albicans* are temperature, pH, CO_2, carbohydrates, presence of serum, inoculum size and media composition such as nitrogen or carbon starvation, (Beheshti et al., 1975; Hagihara et al., 1988; Hudson et al., 2004; Joshi et al., 1975; Senet, 1997). Most of these factors impose a stress on the fungus (Brown and Gow, 1999). Yeast–hyphal morphogenesis is regulated by a complex network of signaling pathways (Arigmon et al., 2007). Hyphal growth is stimulated by activation of the transcription factors Cph1p and Efg1p which belong to the mitogen-activated protein kinase (MAPK) and Ras–cAMP pathways, respectively (Brown and Gow, 1999), whereas it is inhibited by transcriptional repressors Tup1, Ngr1 and Rfg1 (Arigmon et al., 2007). Two quorum sensing molecules, tyrosol and farnesol, produced by *C. albicans*, were also found to regulate morphogenesis. Tyrosol stimulates germ tube formation (Chen et al., 2004) while farnesol inhibits it (Hornby et al., 2001).

Cell wall

In most fungi, the cell wall is the outermost layer and plays a major role in maintaining the characteristic shape of the fungus and virulence or pathogenicity of the organism. It is responsible for interactions with the external world including inert surfaces and other biological forms (Chaffin et al., 1998).

Transmission electron microscopy has shown the existence of several layers in the cell wall. The cell wall of *C. albicans* is mainly composed of carbohydrates (approximately 80 to 90%). It also contains proteins (6-25%) and minor amounts of lipids (1–7%). The carbohydrate contents are (i) branched polymers of glucose containing β–1,3 and β–1,6 linkages (β–glucans), (ii)

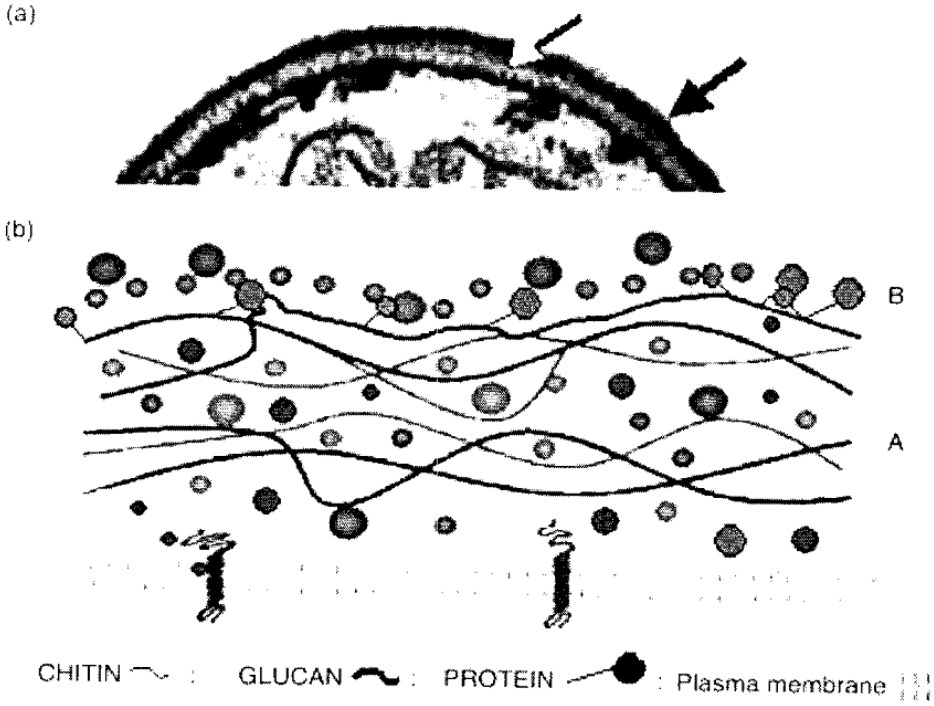

Figure 1.1: Structure and schematic representation of the architecture of *C. albicans* cell wall (Ruiz-Herrera, 2006). (a) Electron micrograph of a median cell section. Inner layer of the wall is made mainly of polysaccharides (β–glucans and chitins) and small amounts of proteins. (b) The electron dense outer layer is built mostly of different types of mannoproteins linked to either β–1,3 or β-1,6–glucan chains by covalent bonds; glucans in turn are linked to chitin microfibrils and, together with some proteins, give rise to basic composite.

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). The percent composition of β-glucans, chitins, and mannan vary according to the morphological form of *C. albicans*.

Among these carbohydrates, β-glucans and chitin form the structural components of the wall that provide rigidity, and are more concentrated in the inner wall layer. Mannose polymers (mannan) that exist as mannoproteins are dominant in the outermost cell wall layer, and are the main materials of cell wall matrix (Chaffin et al., 1998). These mannoproteins appear as a dense network of radially projecting fibrils, referred to as fimbriae (Cassone, 1989). Both the yeast and hyphal forms of *C. albicans* exhibit this characteristic feature (Bobichon et al., 1994). The major structural sub unit of fimbriae is a glycoprotein, and these components mediate the adherence of *C. albicans* to glycosphingolipid receptors on human epithelial cells (Chaffin et al., 1998).

Physical interactions between the fungus and the host are mediated by proteins, mannoproteins, chitins, β–glucans, and lipids present in the cell wall as they are components of the outer most surface of the organism. Mannoproteins are the major mediators of adhesion. These consist of O-glycosylated oligosaccharides and N-glycosylated polysaccharide moieties that are important in host–fungal interactions and virulence (Shibata et al., 2007). The mannans consist of acid-stable and acid-labile regions, which are connected by a phosphodiester group. The acid-stable region is composed of α–1,6-linked mannopyranose units that form the backbone. Many branches of α–1,2, α–1,3, and/or β–1,2 linked mannopyranose units are/is connected to the second carbon of the large parts of α–1,6-linked mannopyranose units in the backbone moiety. The acid-labile region is composed of β –1,2–linked oligomannosyl residues that are linked to the phosphate group via the reducing terminal group of each oligosaccharide residue. This region is connected to the acid-stable region with another linkage of the phosphate group in a diesterified form (Shibata et al., 1992).

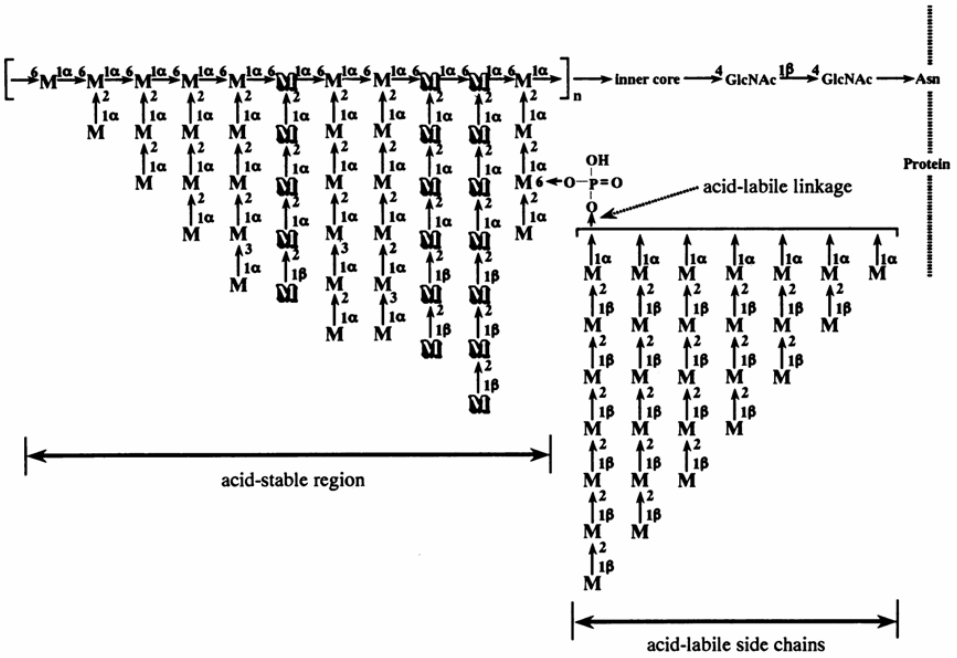

Figure 1.2: Representative structure of *C. albicans* mannans (Shibata et al., 1992). 'M' denotes D-mannopyranose residues.

1.3 Primary attachment and biofilm formation

Biofilms are defined as three dimensional aggregates of microorganisms (either single or multiple species) that are embedded in an exopolysacharide matrix produced by them. The cells are phenotypically different from their planktonic counterparts (Douglas, 2003). The biofilm mode of growth is studied more extensively than the planktonic as a significant proportion of microbial infections in humans involve biofilms. Compared to the planktonic cells, those in biofilms are less susceptible to antimicrobial agents (Mateus et al., 2004; Ramage et al., 2001).

Primary adherence to a surface is considered a prerequisite for biofilm formation and infection (Ahearn et al., 1999). A combination of factors such as strain, substratum, and environmental conditions plays an important role in determining the degree of adherence, with strain properties being dominant (Ahearn et al., 1999). Primary adhesion between bacteria and abiotic surfaces is generally mediated by nonspecific (e.g., hydrophobic) interactions, whereas adhesion to living tissue is accomplished through specific molecular interactions utilizing lectins, ligands, or adhesins (Dunne, 2002). These interactions can form between proteins and carbohydrates or between two proteins.

P. aeruginosa forms biofilms on a variety of biotic and abiotic surfaces. This has become a challenge in clinical settings due to the complexity and increased resistance to antimicrobial therapy attributed to biofilm mode of growth. Cystic fibrosis, endocarditis, device-related infections, and ventilator-associated pneumonia are some of the diseases that are considerably complicated by the formation of bacterial biofilms (Tart and Wozniak, 2008). A number of cellular factors such as flagellum, pili, nonpilus adhesins, and alginate or exopolysaccharides play an important role in adherence and biofilm formation of *P. aeruginosa* (Gilboa-Garber, 1988). Flagella provide motility and chemotaxis functions (Li et al., 2007) as well as play an important role in surface attachment and colonization (O'Toole and Kolter, 1998). Flagella proteins are involved in adhesion to host cells

and molecules *in vitro*. It may bind to mucins, the glycolipid asialoGM1, as well as toll-like receptor 5, inducing inflammation (Doring and Pier, 2008). Also, previous studies have demonstrated that *Pseudomonas* flagellum is variably glycosylated (Schrim et al., 2006).

Type IV pili are long flexible filaments that traverse the outer membrane of gram negative bacteria and contribute to their survival and pathogenesis (Hansen and Forest, 2006) including the biofilm mode of growth (O'Toole and Kolter, 1998). These pili provide a type of surface motility known as twitching motility (Hansen and Forest, 2006) that results from the extension and contraction of type IV pili (Dunne, 2002). They also play an important role in adhesion, autoaggregation, and host cell invasion (Hansen and Forest, 2006). Type IV pili are composed of a single pilin subunit, PilA, and are assembled into long polar surface appendages. The C-terminal receptor binding domain is located at the tip of the pilus and has evolved to retain a structure for binding of both biotic and abiotic surfaces (Glitner et al., 2006; Lee et al., 1994). Mutants defective in flagella motility have been shown to be unable to form a monolayer of cells on a surface, while the mutants deficient in type IV pili form monolayers, but not cellular aggregates. This indicates that flagellar motility is required for primary adhesion and type IV pili are essential for cellular aggregation (Dunne, 2002).

In *P. aeruginosa*, the exopolysaccharide glycocalyx is mainly composed of alginic acid, a linear polymer consisting of β–(1, 4)–linked D–mannuronic acid and L–glucuronic residues. The production of alginic acid by *P. aeruginosa* is upregulated in response to various environmental factors, including high osmolarity, high oxygen tension, ethanol exposure, and nitrogen limitation (Dunne, 2002). It mediates adhesion of bacteria to host cells, tissues, and other bacteria resulting in large aggregates of bacteria, which are firmly attached to substratum (Lyczak, 2000). There is evidence that adherence of bacteria to the respiratory epithelial cells (Doig et al., 1987) was increased in the presence of alginate.

Similarly, *C. albicans* also forms biofilms on implanted bio-materials (stents, shunts, prostheses, implants, endotracheal tubes, pacemakers, and catheters etc.,) and host surfaces (Ramage et al., 2005). *C. albicans* biofilms are characterized by an initial adherence of yeast cells (0-2h), followed by germination and microcolony formation (2-4h), filamentation (4-6h), monolayer development (6-8h), proliferation (8-24h), and maturation (24-48h). Mature biofilms consist of a dense network of yeast cells and hyphae embedded within an exoploymeric material (Ramage et al., 2001).

In *C. albicans*, primary adherence is mediated by adhesins (Calderone and Fonzi, 2001), which are biomolecules that promote adherence to host cells or host ligands. Adhesins are located on the cell wall of the organism aiding it to interact with the environment. These interactions define its pathogenecity and confer the ability to survive in its niche. Expression of adhesins is important in the transition from commensalisms to pathogenicity and is influenced by particular host conditions (Senet, 1998). Adhesins are mosaic proteins with several domains arranged in a standard order: N-terminal secretion signals, domains for ligand binding, N- and O-glycosylated stalks that elevate the binding domains above the wall surface and C-terminal regions. These regions mediate covalent cross linking to the wall matrix through modified glycosylphospatidylinositol (GPI) anchor. The interactions between adhesins and the substrate can be either weak or strong depending on the concentrations of adhesins and/or ligands (Dranginis et al., 2007).

Some of the *C. albicans* adhesins include proteins of the Als family, Eap1p, and Hwp1p. Als adhesins mediate adhesion to epithelia, yeast aggregation, and biofilm formation. The family is encoded at eight loci. The expression of different adhesins varies with the phase of growth and infection. Als1 expression is maximal just after inoculation into fresh growth medium and Als3 expression is maximal when germ tubes are microscopically visible. Als1p is the most widely expressed member of the family and contributes to adhesion and colonization (in endothelia and epithelia), whereas Als3p is also important for biofilm formation. Eap1p is a hydrophobic cell

surface protein that mediates binding to plastic. Hwp1p is a glutamine rich, GPI wall–anchored adhesin that participates in a covalent cross link between the *Candida* cells and the host epithelium. The resulting association would be shear resistant, extremely close, and permanent in the absence of proteolysis (Draginis et al., 2007).

1.4 Lectins and carbohydrate inhibition

Lectins are carbohydrate binding proteins that are sugar-specific, and present widely in nature (Garber et al., 1987). Adherence to surface is considered to be a prerequisite for both colonization and infection (Beuth et al., 1987). Adherence is crucial for initiation of infection, since it enables the bacterial enzymes or toxins to act on the host cells and macromolecules at optimal efficiency (Gilboa-Garber et al., 1997). Lectins facilitate adherence to macromolecules and co-aggregation with other bacteria, which may function together in damaging the host (Gilboa-Garber et al., 1997). Specific adhesion is mediated by lectins interacting with defined carbohydrate structures on various mucosal cell surfaces or within tissues (Beuth et al., 1987). Therefore, their role in adhesion and agglutination make them important in both symbiotic and pathogenic interactions (Slifkin and Doyle, 1990).

Many studies have shown that there can be competition among organisms for lectin receptors, and infection with one microorganism may destroy or uncover receptors for another (Uhlenbruck, 1987). For example, *in vitro* experiments with frozen sections of human lung and kidney have demonstrated that adherence of *Streptococcus pneumoniae* and *P. aeruginosa* to human cells was mediated by bacterial lectins (Beuth et al., 1987). It is important to note that the binding of *P. aeruginosa* to cystic fibrosis epithelium was also found to be lectin dependent (Imundo et al., 1995). *P. aeruginosa* produces two cytoplasmic lectins, LecA (PA-I) and LecB (PA-II) that differ in their carbohydrate specificities (Gilboa-Garber, 1982). LecA binds to D-galactose, whereas LecB

exhibits a high specificity for L-fucose and its derivatives (Garber et al., 1987; Gilboa-Garber et al., 2000) and for D-mannose with comparatively lower affinity. Both are present inside the cell (highest activity), in its periplasmic space, on the cell surface, and may also be secreted to the culture medium. The distribution of the lectins in different compartments of the cell changes with the environmental conditions and the form of growth (Gilboa-Garber, 1988; Glick and Garber, 1983; Nicas and Iglewski, 1985).

Lectin-carbohydrate interaction is carbohydrate and receptor specific, and reversible (Gilboa-Garber et al., 1997). Therefore, attachment inhibition by carbohydrates or heamagglutination activity is used to identify the lectin receptors (Gilboa-Garber, 1972; Glick and Garber, 1983; Wentworth et al., 1991). When lectins were mixed with intact *P. aeruginosa* cells it caused the bacteria to bind to rabbit corneal epithelial cells. This process was reversible with the addition of D-galactose and D-mannose. These sugars had no effect on the adhesion of lectin-free *P. aeruginosa* cells (Wentworth et al., 1991). Moreover, *P. aeruginosa*-induced otitis externa diffusa and respiratory tract infections could be successfully treated by applying a solution containing LecA and LecB specific sugars, which prevents lectin-mediated bacterial adhesion to the corresponding host tissue (Tielker et al., 2005).

The cell wall of *C. albicans* is mainly composed of carbohydrates. Among the three basic carbohydrates, mannose polymers (mannan), which are found in covalent association with proteins (mannoprotein), are the main material of the cell wall matrix. The mannoproteins and proteins bind to host ligands or contribute to hydrophobic interactions (Chaffin et al., 1998). Lectins with different sugar specificities are useful for determining the organization of the polysaccharides in yeast cell walls (Slifkin and Doyle, 1990).

Concavalin A (ConA) is another carbohydrate binding protein (lectin) isolated from jack beans (*Canavalia ensiformis*) (Goto et al., 2002). This lectin has a high affinity to D-mannose and

D-glucose (Portez and Goldstein, 1970). Receptors for ConA are generally either glycoproteins or glycolipids. The oligosaccharide side chains of glycoproteins function as receptor determinants. The saccharide binding specificity of ConA is directed towards the pyranose forms of the monosaccharides, glucose and mannose. Both sugars contain similar hydroxyl group configurations at 3-,4-,and 6- positions. ConA binds α anomers of these glycosides more strongly than the β anomers. There are two main classes of linear oligosaccharides that bind to ConA with different affinities. The first class that includes α(1-3), α(1-4), and α(1-6) oligosaccharides contains a non reducing terminal glucose or mannose residue, and it has no enhanced binding relative to monosaccharide. The second class includes α(1-2)-mannosyl oligosaccharides and shows enhanced binding (Brewer and Bhattacharyya, 1986). Researchers found two important mechanisms that enhance the binding of saccharides and glycopeptides to ConA: (i) clustering multiple α(1-2)-linked mannose (or glucose or GlcNac) residues in a molecule and (ii) binding of the trisaccharide moiety in molecules to the extended binding site of ConA (Brewer and Bhattacharyya, 1986). The interaction of ConA with the cell walls of *Candida* species has been demonstrated. The wide spectrum of ConA binding sites is associated primarily with mannan receptors on their cell surfaces (Goto et al, 2002). Among the monosaccharides, methyl-α-D-mannopyranoside is the most potent inhibitor of ConA-dextran complex formation, whereas the mannotriose 3,6-di-o-(α-D-mannoyl)-α-D-mannoside was found (Goto et al., 2002) to be the most potent oligosaccharide (more potent than Methyl-α-D-mannopyranoside).

1.5 *Pseudomonas–Candida* interactions

P. aeruginosa and *C. albicans* have shown antagonistic relationships both *in vivo* (Kerr, 1994) and *in vitro* (El Azizi et al., 2004; Hogan and Kolter, 2002; Hogan et al., 2004; Kerr, 1994; Kerr et al., 1999). The first report of antifungal activity of *P. aeruginosa* was made by Jonathan

Kerr in 1994. Although *P. aeruginosa* suppressed the growth of *C. albicans* in cystic fibrosis patients, regrowth of *C. albicans* was seen when *P. aeruginosa* was eradicated (Kerr, 1994). Following this observation, the same group studied the inhibitory activity of *P. aeruginosa* on *C. albicans* and demonstrated that different phenazines produced by *P. aeruginosa*, such as pyocyanin and 1-hydroxyphenazine, are reasons for such antagonistic interactions (Kerr, 1994; Kerr et al., 1999).

A study conducted by Hogan and Kolter (2002) demonstrated that *P. aeruginosa* forms a dense biofilm on *C. albicans* filaments and subsequently kills the fungus whereas it neither binds nor kills the yeast form of *C. albicans*. This could be due to the differences in the cell wall of *C. albicans* yeast and hyphal forms. Another study conducted by Hogan et al. (2004) demonstrated that a quorum sensing molecule, 3-oxo-C(12) Homoserine lactone, of *P. aeruginosa,* inhibits *C. albicans* filamentation, which is a prerequisite for adherence, biofilm formation, and subsequent infection.

The first clinical study to identify *P. aeruginosa–C. albicans* interactions was done by Azoulay et al. (2006). They identified *Candida* spp. tracheobronchial colonization as an independent risk factor for *P. aeruginosa* ventilator associated pneumonia (VAP). Following this observation, Saad et al. (2007) conducted a retrospective case-control study to determine the impact of antifungal treatment on *P. aeruginosa* VAP or tracheobronchial colonization in patients with *Candida* spp. tracheobronchial colonization. Their results suggest that antifungal treatment is associated with reduced risk for VAP or tracheobronchial colonization related to *P. aeruginosa*.

Another group investigated the *in vivo* anti-*Candidal* activity of *P. aeruginosa* in mice with subcutaneous infection and demonstrated that *P. aeruginosa* strains inhibit growth of *Candida* strains completely (Kaleli et al., 2006). Moreover, El- Azizi et al. (2004) reported that *C. albicans* biofilms could hold other organisms including *P. aeruginosa* within the endotracheal tube.

1.6 Rationale

As discussed above, *P. aeruginosa* and *C. albicans* have shown antagonistic interactions in many instances. Although such interactions have been associated with several factors, *Pseudomonas–Candida* interactions remain complicated due to the presence of multiple virulence factors and variability in expression of these virulence factors depending on environmental conditions.

Growth, germ tube formation, primary adherence, and biofilm formation are well known virulence factors of *C. albicans*. Growth in any form is an essential step for pathogenesis. Morphological changes in *C. albicans* in response to environmental factors have been related to its pathogenesis (Liu, 2001). Further, germ tube formation is important for tissue invasion (Rodrigues et al., 1999; Senet, 1998) and filaments adhere more and secrete more hydrolytic enzymes (Senet, 1998). Studies have shown that there is a correlation between relative virulence and the ability to adhere to epithelial cells or other substrata (Beuth et al., 1987; King et al 1980; Rodriguez et al., 1999). Moreover, hydrophobic interactions are important in adherence of microorganisms to eukaryotic cells and inert surfaces, and germ tube formation was associated with a significant rise in cell surface hydrophobicity (Rodrigues et al., 1999).

The cell wall of *C. albicans* is mainly composed of carbohydrates. Among these, the mannose polymers (mannan), which are found in covalent association with proteins (mannoprotein), are the main material of the cell wall matrix. The mannoproteins and proteins bind to host ligands or contribute to hydrophobic interactions (Chaffin et al., 1998). Therefore, it is possible that lectins of *P. aeruginosa* bind to these mannoproteins, preventing the adherence of *C. albicans* to substrata such as epithelial cells and prosthetic devices. Another possibility is that both organisms compete for host receptors (Yu et al., 1994) and *P. aeruginosa* prevents *C. albicans* adherence and progression by competitive inhibition. This may down-regulate the expression of virulence genes of *C. albicans*. Studies have shown that there can be competition among organisms for lectin

receptors, and infection with one microorganism may destroy or uncover receptors for another (Uhlenbruck, 1987).

A significant proportion of human microbial infections including *Pseudomonas* and *Candida* infections involve biofilms. Although there are antibiotics and antifungals available for treatment of infections caused by both organisms (Fowler and Jones, 2002), the drug resistance and multi-factorial nature of virulence in both organisms have made preventing and treating these infections increasingly challenging. The latest approach to prevent biofilm formation is to reduce or inhibit the primary attachment of microorganisms, which is considered an important step in pathogenesis (Bavington and Page, 2005).

The objective of this study is to widen the understanding of the interactions among these two organisms. Our study involved two different approaches. The first approach is from an ecological standpoint, where we looked at the effect of *P. aeruginosa* on growth, viability and morphogenesis of *C. albicans*. The other was a molecular approach to identify adhesins and receptors involved in the attachment of *P. aeruginosa* to *C. albicans*. An in-depth understanding of interactions between these organisms in conjunction with unraveling the attachment mechanism will facilitate vital therapeutic measures.

CHAPTER 2: MATERIALS AND METHODS

2.1 Media and chemicals

All chemicals used during this study were obtained from Sigma (St. Louis, MO) or Difco (BD, Franklin Lakes, NJ). Restriction enzymes were purchased from New England Biolabs (Ipswich, MA).

Growth media

Both bacteria and fungi were maintained in glycerol stocks at -76 $^{\circ}$C. Nutrient agar (NA), Nutrient Broth (NB), Luria Bertani (LB) agar and broth were used as growth media for bacteria unless specified otherwise. Yeast Peptone Dextrose (YPD) agar, and Sabouraud's dextrose (SAB) agar and broth were used for fungi. SAB broth was also used for mixed culture experiments. RPMI–1640 medium was used to induce germination in *Candida*. Growth media for the PAO1 genomic DNA library was supplemented with 15μg/ml tetracycline (Tet15).

Attachment assay media

Attachment assay media were prepared by growing the respective bacteria at 37 $^{\circ}$C in NB to late stationary phase and filter-sterilizing the medium. These are referred to as conditioned NB of PAO1, PAK, *lecB*, and LE392.

Table 2.1: Different strains of bacteria and fungi used in this study

Microorganism	**Reference**
Pseudomonas aeruginosa (PAO1)	GSU glycerol stock collection
Pseudomonas aeruginosa (PAK)	Sonowane et al., 2006
Pseudomonas aeruginosa (*lecB* mutant)	Sonowane et al., 2006
Pseudomonas aeruginosa (PAO1) genomic DNA library	Lightfoot and Lam, 1992
Escherichia coli (LE392)	Provided by Dr. William Fonzi
C. albicans (CAF2)	Provided by Dr. William Fonzi
C. albicans (*tup1* mutant)	Provided by Dr. William Fonzi

2.2 Growth dynamics: *Pseudomonas–Candida* mixed culture

Effect of *P. aeruginosa* on growth and viability of CAF2 and *tup1* mutant

One colony of PAO1 and CAF2 were inoculated into 30ml of SAB broth in a 125ml flask and incubated overnight at 180rpm at 37 $^{\circ}$C and 30 $^{\circ}$C, respectively. *C. albicans tup1* mutant was inoculated into 5ml of SAB broth in a 20ml flask and incubated overnight at 180rpm at 37 $^{\circ}$C. Mixed cultures were prepared by mixing 2ml of PAO1 with 2ml of fresh SAB broth and 2ml of *C. albicans* (CAF2 and *tup1* mutant). Controls were prepared by mixing 2ml of filter sterilized conditioned SAB broth with 2ml of fresh SAB broth and 2ml of *C. albicans* (CAF2 or *tup1* mutant). PAO1 and CAF2 mixed culture was incubated at 30 $^{\circ}$C, whereas PAO1 and *tup1* mutant mixed culture was incubated at 37 $^{\circ}$C. Viable *Candida* counts were taken by serially diluting 100μl volumes of both mixed and control cultures in Phosphate Buffered Saline (PBS) and plating on SAB agar supplemented with tetracycline (60μg/ml), gentamycin (30μg/ml), and chloramphenicol (30μg/ml) from 0 to 72h at 24h intervals.

Effect of *P. aeruginosa* on growth, viability, and morphogenesis of CAF2

In order to prepare the bacterial inoculum, one colony of PAO1 from a LB plate was inoculated into 30ml of SAB broth and incubated overnight at 37 $^{\circ}$C at 180rpm. Cells were harvested by centrifugation at 6000rpm for 10 minutes and washed three times in PBS for 10 minutes at 6000rpm before resuspending in fresh SAB broth. The OD_{600nm} was adjusted to get approximately 2×10^{7} CFU/ml.

CAF2 inoculum was prepared by inoculating one colony of CAF2 from a SAB plate into 30ml of SAB broth and incubating overnight at 30 $^{\circ}$C at 180rpm. CAF2 cells were harvested by centrifugation at 6000rpm for 10 minutes and washed three times in PBS for 10 minutes at 6000rpm and re-suspended in RPMI–1640 to get approximately 4×10^{6} CFU/ml. The bacterial and fungal cultures were mixed in the following combinations in three flasks.

- 5ml of CAF2 in RPMI–1640 and 5ml of fresh SAB broth
- 5ml of CAF2 in RPMI–1640 and 5ml of PAO1 in fresh SAB broth
- 5ml of CAF2 in RPMI–1640 and 5ml of filter sterilized conditioned SAB broth

Fresh SAB broth was used to induce growth, whereas RPMI–1640 medium was used to induce germination. Filter sterilized conditioned SAB broth was used to see if the presence of PAO1 extracellular factors affect growth, viability and morphogenesis of CAF2. All three flasks were incubated at 37 $^{\circ}$C. At 0, 2, 4, and 24h time intervals, 100μl from each was mixed with 0.4% tryphan blue and kept at room temperature for 15 minutes. The live and dead yeasts and germ tubes were counted using a heamocytometer, under a light microscope at 40X objective. Digital photographs were taken with an Olympus DP-12 camera attached to the microscope.

2.3 *P. aeruginosa*–*C. albicans* attachment assay

P. aeruginosa inoculum

A flask of fresh NB medium was inoculated from overnight cultures of *P. aeruginosa* (PAO1, PAK, *lecB* mutant) and incubated at 37 $^{\circ}$C at 180rpm until it reached an OD_{600nm} of 0.730 (late-log phase) and cells were harvested by centrifugation at 6000rpm for 10 minutes. Bacterial cells were re-suspended in conditioned NB of the respective organism to an OD_{600nm} of 0.025 to get approximately 1×10^7 CFU/ml.

C. albicans tup1 mutant inoculum

The *tup1* mutant was grown in 5ml of SAB broth overnight at 37 $^{\circ}$C at 180rpm. Next day cells were harvested by centrifuging at 6000rpm and washed three times with 5ml of PBS. The pellet was crushed with a sterile glass rod to get a smooth suspension. OD_{600nm} was adjusted to 1.4 and 100μl of the suspension was taken for mixed cultures. The final concentration of t*up1* mutant was approximately 2×10^6 cells/ml.

C. albicans CAF2 inoculum

C. albicans CAF2 was grown in SAB broth overnight at 30 $^{\circ}$C at 180rpm. The next day, cells were harvested by centrifuging at 6000rpm and washed three times with RPMI–1640. Equal volumes of CAF2 cells were inoculated into two 15ml flasks of RPMI–1640. One flask was incubated at 30 $^{\circ}$C and the other was incubated at 37 $^{\circ}$C for 3h at 180rpm to get yeast and hyphal forms respectively. After 3h, cells from both flasks were harvested and washed three times in PBS and resuspended to approximately 2×10^6 cells/ml.

Mixed culture

Two milliliters of *P. aeruginosa* was mixed with 100μl of *C. albicans* and incubated at 37 $^{\circ}$C for 20 minutes, statically .

Fixing and counting

One hundred microliters of the mixed culture was fixed with 100μl of 37% formaldehyde overnight at 4 $^{\circ}$C. Subsequently, nine microliters were trapped under a cover slip and the number of hyphae and yeast colonized by *P. aeruginosa* was counted using a 100X oil immersion objective on a light microscope. From this, the percentage of *C. albicans* hyphal segments colonized by *P. aeruginosa* (referred to as percent attachment) was calculated. For each sample, at least 600 hyphal segments were counted and each experiment was performed in triplicate. The percent attachment was determined for the following:

- yeast and hyphal forms of CAF2 strain of *C. albicans*
- *tup1* mutant strain of *C. albicans*
- *P. aeruginosa* harvested from different phases of growth
- different strains of *P. aeruginosa* such as PAO1, PAK, and *lecB*

2.4 Attachment inhibition by carbohydrates and ConA

Inhibition by carbohydrates

Pseudomonas and *Candida* inocula as well as mixed cultures were prepared as same as for the attachment assay. Different sugars such as D-mannose, L-fucose, N-acetylglucosamine (GlcNac), D-galactose, and D-glucose were added at 1M final concentration to the *P. aeruginosa* culture

prior to mixing with *C. albicans*. Fixing and determination of the percent attachment were done as described above.

Inhibition by para-nitrophenyl-α-fucoside

Pseudomonas and *Candida* inocula as well as mixed cultures were prepared as same as for the attachment assay with following modifications: a fucose derivative, para-nitrophenyl-α-fucoside was added to the tube with *P. aeruginosa* at 4mM final concentration prior to adding *C. albicans.* A control was prepared with an equal volume of PBS. Fixing and determination of the percent attachment were done as described above.

Inhibition by ConA

Pseudomonas and *Candida* inocula as well as mixed cultures were prepared as same as for the attachment assay with following modifications: One milliliter of each culture was pelleted at 6000rpm for 10 minutes, with each sample collected in duplicate. One pellet of each was treated with 1mg/ml ConA and the other was treated with an equal volume of PBS. The samples were incubated at 37 $^{\circ}$C for 1h. All samples were washed three times in PBS. PAO1 was re-suspended in filter sterilized conditioned NB to an OD_{600nm} of 0.025 and the *tup1* mutant was re-suspended in PBS to a final OD_{600nm} of 1.4. The standard attachment assay was performed for ConA treated cells by mixing them in following combinations:

- Tube 1: 2ml PBS treated PAO1 and 100μl PBS treated *tup1*
- Tube 2: 2ml PBS treated PAO1 and 100μl ConA treated *tup1*
- Tube 3: 2ml ConA treated PAO1 and 100μl of PBS treated *tup1*
- Tube 4: 2ml ConA treated PAO1 and 100μl ConA treated *tup1*

All four tubes were incubated at 37 $^{\circ}$C for 20 minutes. Fixing and determination of the percent attachment were done as previously described.

2.5 Screening of PAO1 genomic library for attachment

A genomic library of PAO1 (Lightfoot and Lam, 1991) constructed in *E. coli* LE392 was screened to isolate genomic DNA clones that conferred on *E.coli* the ability to attach to *C. albicans* hyphae. Eleven pools of the PAO1 genomic library were received and all were tested by three different panning assays until a reliable method was found. The host strain LE392 was used as the control.

Preliminary experiments

For all of the attachment assays performed in this section, inoculum preparation, attachment assay, fixing and counting was done as in previous experiments except that the assay medium used was conditioned NB of LE392. Firstly it was confirmed that the host strain (LE392) used to construct the PAO1 genomic library does not bind to hyphae of *C. albicans tup1* mutant. Also, prior to panning by filtration, 12μm polycarbonate filters, were screened for their efficiency to retain hyphae of *C. albicans tup1* mutant and pass LE392 and PAO1. Finally NB supplemented with 15μg/ml tetracycline and 30μg/ml fluconazole ($NB_{Tet15+Fcz30}$) was tested for its ability to support the growth of the PAO1 genomic library.

Comparison of percent attachment of PAO1 and LE392

Both PAO1 and LE392 cultures were grown at 37 $^{\circ}$C to the late-log phase and the standard attachment assay was performed with both bacteria. Percent attachment was determined for both bacteria as previously described.

Determining the filter efficiency to pass PAO1 and LE392

Bacteria were grown as previously described and initial inoculum concentration was determined using aerobic plate counts on NA. One milliliter of the same inoculum was filtered through a 12μm polycarbonate filter and the filter was washed three times with 10ml PBS and transferred to a 50ml Falcon tube with 1ml PBS and vortexed well to recover the cells. This suspension was serially diluted and plated on NA to get the background or the amount of bacteria retained by the filter. The standard attachment assay was performed with the same inocula along with hyphae of the *tup1* mutant. One milliliter of mixed cultures was filtered and treated similar to LE392 and PAO1 bacterial cultures to determine the increase in bacterial retention due to attachment to *tup1* hyphae.

Determining the filter efficiency to retain the *C. albicans tup1* mutant

Inoculum was prepared as for attachment experiments without bacteria (i.e., 100μl hyphae in 2ml conditioned medium of LE392), and the inoculum concentration was measured by plating a serial decimal dilution on SAB plates. In order to determine the amount of hyphae retained on the filter, 1ml from a tube containing 100μl hyphae in 2ml conditioned medium of LE392 was filtered through the 12μm polycarbonate filter. Then the filter was washed three times, transferred to a 50ml falcon tube containing 1ml PBS, and vortexed well to recover the cells. This suspension was serially diluted and plated on SAB agar to determine the amount of hyphae retained by the filter. Also, the filtrate was serially diluted and plated on SAB agar.

Growth of the library on NB supplemented with tetracycline and fluconazole

A flask of 10ml NB and a flask of NB supplemented with 15μg/ml tetracycline and 30μg/ml flucanazole ($NB_{Tet15+Fcz30}$) were inoculated with an overnight culture of the library. The cultures were monitored for 6h with Klett meter readings to confirm synchrony in growth.

Panning experiments

Assay 1: Panning by filtration

The eleven pools of PAO1 genomic library were grown to early stationary phase and diluted in spent medium of LE392 to an OD_{600nm} of 0.025, mixed with 100μl of *C. albicans tup1* mutant hyphae, and incubated for 20 minutes at 37 $^{\circ}$C, statically. One milliliter of the mixed culture was filtered through 12μm polycarbonate filters and washed three times with 10ml of PBS. The filter was removed aseptically and transferred to a 50ml Falcon tube containing 1ml PBS. The filter was vortexed well to detach hyphae from it and 100μl was taken for viable bacterial counts on $NA_{Tet15+Fcz30}$. The remaining 900μl was inoculated to fresh $NB_{Tet15+Fcz30}$ to grow the bacterial inoculum for the next round of panning . Attachment, filtering and counting were continued until the plate counts became higher and approximately equals to the plate counts of PAO1 treated in the same manner. This would indicate that the inoculum is enriched with a clone or clones that carry the PAO1 genes required for attachment. The bacterial inoculum was filtered without hyphae to determine the background count at each round of panning.

Assay 2: Panning by centrifugation and washing

Different pools of the genomic library were grown to early stationary phase and diluted in spent medium of LE392 to an OD_{600nm} of 0.025 and mixed with 100μl of *C. albicans tup1* mutant hyphae and incubated for 20 minutes at 37 $^{\circ}$C, statically. One milliliter volumes were transferred to two eppendorf tubes and centrifuged at 500rpm for 5minutes. The supernatant was decanted and the pellet was washed twice with 1ml of PBS and re-suspended in 1ml of PBS. A volume of 100μl from this suspension was taken for plate counts as described previously and the remaining was inoculated to fresh $NB_{Tet15+Fcz30}$. A background control was done for bacteria without hyphae of *C. albicans tup1* mutant.

Assay 3: Plate assay

One milliliter of an overnight culture of *C. albicans tup1* mutant was added to each well of a 12 well polystyrene plate and incubated at 37 $^{\circ}$C for 1h, statically, to allow the hyphae to attach to plastic. After 1h, the medium was aspirated and the wells were washed gently with 1ml of PBS to remove loosely adhered hyphae. All 11 pools of the genomic library were grown to stationary phase and diluted in conditioned medium of LE392 to an OD_{600nm} of 0.100, and 1ml of each pool was added to each well of a 12 well plate. Cultures were incubated at 37 $^{\circ}$C at 60rpm for 1h to allow attachment of bacteria to the fungus. After 1h, each well was washed gently with 1ml of PBS to remove the bacteria not attached to hyphae. One milliliter of fresh $NB_{Tet15+Fcz30}$ was added to each well and incubated at 37 $^{\circ}$C at 180rpm overnight to allow the growth of bacteria that are attached to hyphae. Subculturing to fresh media was done to generate the inocula for the next round of panning. Six cycles of panning were done to each pool of the genomic library, and at the end of the sixth cycle, the inocula were used to perform the attachment assay as previously described.

Final screening assay

Inocula derived from pools 2, 3, 6, 9, and 10 that had higher percent attachment at the end of the sixth cycle of panning using the plate assay were used for the final screening. Attachment was done as previously described, and the mixed cultures were filtered through 12μm polycarbonate filters and washed gently with 3ml of PBS. The filters were removed aseptically and the cell sides of the filters were touched on the surface of LB agar supplemented with 15μg/ml tetracycline (LB_{Tet15}). From these, visible clumps of hyphae were picked and transferred to another LB_{Tet15} agar plate followed by an overnight incubation at 37 $^{\circ}$C. The following day, the bacterial colonies overlapping *C. albicans* colonies were selected and re-streaked to obtain pure cultures. From each pool, at least

four pure cultures were tested for attachment to hyphae of *C. albicans tup1* mutant. Among these, one clone designated as 9a that consistently exhibited an increase in attachment, approximately 7%, to hyphae of *C. albicans tup1* mutant was selected for genetic analysis. As a first step, the effect of D-mannose on attachment of clone 9a to hyphae of *C. albicans tup1* mutant was tested.

Genetic analysis of clone 9a

Cosmid DNA extraction and purification

Cosmids were extracted following standard molecular protocols from Sambrook et al. (1989) "for Plasmid DNA extraction by Alkaline lysis with SDS (Midi preparation)" with the following modifications. In order to increase yield, after the addition of lysis solution 1, lysozyme (20μl of 25mg/ml) was added to 180μl of cosmid containing cell suspension, and allowed to stand at room temperature for 10 minutes. After extraction, cosmid DNA preparation was treated with 20μg/ml RNaseA for 10 minutes at room temperature.

Extraction of Cosmid DNA after RNase A digestion

A 100μl aliquot of the RNase digested sample was mixed with 100μl of phenol:chloroform:isoamyl alcohol (25:24:1) and centrifuged at 10,000rpm for 5 minutes at 4 $^{\circ}$C. The aqueous upper layer was transferred to a fresh tube and re-extracted with 100μl of phenol:chloroform:isoamyl alcohol (25:24:1). The aqueous phase was transferred to a fresh tube and 10μl of 3M sodium acetate (pH 5.4) and 100μl of isopropanol was added, mixed, and incubated at -20 $^{\circ}$C for 15 minutes. The sample was then centrifuged for 10 minutes at 10,000rpm at room temperature and the supernatant was discarded. The pellet was washed twice in 1ml of 70% ethanol, air dried and dissolved in 40μl of TE buffer (100mM Tris-Cl, 10mM EDTA). The DNA concentration was calculated based on the OD_{260nm}.

Genetic analysis

Restriction digestion, agarose gel electrophoresis, preparation of eletrocompetent cells and transformation were carried out according to standard protocols (Sambrook et al., 1989) and manufacturers' instructions. After transformation, the cells were plated on LB_{Tet15} agar to select transformed colonies.

Confirmation of transformant phenotype

In order to confirm that the phenotype of clone 9a is attributed to the cosmid with the PAO1 genomic DNA insert, the cosmids were extracted and transformed into the host strain LE392 by electroporation using a Bio-Rad Gene Pulser. Transformants were screened for their ability to attach to hyphae of *C. albicans tup1* mutant and compared with the attachment ability of LE392 and clone 9a.

Sequencing

Prior to sequencing, the cosmid DNA was purified with QIAGEN Plasmid Mini kit using QIAGEN-tip-20 columns according to the manufacturer's instructions. The DNA was dissolved in sterile deionized water and quantified by Nanodrop. Sequencing was carried out at the Georgetown University's sequencing facility.

Primers used for sequencing

Forward and reverse primers were designed to encompass the *Eco*RI site of the cosmid pLAFR using the Primer3 software (http://frodo.wi.mit.edu/).

Table 2.2: Primers used in this study

Primer	**Sequence**
Forward	5' ggcattcttggcatagtggt 3'
Reverse	5'caagttcgacgacacgaaaa 3'

Table 2.3: Transposon mutants selected from the University of Washington's Genome Center.

ID	**Location**	**PA ORF**	**Gene Product**	**Gene**
2061	lacZbp02q2E05	PA0413	probable component of chemotactic signal transduction system	*pilL* *chpA*
15834	lacZwp06q3C03	PA0414	probable methylesterase	*chpB*
1005	lacZbp01q2F07	PA0415	probable chemotaxis protein	*chpC*
20573	lacZwp09q1H05	PA0415	probable chemotaxis protein	*chpC*
4799	lacZwp01q2D05	PA0419	conserved hypothetical protein	*yggJ*
19815	lacZwp08q3F07	PA0421	hypothetical protein	
20890	lacZwp09q1C07	PA0423	conserved hypothetical protein	*yceI*

Analysis of PAO1 transposon mutants for attachment to *C. albicans tup1* mutants

Seven PAO1 transposon mutants were selected from the available mutant collection in the University of Washington Genome Center and tested their attachment to the *C. albicans tup1* mutant. As shown in Table 2.3, these mutants represented mutations in the *ChpA,B,C,D,E* operon and three other ORFs that encode hypothetical proteins. Inoculum preparation and attachment experiments were done as previously described.

CHAPTER 3: RESULTS

3.1 Growth dynamics: *Pseudomonas–Candida* mixed culture

Effect of *P. aeruginosa* on growth and viability of CAF2 and *tup1* mutant:

Pseudomonas aeruginosa (PAO1) and *Candida albicans* (CAF2 and *tup1* mutants) were grown together at two different temperatures, 30 ° and 37 ° C, to study the effect of PAO1 on growth and viability of *C. albicans*. Overnight cultures of PAO1 and *C. albicans* were mixed with fresh SAB broth to obtain a 1:1:1 ratio. The control contained filter-sterilized, conditioned medium of PAO1 instead of PAO1 cells grown to stationary phase. Fresh SAB broth was added in order to support growth of both organisms.

Figure 3.1 shows the viable count of CAF2 in the mixed culture and control at 30 ° C. In mixed cultures, total inhibition of CAF2 growth up to 48h was followed by the onset of death. The *Candida* only control culture showed rapid growth during the first 24h, before cultures reached the stationary phase. The difference in slopes of control and the mixed culture gives a measure of fungal death rate enhanced by PAO1. When the same experiment was repeated with the constitutively filamentous *tup1* mutant, killing began around 24h as shown in Fig. 3.2.

Effect of *P. aeruginosa* on growth, viability, and morphogenesis of CAF2

Based on initial results, the effect of live PAO1 as well as the conditioned medium of PAO1 on growth, viability, and morphogenesis of CAF2 was studied over the first 24h at hyphal inducing condition. Overnight cultures of CAF2 cells were washed and diluted in RPMI–1640 medium and

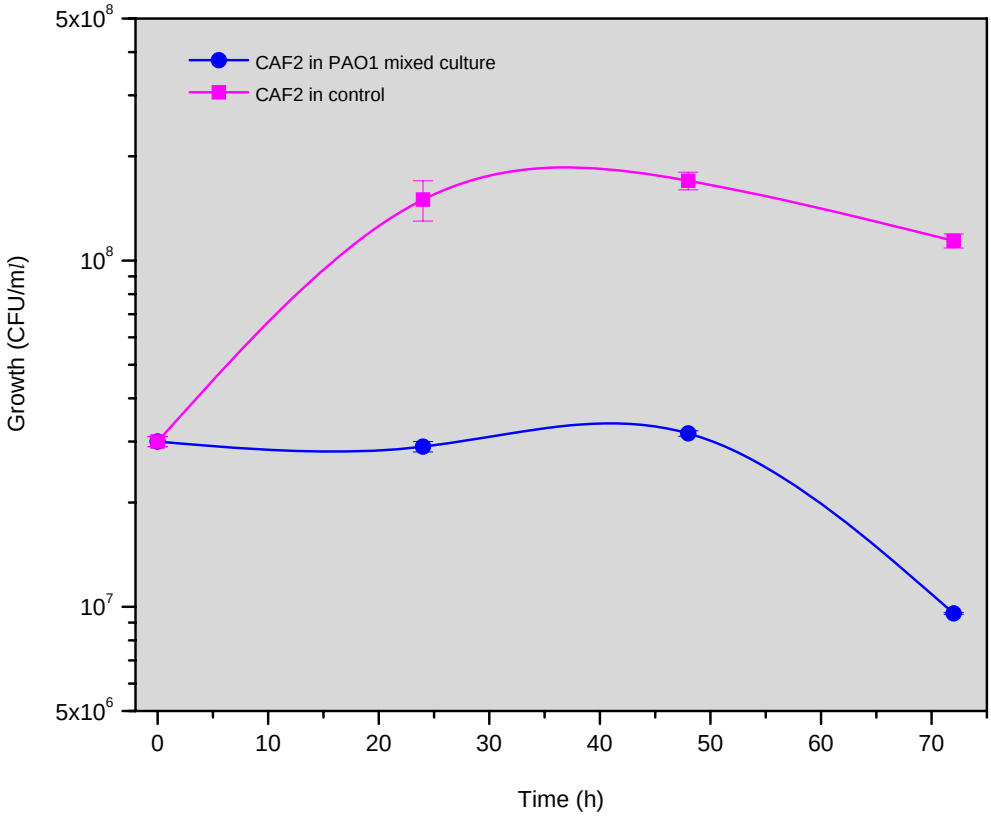

Figure 3.1: Effect of *P. aeruginosa* on growth and viability of *C. albicans* (CAF2) at 30 °. In the control CAF2 grew rapidly during the first 24h, whereas in the mixed culture, growth of CAF2 was totally inhibited up to 48h followed by death. Error bars represent standard deviation in triplicate samples.

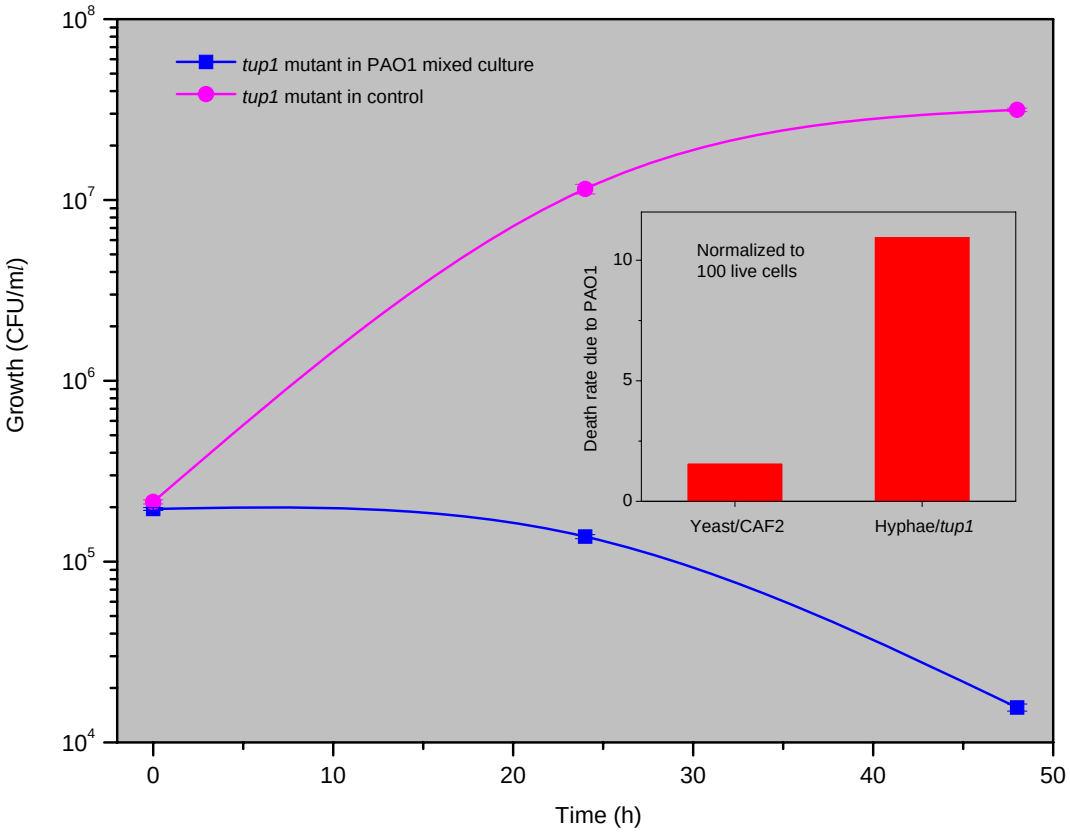

Figure 3.2: Effect of *P. aeruginosa* on growth and viability of *C. albicans* (*tup1* mutant) at 37 °C. Error bars represent standard deviation in triplicate samples. Inset shows the death rates of CAF2 and *tup1* mutant induced by *P. aeruginosa* (normalized to 100 live cells).

overnight cultures of PAO1 cells were washed and diluted in SAB broth to get CAF2 and PAO1 concentrations of 4×10^6 and 2×10^7 CFU/ml, respectively. RPMI–1640 was used to dilute CAF2 cells as it is a medium conducive for germination. Fresh SAB broth was used as it supports the growth of both CAF2 and PAO1. CAF2 diluted in RPMI–1640 medium was mixed with i)fresh SAB broth, ii)PAO1 diluted in fresh SAB broth, and iii) filter sterilized conditioned SAB broth of PAO1, at 1:1 ratio in 3 separate flasks. Conditioned SAB broth was used as an additional control to verify if the extracellular factors produced by PAO1 has an effect on growth, viability, and morphogenesis in the absence of live PAO1 cells. All flasks were incubated at 37 $^\circ$C for 24h. At different time intervals (0, 2, 4, and 24h), 100μl from each flask was stained with 0.4% tryphan blue to obtain total viable and differential (yeast and hyphal) counts using a heamocytometer. The total viable count of CAF2 increased with time (Fig. 3.3) in the control, mixed culture, and in the filter sterilized conditioned SAB broth of PAO1. However, the increase in viable count was lower in the mixed culture where live PAO1 cells were present.

The morphological changes of CAF2 at different time intervals are shown in figures 3.4 through 3.6 for all three CAF2 cultures. CAF2 cells in the control and the mixed culture germinated (Figs. 3.4 and 3.5) whereas the cells in the filter-sterilized conditioned SAB broth of PAO1 did not germinate even at 24h (Fig. 3.6). At 2h, the percentage of germ tubes was higher in the control whereas in the mixed culture it was lower, and in the conditioned medium there were none (Fig. 3.7). As summarized in Fig. 3.7, during the period from 4 to 24h, the yeast to germ tube ratio increased in both the control and the mixed culture.

3.2 Attachment of *P. aeruginosa* to *C. albicans*

The percent attachment of *P. aeruginosa* to *C.albicans* was tested as described in **Materials and Methods**. Firstly, the attachment of *P. aeruginosa* was tested with different mor-

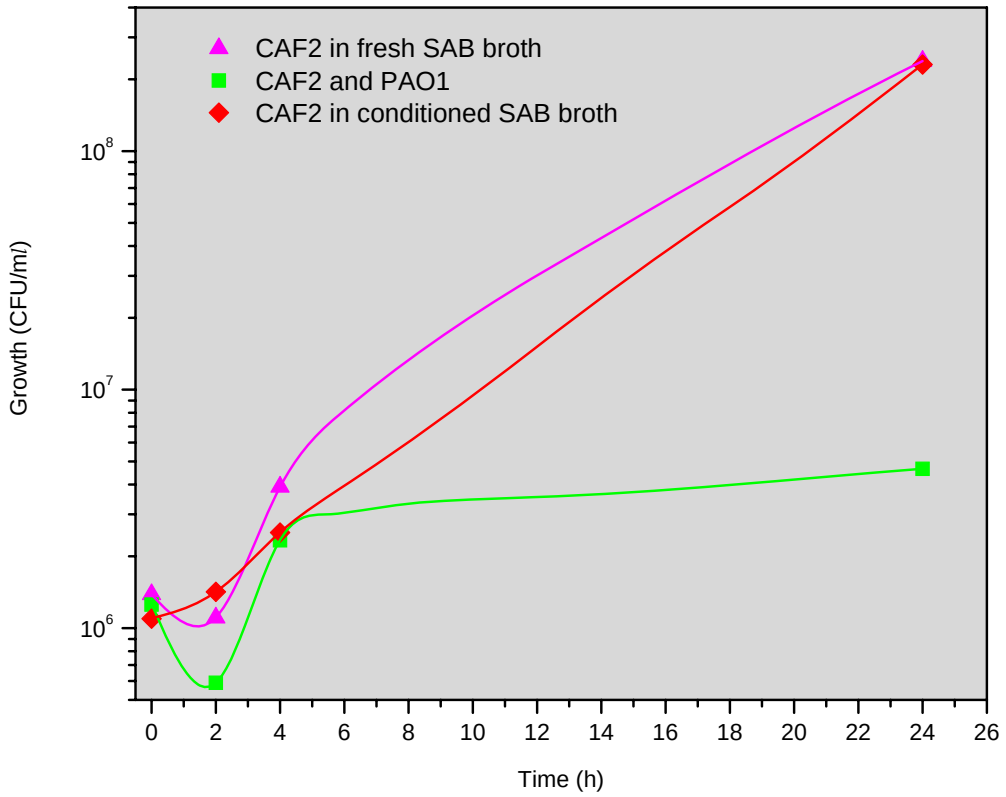

Figure 3.3: Total viable count of CAF2 under hyphal inducing conditions. Presence of live PAO1 cells inhibits the growth of CAF2. CAF2 mixed in conditioned SAB broth serves as an additional control to study the effect of extracellular products of PAO1 on the growth and morphogenesis of *C. albicans*.

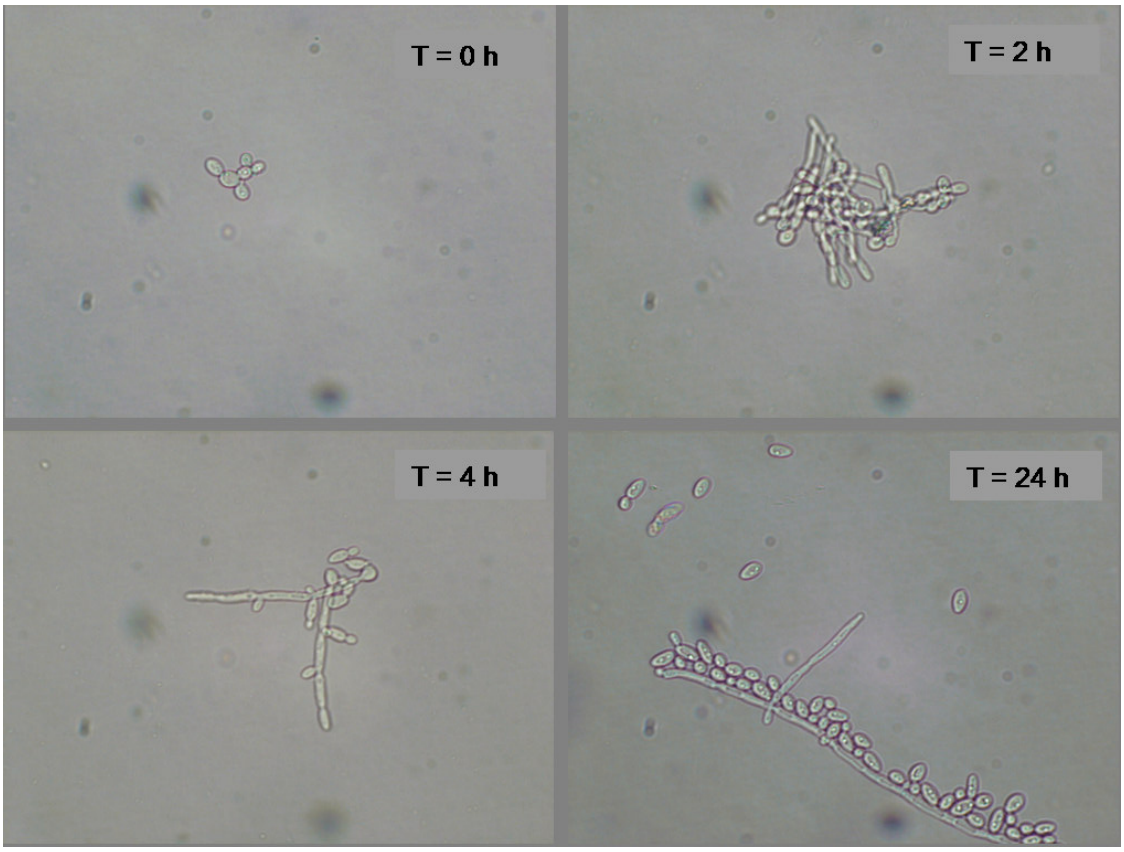

Figure 3.4: Morphogenesis of *C. albicans* (CAF2) in RPMI–1640 and fresh SAB broth. At 2h, germ tubes were present. Reversion from germ tubes to yeast form has occurred at 24h. Digital photographs were taken with an Olympus DP12 camera attached to a light microscope at 40X objective.

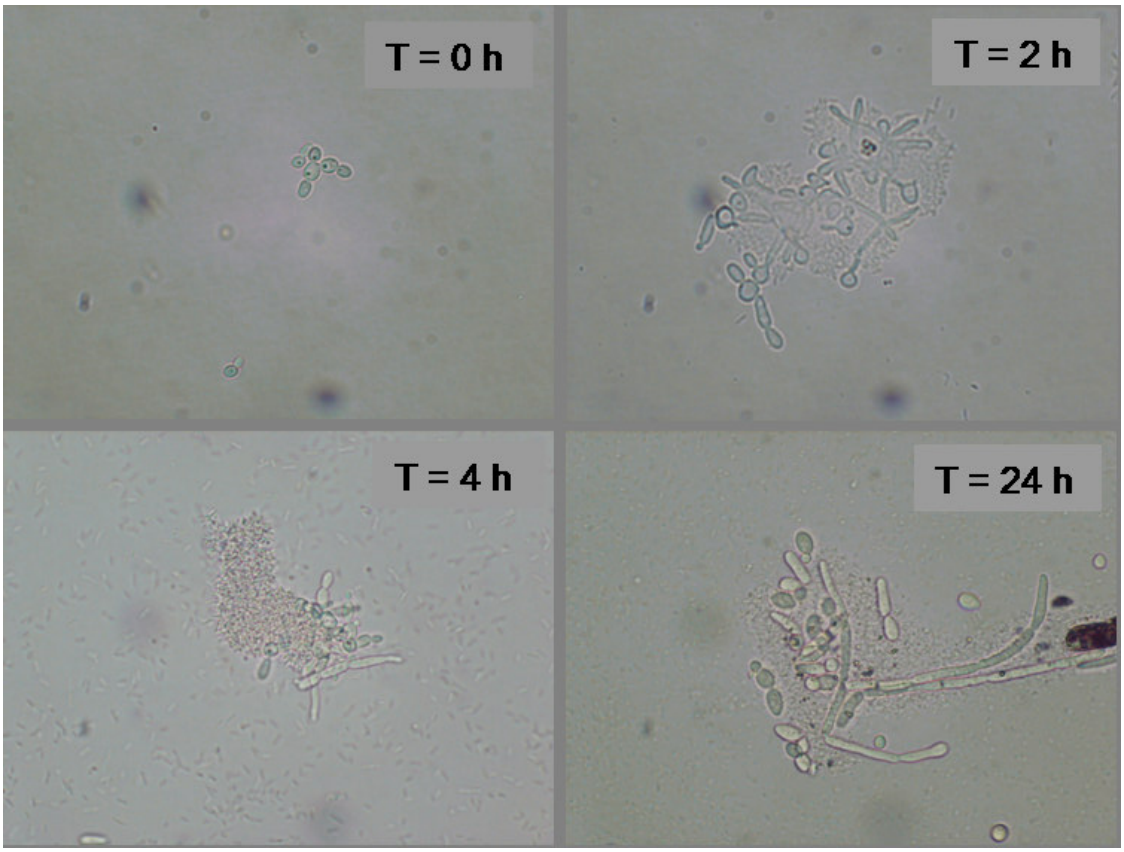

Figure 3.5: Morphogenesis of *C. albicans* (CAF2) in RPMI–1640 with live PAO1 cells in fresh SAB broth. At 2h, germ tubes were present and PAO1 cells attached to germ tubes. Dark or blue cells represent the dead CAF2 cells. Digital photographs were taken with an Olympus DP12 camera attached to a light microscope at 40X objective.

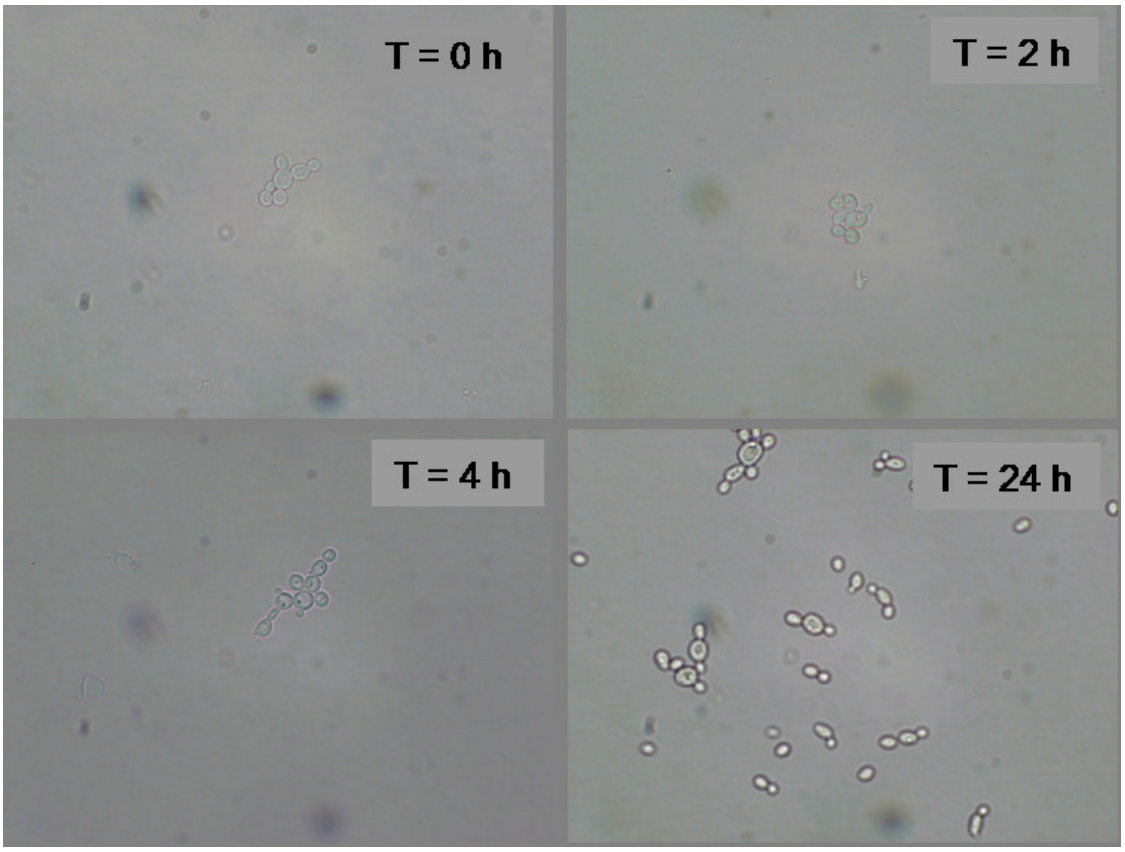

Figure 3.6: Morphogenesis of *C. albicans* (CAF2) in RPMI–1640 and filter-sterilized conditioned SAB broth of PAO1. The germination observed during the 24h period was insignificant. Digital photographs were taken with an Olympus DP12 camera attached to a light microscope at 40X objective.

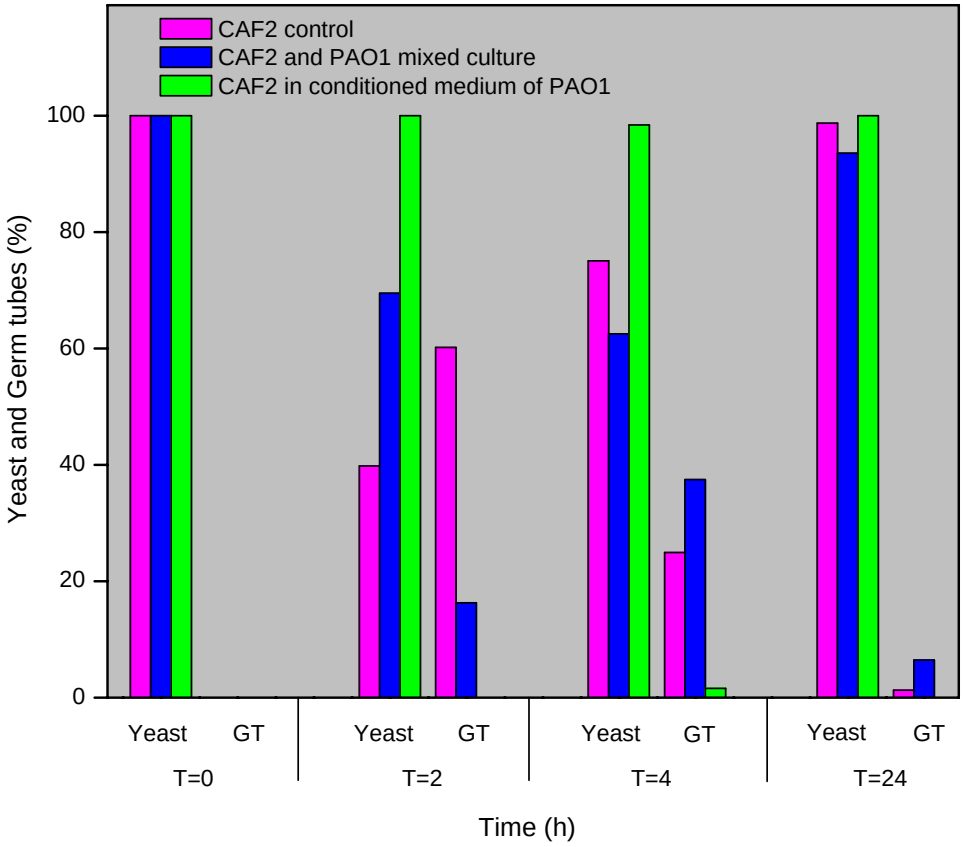

Figure 3.7: Effect of *P. aeruginosa* PAO1 and conditioned SAB broth of PAO1 on morphogenesis of *C. albicans* CAF2. At 2h, germination of CAF2 cells in fresh SAB broth was significantly high. CAF2 cells grown with PAO1 cells also germinated but at a lower rate. Those in the conditioned medium did not germinate from 0-24h.

phological forms and strains of *C. albicans* including the yeast form of CAF2, the filamentous form of CAF2 and the constitutively filamentous *tup1* mutant. The percent attachment to yeast was negligible compared to that of hyphae of CAF2 and *tup1* mutant (Fig. 3.8).

In order to see if the growth phase of *P. aeruginosa* has an effect on its attachment to *C. albicans*, *P. aeruginosa* was harvested from the same flask at different phases of growth and percent attachment was determined. Figure 3.9 shows that the percent attachment was higher when cells were harvested from mid-stationary phase compared to cells harvested from late-log phase and late-stationary phase.

To determine if LecB, a lectin produced by *P. aeruginosa*, plays a role in attachment to *C. albicans* hyphae, the percent attachment of a *lecB* mutant and its parental wild type strain, PAK, were compared. The conditioned medium of the *lecB* mutant and PAK was used as the assay medium for the *lecB* mutant and PAK strains, respectively. As shown in Fig. 3.10, the percent attachment was two–fold, greater for PAK compared to *lecB* mutant.

3.3 Attachment inhibition by carbohydrates and ConA

To determine if LecB had a direct role in attachment of *Pseudomonas* to *Candida* hyphae and to better understand the receptor-ligand specificities in this attachment mechanism, carbohydrate inhibition assays were performed. Firstly, different carbohydrates such as D-galactose, N-acetylglucosamine (GlcNac), L-fucose, D-mannose and D-glucose were tested at 1M final concentration. These were added to the attachment assay tube containing *P. aeruginosa* culture prior to mixing of hyphae and the assay was performed as described in **Materials and Methods**. A negative control was done by adding PBS instead of carbohydrates. As shown in Fig. 3.11, D-glucose and D-mannose inhibited attachment by approximately five to six–fold compared to the control and the other carbohydrates tested. The direct involvement of LecB can be proved only

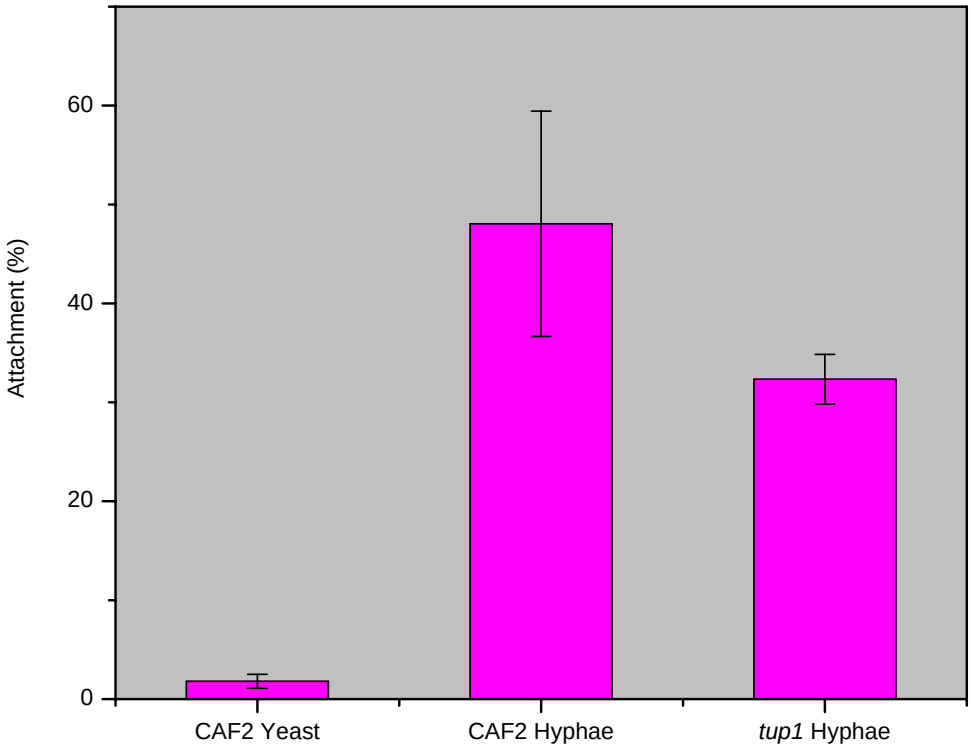

Figure 3.8: Comparison of attachment of *P. aeruginosa* to yeast and hyphal forms of CAF2 and hyphae of *tup1* mutant. The attachment to yeast form is insignificant compared to hyphal forms irrespective of the strain. Error bars represent standard deviation in triplicate samples.

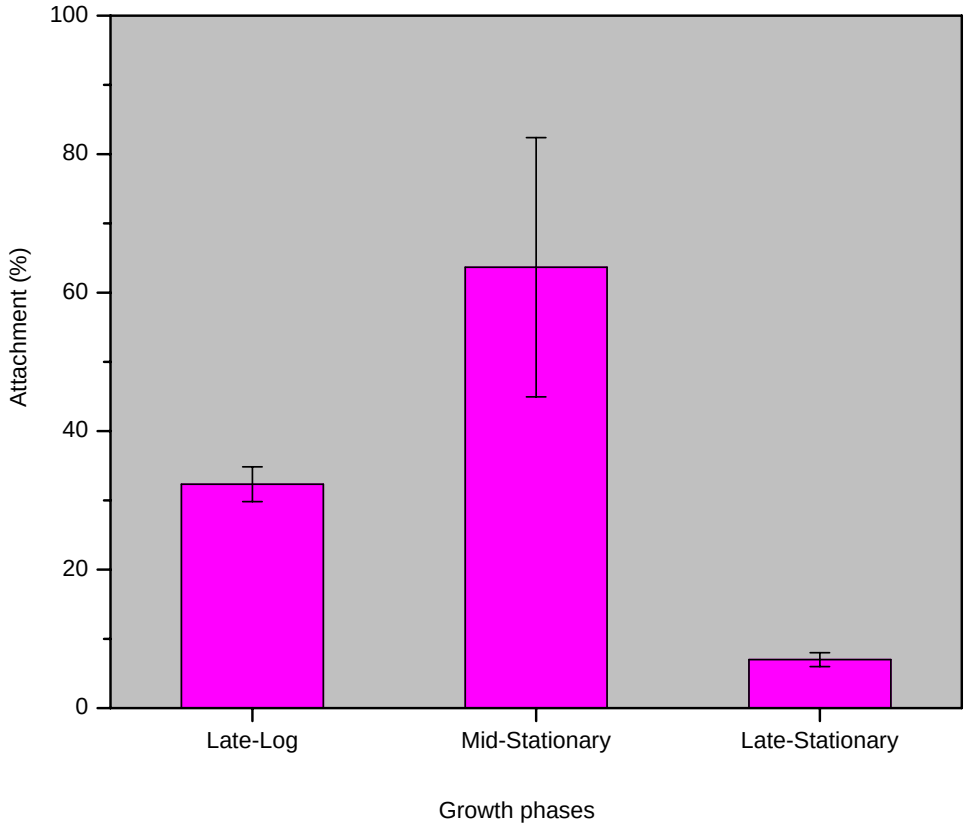

Figure 3.9: Comparison of attachment when *P. aeruginosa* was harvested at different phases of growth. Mid-stationary phase cells showed the highest attachment whereas late-stationary phase cells attached least. Error bars represent standard deviation in triplicate samples.

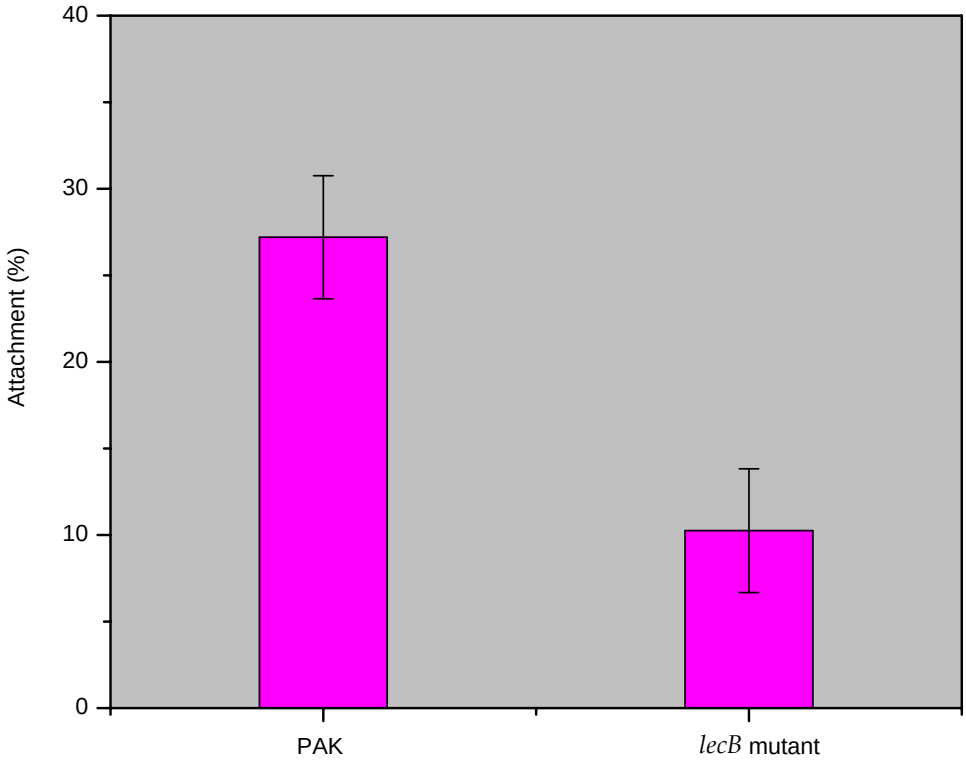

Figure 3.10: Comparison of PAK and *lecB* mutant for attachment to *C. albicans* (*tup1* mutant). The attachment of PAK was two–fold greater than *lecB* mutant. Error bars represent standard deviation in triplicate samples.

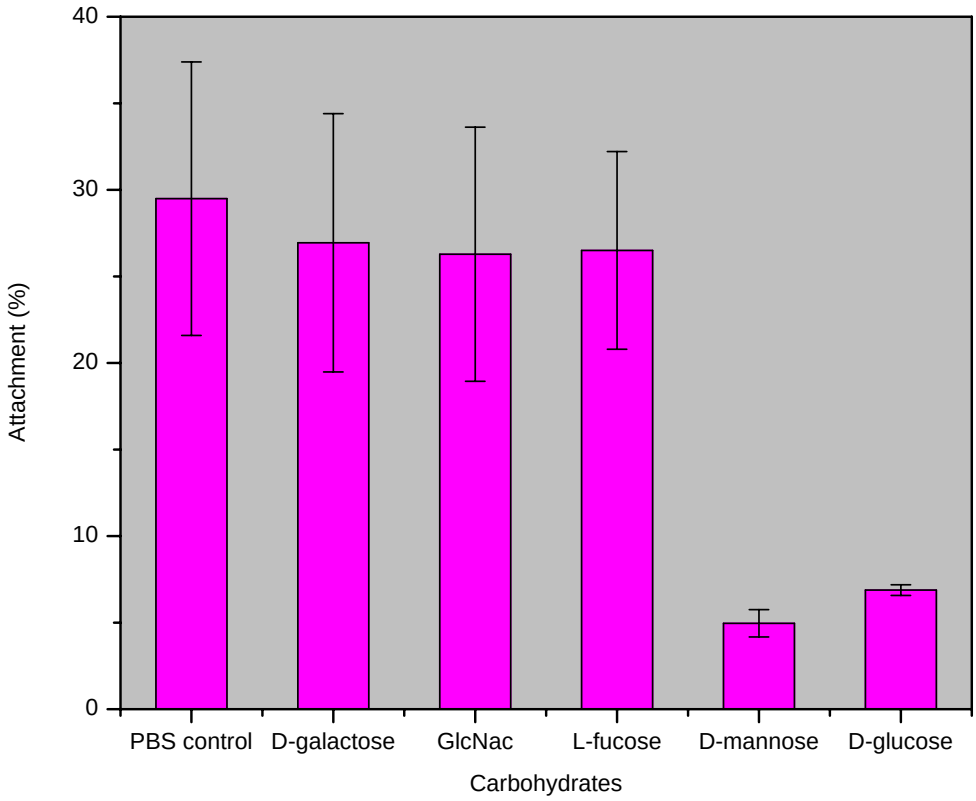

Figure 3.11: Effect of different carbohydrates on attachment of *P. aeruginosa* to *C. albicans* (*tup1* mutant). Attachment was inhibited by D-glucose and D-mannose approximately five to six–fold, compared to the PBS control and other carbohydrates. Error bars represent standard deviation in triplicate samples.

if L-fucose also inhibits attachment as L-fucose has a higher affinity to LecB than D-mannose. Therefore, to completely rule out the direct involvement of LecB, another carbohydrate derivative para-nitrophenyl–α–fucoside (pNPF) which has a higher affinity to LecB than L-fucose was tested for inhibition of attachment at a final concentration of 4mM. As shown in Fig. 3.12, there was no significant inhibition compared to the control. Finally, to determine if the inhibition by D-mannose was due to the blocking of lectin receptors on *P. aeruginosa* or *C. albicans*, these cells were treated with ConA which is a mannose specific lectin. Both bacterial and fungal cells were pretreated with 1mg/ml ConA for 1h and washed three times before mixing them for the attachment assay. The control consisted of cells that were not treated with ConA. As shown in Fig. 3.13, there was no significant inhibition due to treatment with ConA.

3.4 Screening of PAO1 genomic library for attachment

Preliminary tests

A *P. aeruginosa* PAO1 genomic library (Lightfoot and Lam 1999) was screened by three different panning assays to isolate clones that had a higher attachment to *C. albicans* hyphae. Firstly, the wild type *E. coli* LE392, which is the host strain used to construct the genomic library, was tested for its attachment to *C. albicans* by the standard procedure described in **Materials and Methods**. Prior to this, growth of PAO1 and LE392 was tested in different media and NB was selected as the favorable medium for both organisms. The attachment assay was performed in conditioned NB of the respective bacteria. As shown in Fig. 3.14, attachment of LE392 was insignificant compared to the attachment of PAO1.

Prior to performing the screening assay using 12μm polycarbonate filters, the efficiency of the filter to retain *C. albicans* hyphae and to pass LE392 and PAO1 was tested. To determine the number of hyphae retained, after filtering the inoculum, the filter was put in 1ml of PBS and

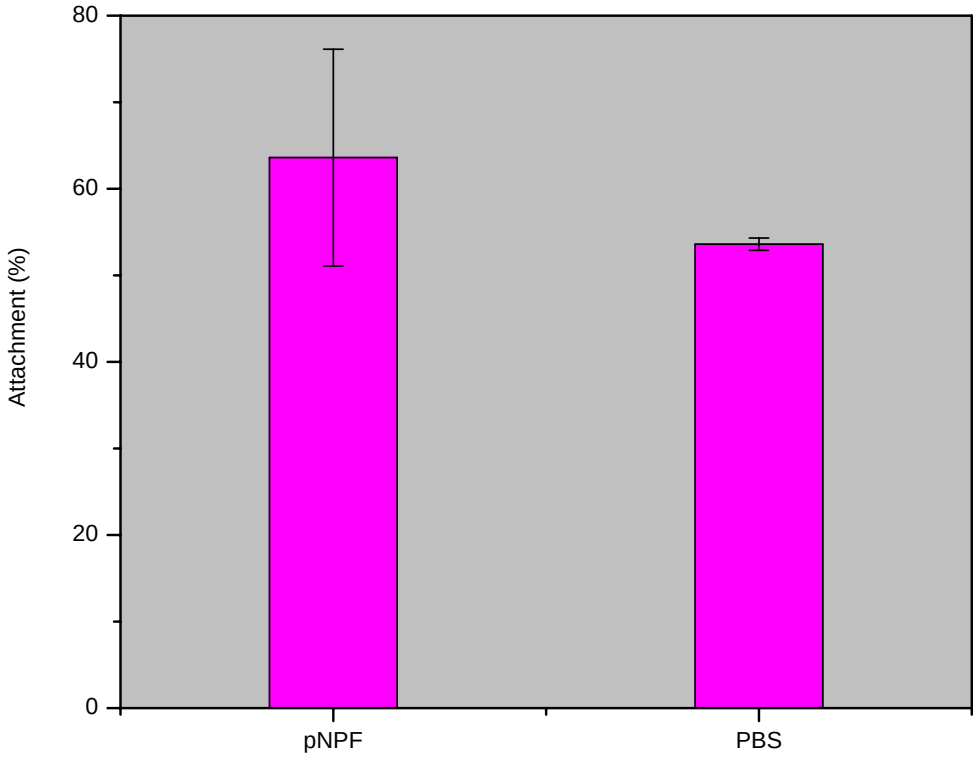

Figure 3.12: Effect of para-nitrophenyl–α–fucoside (pNPF) on attachment of *P. aeruginosa* to *C. albicans* (*tup1* mutant). No significant inhibition in attachment was observed compared to the PBS control. Error bars represent standard deviation in triplicate samples.

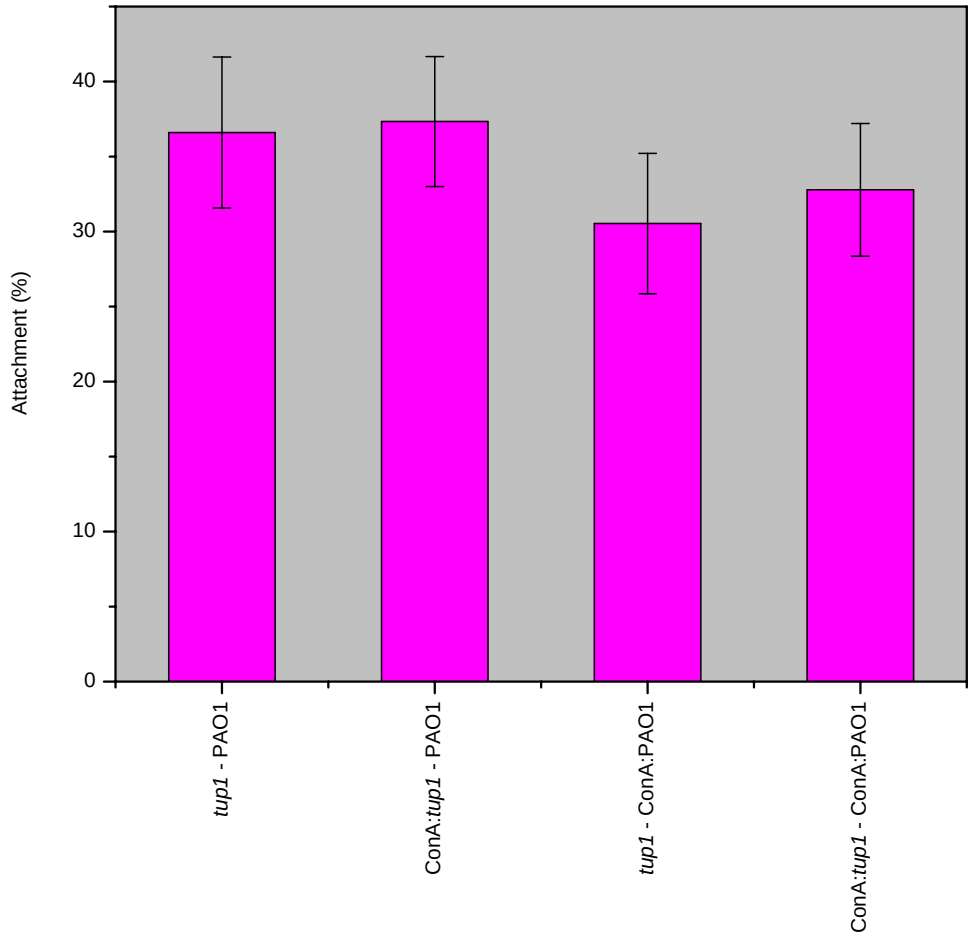

Figure 3.13: The effect of pre-treatment of *P. aeruginosa* (PAO1) and *C. albicans* (*tup1* mutant) cells with ConA on attachment. There was no significant inhibition in attachment due to ConA treatment. Error bars represent standard deviation in triplicate samples.

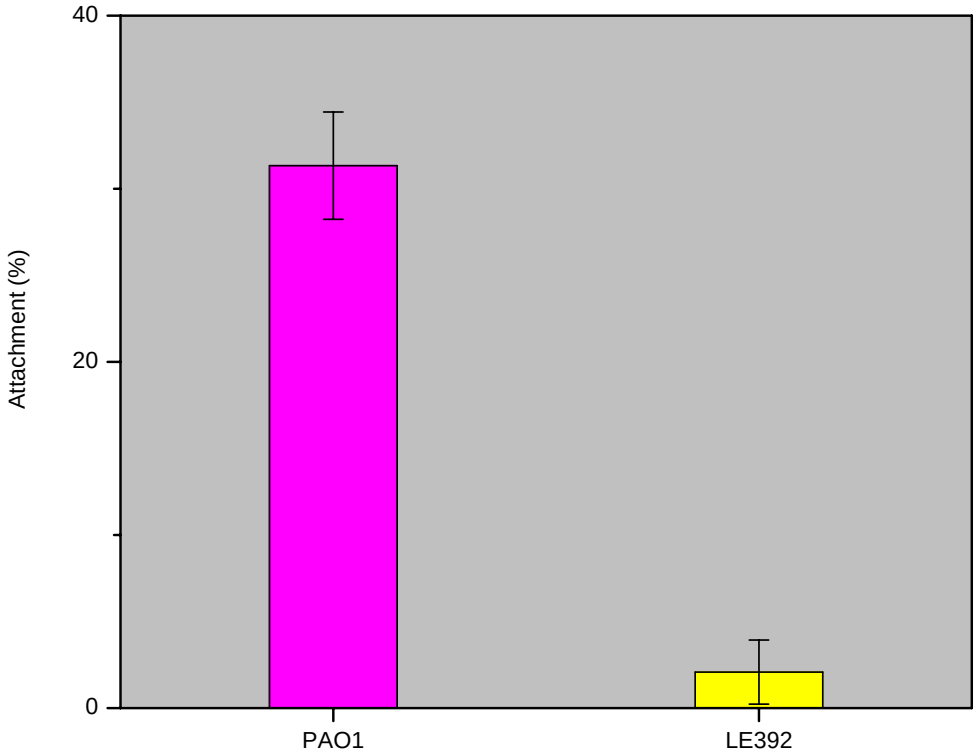

Figure 3.14: Comparison of attachment of PAO1 and LE392 to *C. albicans* (*tup1* mutant). PAO1 attachment was approximately one order higher. Error bars represent standard deviation in triplicate samples.

vortexed well to detach the cells from the filter. This, as well as the filtrate and the inoculum, were serially diluted and plated on SAB agar plates. As shown in Fig. 3.15, approximately 93% of hyphae were retained on the filter and approximately 4% was in the filtrate. Both bacteria were also filtered and the amounts retained on filters were determined as described in **Materials and Methods**. Around 0.1% of PAO1 and 0.003% of LE392 from the original inoculum were retained on the filters (Fig. 3.16).

The same bacterial inocula were used to perform the attachment assay as described in **Materials and Methods** and the solutions were filtered and plated. This was done to confirm that the difference in attachment ability of PAO1 and LE392 bacteria to *tup1* mutant hyphae can be detected by this assay as well. As shown in Fig. 3.16, plate counts of the filter retentate from PAO1 mixed cultures was six–fold higher compared to its control, whereas that of LE392 was not significantly different from its control. To confirm that supplementing the growth medium with tetracycline and fluconazole does not interfere with growth of the PAO1 genomic library, the growth of the library was tested in medium with and without supplements. As seen in Fig. 3.17, there was no adverse effect due to supplementation.

Panning Assays

Three panning assays were designed to enrich the genomic library pools with a clone possessing higher attachment ability to *C. albicans* hyphae. In the filtration assay, the plate count was expected to increase at the end of each round. In contradiction, plate counts from all the pools decreased after the second round of panning and this assay was discontinued after four rounds of panning. The next approach was to pan by centrifugation and washing. This method was not satisfactory as the bacteria (without hyphae) were sedimenting even at very low rpm values, and a large amount of hyphae were lost during each washing step. Finally, a plate screening assay was

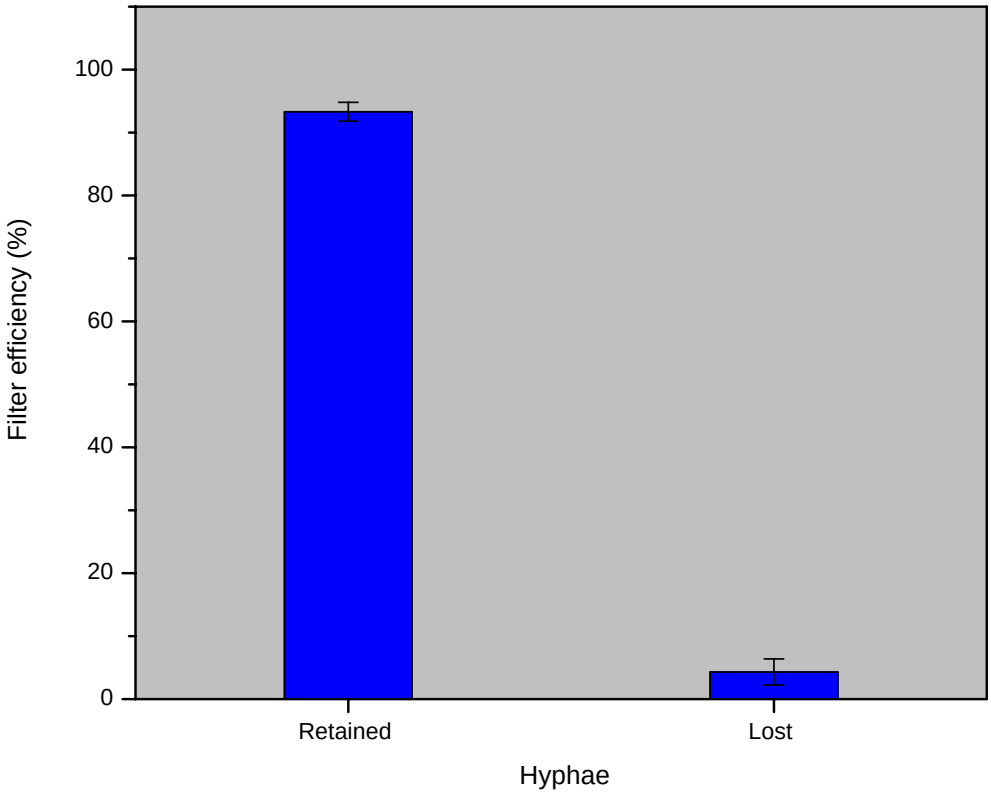

Figure 3.15: Efficiency of 12μm polycarbonate filters to retain hyphae of *C. albicans tup1* mutant. The filter had a high efficiency in retaining the hyphae which qualified it to be used for the screening assay. Error bars represent standard deviation in triplicate samples.

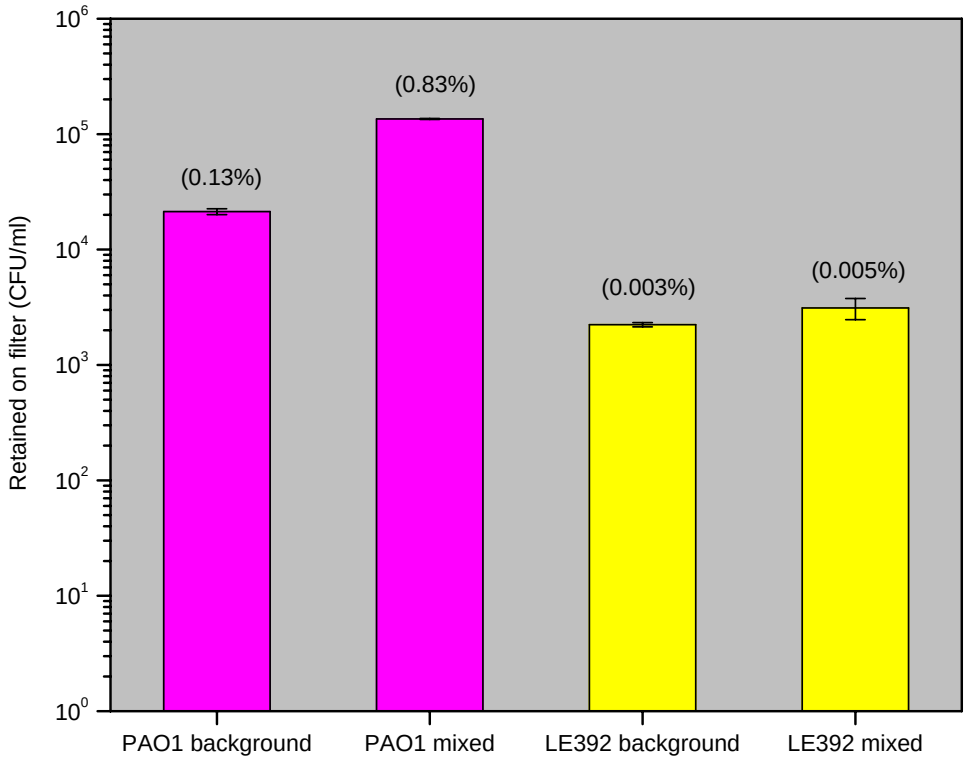

Figure 3.16: Retention of bacteria on 12μm polycarbonate filters. These filters have excellent low retention efficiency (shown within parentheses) for LE392 and PAO1. Also, the difference in attachment of LE392 and PAO1 to *C. albicans* (*tup1* mutant) can be detected. Error bars represent standard deviation in triplicate samples.

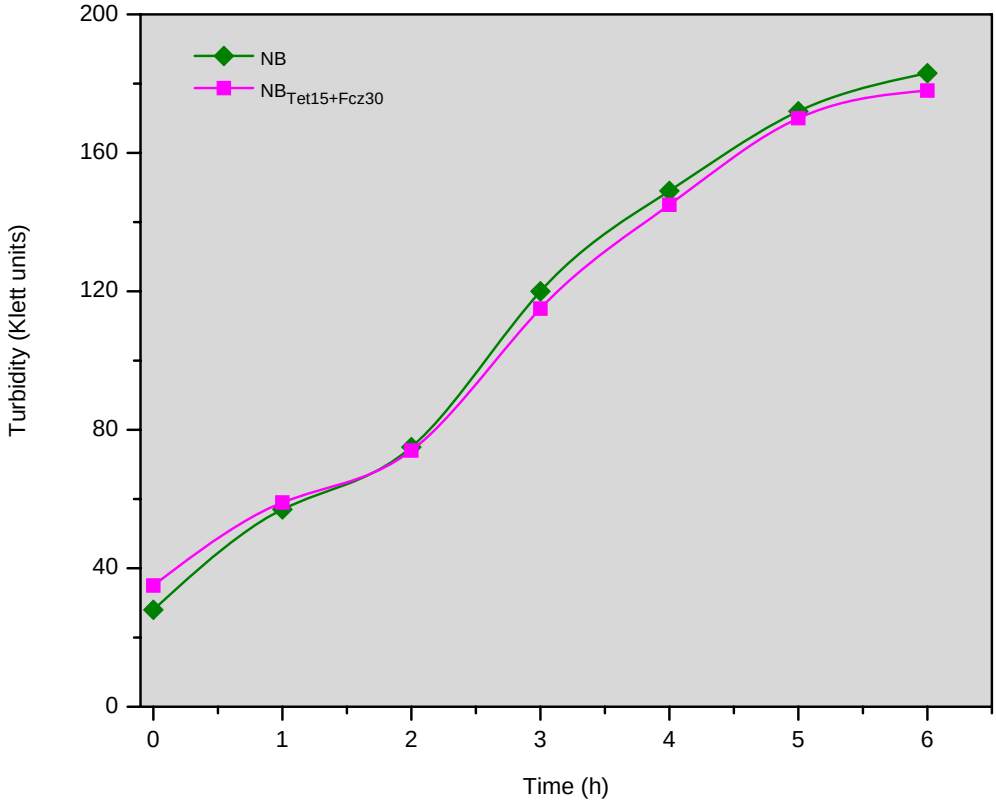

Figure 3.17: Growth of the PAO1 genomic library in NB supplemented with 15μg/ml tetracycline and 30μg/ml fluconazole. The supplements had no adverse effect.

designed. Overnight cultures of the *C. albicans tup1* mutant were placed in each well of a 12 well polystyrene plate and incubated 1h statically at 37 $^{\circ}$C in order to attach the hyphae to the plastic. After one hour, the medium was aspirated and the wells were washed gently with 1ml of PBS to remove loosely adhered hyphae. All 11 pools of the genomic library were grown up to stationary phase and diluted in conditioned NB of LE392 to an OD600 of 0.100 and 1ml of each pool was added to each well in the same 12 well plate. The plate was incubated at 37 $^{\circ}$C at 60rpm for one hour to allow attachment of bacteria to the hyphae. The supernatant was removed and the wells were washed gently with 1ml of PBS to remove unattached bacteria. One ml of fresh $NB_{Tet15+Fcz30}$ was added to the wells and the plate was incubated at 37 $^{\circ}$C at 60rpm overnight to allow the attached bacterial cells to grow. These were subcultured again to $NB_{Tet15+Fcz30}$ to prepare the inoculum for the next round of panning. Six cycles of panning were done on each pool and after the sixth cycle, the inocula were used to perform the attachment assay as before. As shown in Table 3.1, pools 2, 3, 6, 9 and 10 showed an increase in attachment.

Final screening assay

Inocula derived from pools, 2, 3, 6, 9, and 10 after the sixth cycle of panning were used for the final screening. Attachment was done as before and the mixed culture was filtered through 12μm polycarbonate filter and washed gently with 3ml of PBS. The filters were removed aseptically, and the upper surface of the filters was applied to the surface of LB agar supplemented with 15μg/ml tetracycline (LB_{Tet15}). Visible clumps of hyphae were picked and transferred to another LB_{Tet15} agar plate, and incubated at 37 $^{\circ}$C overnight. The following day the bacterial colonies overlapping *C. albicans* colonies were selected and re-streaked to obtain pure cultures. These pure cultures, were retested for their ability to attach by the standard procedure. Among these pure cultures, only one clone could be isolated which exhibited approximately 7% attachment (Fig. 3.18). In order

Table 3.1: The percent attachment of different pools at the final screening step of the plate assay. Among these pools: 2, 3, 6, 9, and 10 had a higher attachment compared to the control.

Pool Number	**Attachment %**
1	4
2	7
3	6.75
4	2
5	0
6	8.6
7	4.6
8	3.3
9	7.6
10	14.6
11	4.3

to confirm it was not a false positive result, the assay was repeated several times with consistent results. This clone was named as 9a. The effect of D-mannose on attachment of clone 9a to *tup1* hyphae was tested. As shown in Fig. 3.19, there was no significant inhibition of attachment.

Genetic analysis of clone 9a

Clone 9a was subjected to genetic analysis in order to identify the PAO1 genes present. Plasmid extraction and purification, agarose gel electrophoresis, transformation and sequencing were done according to standard protocols and manufacturers instructions. Firstly, the cosmid DNA was extracted from clone 9a and transformed to the host LE392 by electroporation. The transformed cells were retested for its attachment ability to confirm that the phenotype was conferred by the cosmid which contains PAO1 genes and not due to a mutation in the original host strain. As shown in Fig. 3.20, the attachment of the transformant was equal to the originally isolated clone 9a. The purified cosmid DNA was digested with *Xho*I and *Eco*RI restriction enzymes to estimate the size of the insert. The *Eco*RI digest showed that the insert is between 17-18kb in size (Fig. 3.21). The Cosmid DNA was further purified by QIAGEN mini prep kits and used for sequencing. The insert was sequenced from both ends using Forward and Reverse primers designed to encompass the *Eco*RI site of the pLAFR vector, designed using the Primer3 (Rozen and Skaletsky, 2000) software. The forward and reverse sequences were aligned with the available *P. aeruginosa* nucleotide sequence database by using Basic Local Alignment and Search Tool (BLAST). Based on the sequence data the insert was 17.3kb long and contained nucleotides 458565 to 475917 of the PAO1 genome (Fig. 3.22). To further confirm the involvement of these genes, seven PAO1 mutants representing mutations in *ChpA,B,C,D,E* operon and three other ORFs were selected from the transposon mutant pool available from University of Washington Genome Center, and tested for their attachment ability. As shown in Fig. 3.23, all of the mutants tested had a lower attachment.

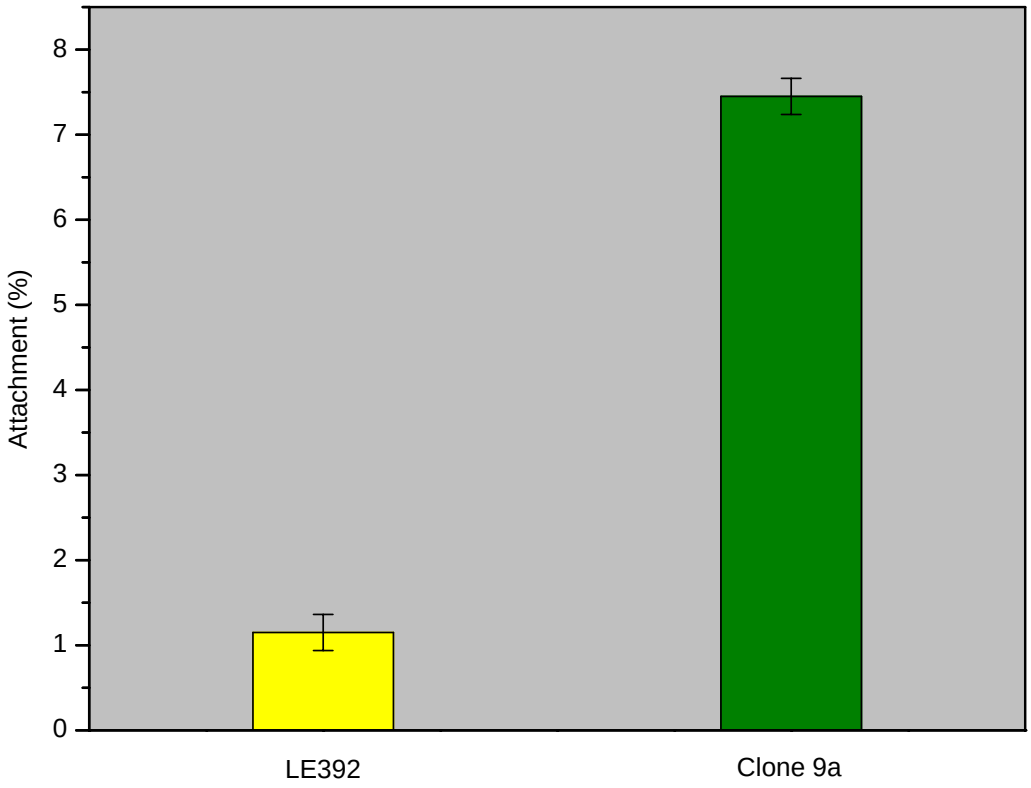

Figure 3.18: The attachment of clone 9a compared with the host strain LE392. Error bars represent standard deviation in triplicate samples.

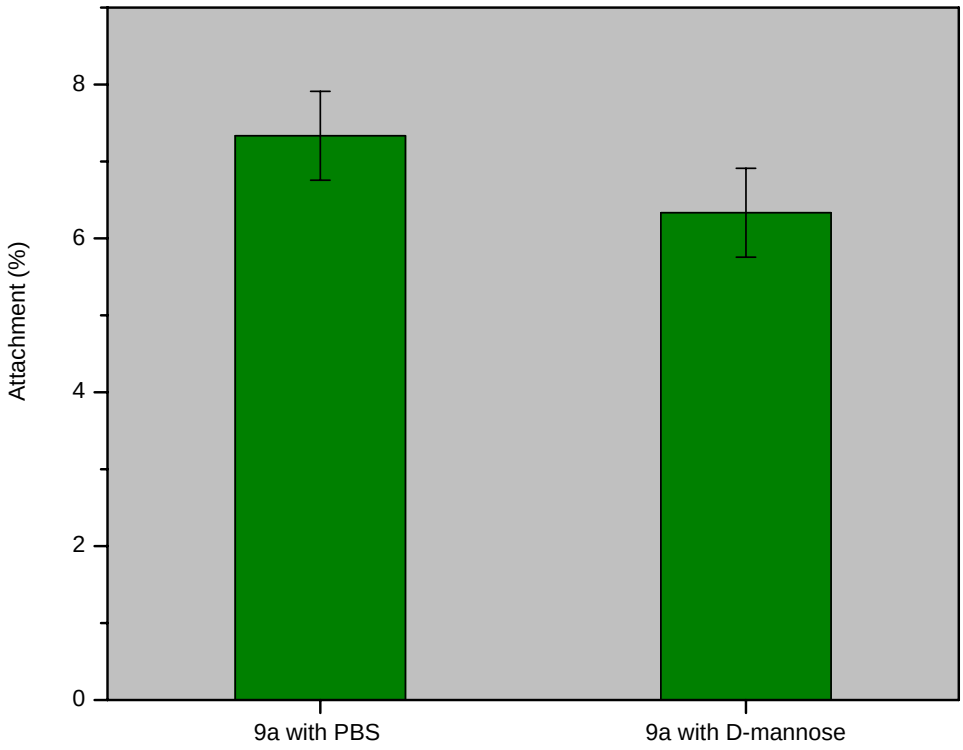

Figure 3.19: Effect of D-mannose on attachment of clone 9a to *C. albicans* (*tup1* mutant). No significant inhibition was observed. Error bars represent standard deviation in triplicate samples.

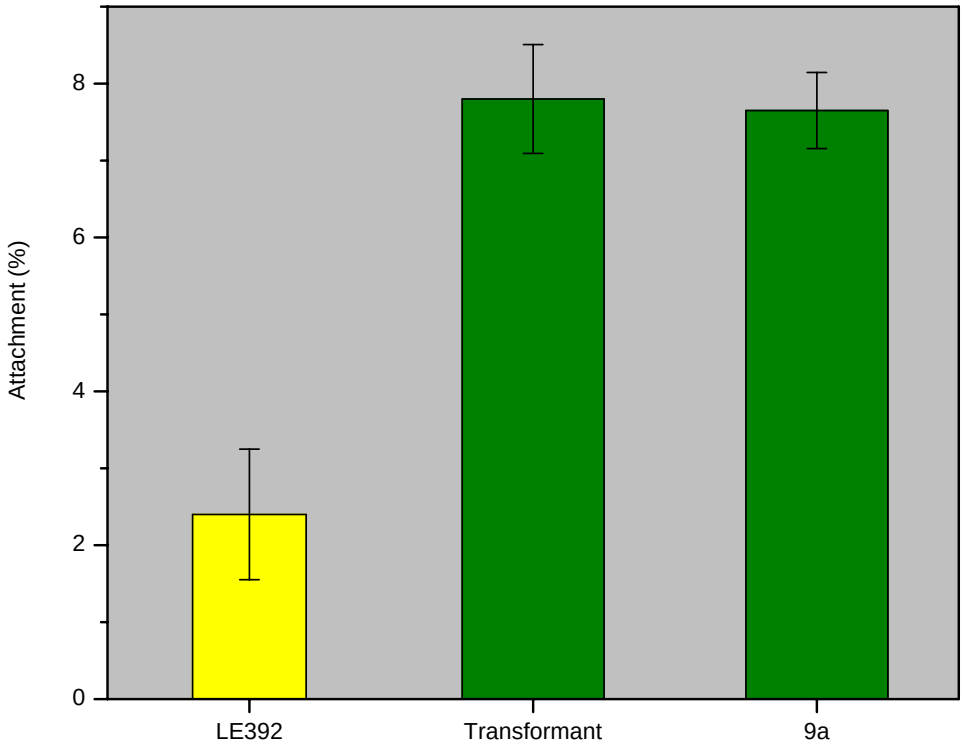

Figure 3.20: Comparison of attachment ability of the transformant with the host strain LE392 and clone 9a. The attachment of both 9a and the transformant was similar. Error bars represent standard deviation in triplicate samples.

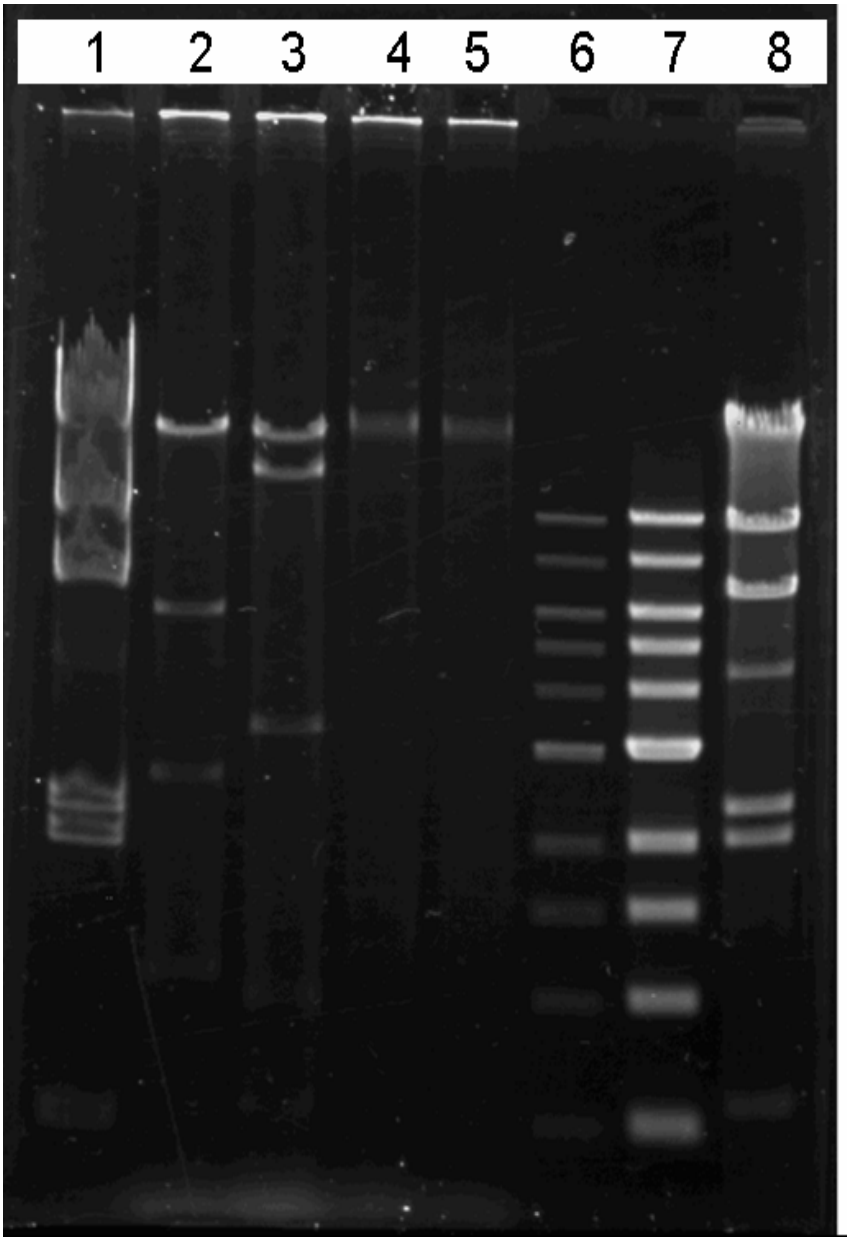

Figure 3.21: Agarose gel electrophoresis results of clone 9a. Lanes 1 and 8: 23kb λ *Hin*dIII marker, Lane 2: *Xho*I digested cosmid DNA, Lane 3: *Eco*RI digested cosmid DNA, Lanes 4 and 5: uncut cosmid DNA, Lanes 6 and 7: 1kb marker. Estimated size of the insert is between 17–18Kb.

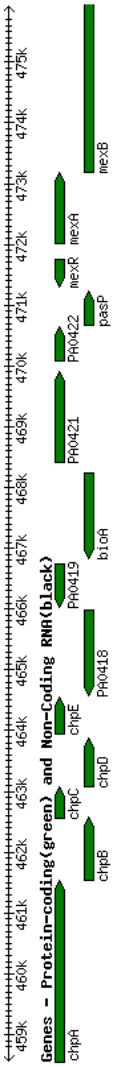

Figure 3.22: The region of the PAO1 genome present in clone 9a. The insert is 17.3kb long and contained nucleotides 458565 to 475917 of the PAO1 genome including some genes of the *Pil-Chp* gene cluster.

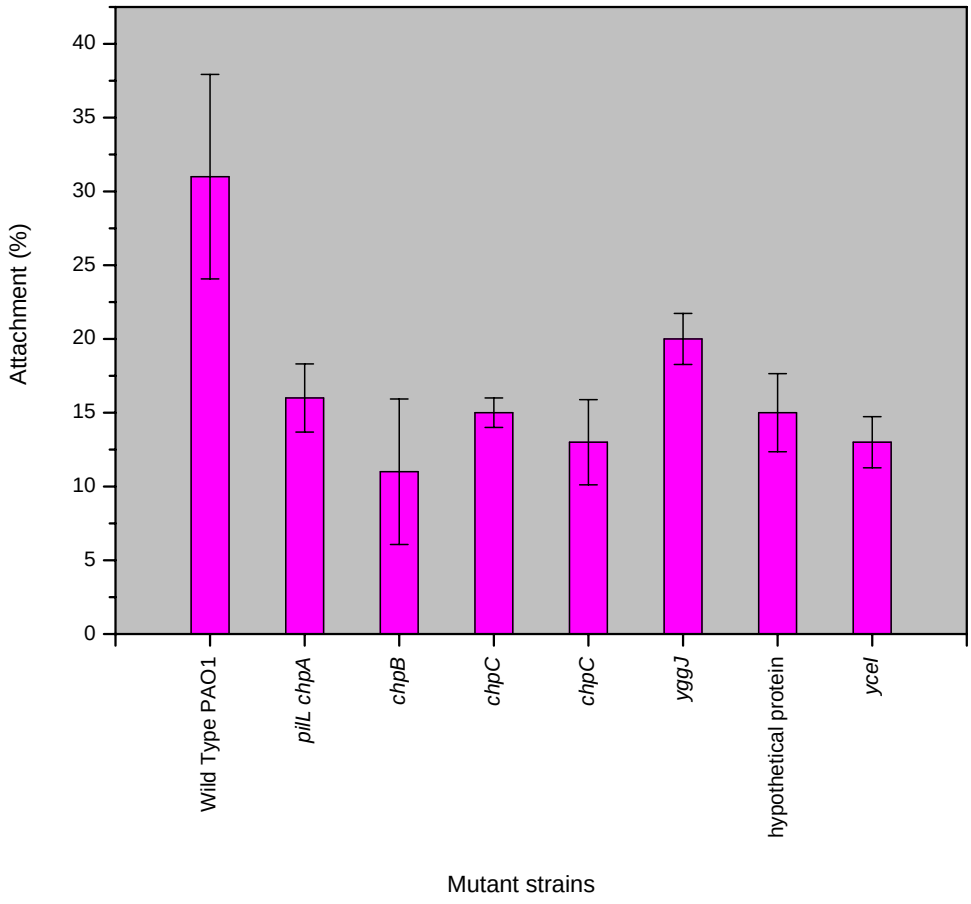

Figure 3.23: Comparison of attachment ability of transposon mutants of PAO1 with the wild type. All of the tested mutants showed a reduction in attachment. Error bars represent standard deviation in triplicate samples.

CHAPTER 4: DISCUSSION

Pseudomonas aeruginosa and *Candida albicans* share many functional characters. Being opportunistic pathogens in immunocompromised patients, the ability to form biofilms on prosthetic devices, and their increased drug resistance are some of the many common traits shared by both organisms (Aaron et al., 2002; Chandra et al., 2001; Douglas, 2003; Ramage et al., 2001). *P. aeruginosa* is one of the most common pathogens found in cystic fibrosis lungs and accounts for the majority of morbidity and mortality among these patients (Lyczak, 2000). *C. albicans* is the fourth most common blood stream pathogen and the third leading cause of catheter related infections (Ramage et al., 2002). It causes a variety of superficial, mucosal, and systemic infections (Douglas, 2003). Although there are antibiotics and antifungals available for such infections, treatments have become increasingly difficult with the emergence of antibiotic resistant strains of these organisms. This warrants the study of alternative approaches.

A novel approach to prevent biofilm formation is to reduce or inhibit the primary attachment of microorganisms. Attachment can be inhibited by various methods such as competitive inhibition of receptors or by modifying the substratum in such a way that organisms cannot bind. Therefore, an in-depth knowledge of receptor-ligand specificity of these pathogens and their behaviors in different ecological conditions is required to formulate preventive or therapeutic measures. This work focuses on growth dynamics of *C. albicans* in a mixed population of *P. aeruginosa* and *C. albicans*, and their receptor and ligand specificities, both from ecological and molecular point of views.

4.1 Effect of *P. aeruginosa* on growth and viability of *C. albicans*

The effect of *P. aeruginosa* (PAO1) on growth and viability of yeast (CAF2) and hyphal (*tup1* mutant) forms of *C. albicans* was studied. The results of this study are shown in Figs. 3.1 and 3.2. Growth of yeast form of CAF2 and hyphal form of *tup1* mutant strains of *C. albicans* was inhibited at temperatures of 30 $^\circ$ and 37 $^\circ$C, respectively. Growth inhibition of the hyphal form of CAF2 is discussed elsewhere in this dissertation. My data indicate that the rate of killing differed from one morphological form to the other. At 37 $^\circ$C, where constitutively filamentous *tup1* mutant was tested, killing began as early as 24h, whereas, the yeast form (at 30 $^\circ$C) survived 48h before the onset of death. The steep slope after 48h in the mixed culture of CAF2 indicates that the death rate is enhanced by the presence of PAO1. This can be attributed to the depletion of nutrients due to competition by both members and accumulation of toxic byproducts. The difference in slopes of the control and the mixed cultures gives a measure of fungal death rate enhanced by PAO1. Further, as shown in the inset to Fig. 3.2, the death rate induced by *P. aeruginosa* in hyphal form is significantly high compared to yeast form. This indicates that the killing effect of *P. aeruginosa* is more pronounce on the hyphal form through the attachment of *P. aeruginosa* to fungal hyphae.

Hogan and Kolter (2002) demonstrated that *P. aeruginosa* binds to *C. albicans* hyphae and kills the fungus, whereas it neither binds nor kills the yeast form of *C. albicans*. Kerr et al. (1999) demonstrated that pyocyanin and 1-hydroxyphenazine produced by *P. aeruginosa* inhibit the growth of *C. albicans*. A recent study by Brand et al. (2008) found that *Pseudomonas* culture filtrates contained heat labile soluble factors that killed *C. albicans* hyphae, and hyphal killing involves both contact mediated and soluble factors. These findings validate our results. A speculation that arises from the results is that if the yeast and hyphae inducing conditions (i.e. 30 $^\circ$ and 37 $^\circ$C) have an effect on enhancing the expression of antifungal factors in *P. aeruginosa*. It

was concluded in a recent study (Brand et al., 2008) that constitutively filamentous *tup1* mutant is hypersensitive to killing by *P. aeruginosa* under hyphal inducing conditions while it is almost resistant to killing under yeast inducing conditions. In other words, the latter condition does not enhance expression of antifungal factors in *P. aeruginosa* or a hyphal–specific property required for susceptibility is not induced in *tup1* mutant.

Based on initial results, a different experiment was carried out to study the effect of *P. aeruginosa* on growth of CAF2 at 37 $^{\circ}$C. As described in **Materials and Methods**, three different growth conditions were used. Results of total viable counts under these conditions are shown in Fig. 3.3. The total viable count of CAF2 increased with time in the control, mixed culture, as well as in the conditioned medium of PAO1. However, the viable count in the culture with live PAO1 cells was significantly lower compared to the others at 24h. Such a large difference (almost 100 times) clearly indicates that the presence of live PAO1 cells inhibited the growth of *C. albicans*. This was further confirmed by the growth rate of CAF2 in conditioned SAB medium without live PAO1 cells. The almost similar rate as in the control implies that extracellular factors produced by PAO1 have not played a role in growth inhibition.

The effect of live PAO1 cells and conditioned SAB broth on the morphogenesis of CAF2 was also studied in the same experiment. Figures 3.4 through 3.6 show photograph images of the morphology of CAF2 cells at different time intervals. The summarized results are shown in Fig. 3.7. As shown in these figures, CAF2 cells in the control and the mixed culture germinated, whereas germination of CAF2 cells in the conditioned SAB broth was insignificant even at 24h. This indicates that extracellular products of PAO1 inhibited germ tube formation. Moreover, at 2h, the percentage of germ tubes was higher in the control, whereas in the mixed culture it was lower, again indicating the effect of *Pseudomonas* on the germination (Fig. 3.7). During the period 4 to 24h, the percentage of yeast cells has increased in both the control and the mixed culture

which may be due to the increase in the cell concentration. The reversion of germ tubes to yeast cells, at 24h, can be seen in Figs. 3.4 and 3.5.

Using a different experimental approach, Hogan et al. (2004) has previously shown that 3-oxo-C(12) Homoserine lactone, a cell-cell signaling molecule produced by *P. aeruginosa*, inhibits *C. albicans* germination without affecting the growth rate. These findings demonstrate that *P. aeruginosa* has an effect on the growth as well as the morphogenesis of *C. albicans,* and that the growth condition has an influence on regulating these factors.

4.2 Attachment of *P. aeruginosa* to *C. albicans*

The attachment of PAO1 to *C. albicans* hyphae and yeast cells is quantitated in Fig. 3.8. The percent attachment of PAO1 to *C. albicans* hyphae was significantly higher irrespective of the fungal strain (hyphae generated from CAF2 and *tup1* mutants), compared to yeast cells. Previous findings on this attachment mechanism by Hogan and Kolter (2002) were reported only for the *tup1* mutant strain. The results of our experiments ruled out the speculation that the attachment is due to a specific character of the *tup1* mutant.

Figure 3.9 shows the results of attachment of PAO1 to *C. albicans* when PAO1 was harvested at different phases of growth. Attachment was higher when PAO1 was harvested from the mid-stationary phase than from either late-log phase or late-stationary phase. This can be attributed to the upregulation of production of adhesins or other extracellular factors in the mid-stationary phase compared to late-log phase and accumulation of toxic products in the late-stationary phase.

The role of LecB in attachment of *P. aeruginosa* to *C. albicans* was studied by using a *lecB* mutant and PAK, its parental wild type strain. The attachment of PAK to *tup1* hyphae was approximately two–fold higher than that of *lecB* mutants (Fig. 3.10). This indicates that

LecB plays a role in this attachment mechanism directly, or indirectly by regulating the expression of other proteins that are responsible for attachment. Previous research has demonstrated that LecB of *P. aeruginosa* plays a major role in adherence (Beuth et al., 1987) and biofilm formation (Imberty et al., 2004; Loris et al., 2003). LecB is L-fucose and D-mannose specific, and *C. albicans* has mannoproteins and candidal lectin-like proteins in its cell wall. Further, numerous studies have shown that there can be competition among organisms for lectin receptors and infection with one microorganism may destroy or uncover receptors for another (Uhlenbruck, 1987).

4.3 Inhibition of attachment by carbohydrates and lectins

To assess whether LecB had a direct role in the attachment, carbohydrate inhibition assays were performed. As shown in (Fig. 3.11), the attachment was approximately five– to six–fold less when incubated with D-glucose and D-mannose, whereas D-galactose, L-fucose, and N-acetylglucosamine showed no effect. Previous research has proved that mannose binding lectins can recognize glucose almost equally well (Loris et al., 2003) and this is consistent with our observations. Also, inhibition of attachment by D-mannose and D-glucose suggests a lectin-carbohydrate interaction.

Garber et al. (1987) studied LecB for its relative affinity to various carbohydrates using equilibrium dialysis and heamagglutination inhibition tests. LecB was found to exhibit a high affinity for L-fucose and its derivatives. Among them p-nitrophenyl-α-L-fucose (pNPF) was the strongest inhibitor followed by L-fucose, L-fucosylamine, L-galactose , D-mannose, and D-fructose. The association constant (Ka) of L-fucose for LecB was 1.5×10^{6} M^{-1} while Ka of D-mannose for LecB was 3.1×10^{2} M^{-1}. It was found that 0.025mM L-fucose was required for inhibition of 2 heamagglutination units. The concentration of D-mannose required for the same degree of heamagglutination inhibition was 5mM, whereas 0.013mM pNPF was enough for the same degree

of inhibition (Garber et al., 1987). Therefore, if LecB is directly involved in the attachment process, the attachment will be inhibited greatly by L-fucose and pNPF. Contradictory to this, there was no such significant inhibition of attachment when treated with L-fucose or pNPF (Figs. 3.11 and 3.12). The differences in inhibition by D-mannose, L-fucose, and pNPF indicate that LecB is not directly involved in this attachment mechanism. This conclusion is supported by the findings of Hogan and Kolter (2002) and Sonowane et al. (2006).

Hogan and Kolter, (2002) analyzed three classes of *P. aeruginosa* mutants in order to investigate the role of *P. aeruginosa* virulence genes on *C. albicans*. These mutants encompassed of i) mutants defective in surface structures, ii) secreted factors, and iii) regulatory molecules. Their results suggested that type IV pili participate in initial attachment to *Candida* hyphae and are not required for biofilm formation at a later time point. Also, mutants lacking type IV pili did not kill hyphae even after 48 hours although they formed biofilms surrounding hyphae. This indicates that type IV pili are directly or indirectly involved in killing of fungal hyphae. Sonowane *et al.* (2006) demonstrated that *pilJ*, which is a member of the operon containing the pili associated genes *pil*G-L, is six–fold less expressed in the *lecB* mutant, and that the *pilJ* mutant was deficient in twitching motility and had no type IV pili. They concluded that LecB is involved in multiple functions such as affecting the expression of PilJ, which in turn affects pilus biogenesis, affects proteolytic activity, and both type II and type IV secretion pathways. They infer that some of the ascribed phenotypes are secondary effects on other systems rather than effects of the lectins themselves.

Inhibition of the attachment by D-mannose may have resulted from blocking the attachment of PAO1 to mannose moieties on *Candida* cell wall. However, this raises the question as to why it cannot bind to yeast form of *Candida* because the cell wall of both hyphae and yeast forms of *Candida* has mannose. A possible explanation is that the PAO1 attaches to a form of mannose which is different in these two morphological forms or some yeast–specific proteins block

the receptors on yeast form of *C. albicans*. However, Brand et al. (2008) showed that the glycosylation status of the *Candida* cell wall affected the rate of contact dependent killing because mutants with severely truncated O-linked but not N-linked mannans were hypersensitive to *Pseudomonas*-mediated killing. In other words, O-linked mannans are protective against killing by *Pseudomonas*. Survival of the yeast form of O-glycosylation mutants was the same as the control strain. Shibata et al. (2007) demonstrated that the morphological transition from yeast to hyphal form induced a significant decrease in the phosphodiesterified acid-labile beta-1,2-linked manno-oligo-saccharides. Brand et al. (2008) also tested 3 hyphal specific proteins, Hyr1p, Hwp1p and Als3p. None of these had an effect on the killing rate.

Concavalin A (ConA) is a carbohydrate binding protein isolated from jack bean *Canavalia ensiformis* (Goto et al., 2002). It has a high affinity for D-mannose and D-glucose (Portez and Goldstein, 1970). The carbohydrate inhibition experiments showed that the attachment of PAO1 to the *tup1* mutant was significantly reduced at the presence of D-mannose and D-glucose, but not D-galactose, L-fucose, and N-acetylglucosamine. Therefore, a reduction in attachment after pretreating the *C. albicans* or PAO1 with ConA would further prove the involvement of mannose residues of *C. albicans* cell wall or bacterial cell surface in this specific interaction. In our observations, the pretreatment of *Candida* and/or PAO1 with ConA did not significantly affect attachment (Fig. 3.13). This could be attributed to receptor specificity as discussed in the **Introduction** chapter. Also, it was found by Goto et al. (2002) that the affinity of ConA for sugars is dependent on its conformation induced by interaction with the binding partner.

4.4 Screening of PAO1 genomic library

Following the results observed in attachment and carbohydrate inhibition studies, we approached the attachment interaction at the molecular level. The purpose of screening the genomic

library was to pinpoint the receptors and ligands involved in the attachment of *P.aeruginosa* to *C. albicans*. Eleven different pools of a PAO1 genomic library were screened for the attachment to hyphae of the *C. albicans tup1* mutant by three different panning assays.

Prior to screening by panning assays, preliminary tests were conducted to rule out any possible false positive or negative results. As shown in Fig. 3.14, it was confirmed that the host strain used to construct the genomic library does not attach to hyphae of the *C. albicans tup1* mutant. Next, as shown in Figs. 3.15 and 3.16, the filter was validated for the ability to retain hyphae and pass the unattached bacteria efficiently. The inability of strain LE392 to attach to hyphae can be detected by the filtering assay as shown in Fig. 3.16. Next, the growth medium used to prepare the inoculum in panning assays was validated. As shown in Fig. 3.17, supplementing the growth medium with tetracycline (15μg/ml) or fluconazole (30μg/ml) has no adverse effect on the growth of the library.

Three different panning assays were designed to screen the genomic library. At the end of this series of screenings, we were able to isolate only a single clone exhibiting a small increase in attachment, which was designated as 9a (Fig. 3.18). Effect of D-mannose on attachment of clone 9a to *C. albicans tup1* hyphae was tested and there was no significant inhibition found as shown in Fig. 3.19. However, the genetic analysis of this clone was continued as it showed a consistent attachment. The phenotype was confirmed by transforming the cosmid back to the host strain LE392 and comparing the attachment ability of the transformant with that of the originally isolated clone 9a and the host LE392 (Figure 3.20).

The cosmid DNA was digested with *Xho*I and *Eco*RI restriction enzymes and run on a 0.7% agarose gel to estimate the size of the insert. As shown in Fig. 3.21, it was around 17-18kb. The insert was sequenced by using Forward and Reverse primers designed to encompass the *Eco*RI site of the pLAFR vector and the sequences were aligned with the available nucleotide sequence

database using the Basic Local Alignment and Search Tool (BLAST). Based on the sequence data it was confirmed that the insert is 17.3kb long and contained nucleotides 458565 to 475917 of the PAO1 genome (Fig. 3.22). In order to assess which genes or operons contained in the cosmid were responsible for conferring adherence, seven transposon mutants representing mutations in *ChpA,B,C,D,E* operon and three other ORFs were tested for their attachment ability. As shown in Fig. 3.23, almost all the mutants had a lower attachment compared to the wild type. These observations indicate that the reduction in attachment is a non specific effect and could be due to a common genetic defect in the background of these transposon mutants. Consequently, it was not possible to attribute the adherence phenotype to specific gene(s) within the cosmid insert.

Failure to isolate a positive clone that completely represents the attachment ability of the wild type PAO1 may be due to several reasons including i) the library is not representative of the entire genome, ii) the panning assays are not effective, and/or iii) the attachment of *P. aeruginosa* to *C. albicans* is multi-factorial. The first two reasons are unlikely as the library has been used by many others with success, and the assays were designed with extreme care to rule out all possible false positive and negative results. The best explanation would be that this attachment is multi-factorial and regulated by more than one mechanism.

BIBLIOGRAPHY

Aaron, S. D., W. Ferris, K. Ramotar, K. Vandemheen, F. Chan, and R. Saginur. 2002. Single and combination antibiotic susceptibilities of planktonic, adherent and biofilm-grown *P. aeruginosa* isolates cultured from sputa of adults with cystic fibrosis. J. Clin. Microbiol. **40**:4172-4179.

Ahearn, D. G., R. N. Borazjani, R. B. Simmons, and M. M. Gabriel. 1999. Primary adhesion of *Pseudomonas aeruginosa* to inanimate polymers including biomaterial. Meth. Enzymol. **310**:551-557.

Argimón, S., J. A. Wishart, R. Leng, S. Macaskill, A. Mavor, T. Alexandris, S. Nicholls, A. W. Knight, B. Enjalbert, R. Walmsley, F. C. Odds, N. A. Gow, and A. J. Brown. 2007. Developmental regulation of an adhesin gene during cellular morphogenesis in the fungal pathogen *Candida albicans*. Eukaryot. Cell **6**:682-692.

Avichezer, D., D. J. Katcoff, N. C. Garber, and N. Gilboa- Garber. 1992. Analysis of the amino acid sequence of the *P. aeruginosa* galactophilic PA-I lectins. The J. Of Biol. Chem. **267**:23023-23027.

Azoulay, E., J. F. Timsit, M. Tafflet, A. de Lassence, M. Darmon, J. R. Zahar, C. Adrie, M. Garrouste-Org, Y. Cohen, B. Mourvillier, and B. Schlemmer. 2006. *Candida* colonization of the respiratory tract and subsequent *Pseudomonas* ventilator-associated pneumonia. Chest **129**:110-117.

Bavington, C., and C. Page. 2005. Stopping Bacterial Adhesion: A Novel Approach to Treating Infections. Respiration **72**:335-344.

Beheshti, F., A. G. Smith, and G. W. Krause. 1975. Germ tube and chlamydospore formation by *C. albicans* on a new medium. J. of Clin. Microbial. **2**:345-348.

Beuth, J., H. L. Ko, G. Uhlenbruck, and G. Pulverer. 1987. Lectin-mediated bacterial adhesion to human tissue. Eur. J. Clin. Microbiol. **6**:591-593.

Bobichon, H., D. Gache, and P. Bouchet. 1994. Ultra rapid cryofixation of *C. albicans*: evidence for a fibrillar reticulated external layer and mannan channels within the cell wall. Cryo-Lett. **15**:161-172.

Brand, A., J. D. Barnes, K. S. Mackenzie, F. C. Odds, and N.A. Gow. 2008. Cell wall glycans and soluble factors determine the interactions between the hyphae of *Candida albicans* and *Pseudomonas aeruginosa*. FEMS Microbiol Lett. 287:48-55.

Brewer, C.F., and L. Bhattacharyya. 1986. Specificity of Concanavalin A binding to asparagine-linked glycopeptides. A nuclear magnetic relaxation dispersion study. J. Biol. Chem. **261**:7306-7310.

Brown, A. J., and N. A. Gow. 1999. Regulatory networks controlling *Candida albicans* morphogenesis. Trends in Microbiol. **7**:333-338.

Cassone, A. 1989. Cell wall of *C. albicans*: its functions and its impact on the host. Curr. Top. Med. Mycol. **3**:248-314.

Calderone, R. A., and W. A. Fonzi. 2001. Virulence factors of *C. albicans.* Trends in Microbiol. 9:327-335.

Chaffin, W. L., J. S. Lopez-Ribot, M. Casanova, D. Gozalbo, and J. P. Martinez. 1998. Cell wall and secreted proteins of *C. albicans*: Identification, Function and expression. Microbiol. And Mol. Biol. Rev. **62**:130-180.

Chandra, J. D., M. Kuhn, P. K. Mukherjee, L. L. Hoyer, T. McCormick, and M. A. Ghannoum. 2001. Biofilm formation by the fungal pathogen *C. albicans*: development, architecture, and drug resistance. J. Bacteriol. **183**:5385-5394.

Chen, H., M. Fujita, Q. Feng, J. Clardy, and G. R. Fink. 2004. Tyrosol is a quorum-sensing molecule in *Candida albicans*. Proc. Natl. Acad. Sci. **101**:5048-5052.

Doig, P., N. R. Smith, T. Todd, and R. T. Irvin. 1987. Characterization of the binding of *Pseduomonas aeruginosa* alginate to human epithelial cells. Infect. Immun. **56**:2788-2793.

Doring, G. and G. B. Pier. 2008. Vaccines and immunotherapy against *P. aeruginosa*. Vaccine **26**:1011-1024.

Douglas, L. J. 2003. *Candida* biofilms and their role in infection. Trends in Microbiol. **11**:30-35.

Dranginis, A. M., J. M. Rauceo, J. E. Coronado, and P. N. Lipke. 2007. A Biochemical Guide to Yeast Adhesins: Glycoproteins for Social and Antisocial Occasions. Microbiol. Mol. Biol. Rev. **71**:282-294.

Dunne, Jr. W. M. 2002. Bacterial adhesion: Seen any good biofilms lately. Clin. microbiol. Rev. **15**:155-166.

El-Azizi, M. A., S. E. Starks, and N. Khardori. 2004. Interactions of *C. albicans* with other *Candida. spp.* and bacteria in the biofilms. J. Appl. Microbiol. **96**:1067-1073.

Fowler, S. and D. S. Jones. 1992. Modified adherence of *Candida albicans* to human buccal epithelial cells *in vitro* following treatment with cationic, non antibiotic antimicrobial agents. Int. J. of Pharm. **86**:193-199.

Garber, N., U. Guempel, N. Gilboa-Garber, and R. J. Doyle.1987. Specificity of the fucose binding lectin *of P. aeruginosa*. FEMS Microbiol. Lett. **48**:331-334.

Gilboa-Garber, N. 1972. Inhibition of broad spectrum hemagglutinin from *P. aeruginosa* by D-Galactose and its derivatives. FEBS Lett. **20**:242-244.

Gilboa-Garber, N. 1982. *P. aeruginosa* lectins. Meth. Enzymol. **83**:378-385.

Gilboa-Garber, N. 1988. *P. aeruginosa* lectins as a model for lectin production, properties, applications and functions. Zbl. Bak. Hyg. **270**:3-15.

Gilboa-Garber, N., D. Avichezer, and N. C. Garber. 1997. Bacterial Lectins: Properties, Structure, Effects, Function and applications. Glycosciences. Chapman Hill, Weinheim. 369-396.

Gilboa-Garber, N., D. J. Katcoff, and N. C. Garber. 2000. Identification and characterization of *P. aeruginosa* PA-IIL lectin gene and protein compared to PA-IL. FEMS Immun. and Med. Microbiol. **29**:53-57.

Glick, J., and N. Garber. 1983. The intracellular localization of *P. aeruginosa* lectins. J. Gen. Microbiol. **129**:3085-3090.

Glitner, C. L., E. J. V. Schaik, G. F. Audette, D. Kao, R. S. Hodges, D. J. Hassett, and R. T. Irvin. 2006. The *Pseudomonas aeruginosa* type IV pilin receptor binding domain function as an adhesion for both biotic and abiotic surfaces. Mol. Microbiol. **59**:1083-1096.

Goto, S., K. Masuda, M. Miura, K. Kanazawa, M. Sasaki, M. Masui, M. Shiramizu, H. Terada, and H. Chuman. 2002. Quantitative estimation of interaction between carbohydrates and concanavalin A by surface plasmon resonance biosensor. Chem. Pharm. Bull. **50**:445-449.

Gow, N. A. R., A. J. P. Brown, and F. C. Odds. 2002. Fungal morphogenesis and host invasion. Curr. Opin. Microbiol. **5**:366-371.

Hagihara, Y., T. Cho, A. Kuroki, T. Suehara, Y. Suehara, and H. Kaminishi. 1988. Aging and germ tube formation in *C. albicans*. Fukuoka Shika Daigaku Gakkai Zasshi. **15**:63-71.

Hansen, J. K., and K. T. Forest. 2006. Type IV pilin structures: Insights on shared architecture, Fiber assembly, Receptor binding and other typ1 II secretion. J Mol Microbiol. Biotechnol. **11**: 192-207.

Hogan, D. A., and R. Kolter. 2002. *Pseudomonas-Candida* Interactions: an ecological role for virulence factors. Science **296**:2229-2232.

Hogan, D. A., A. Vik, and R. Kolter. 2004. A *P. aeruginosa* quorum-sensing molecule influences *C. albicans* morphology. Mol. Microbiol. **54**:1212-1223.

Hornby, J. M., E. C. Jensen, A. D. Lisec, J. J. Tasto, B. Jahnke, R. Shoemaker, P. Dussault, and K. W. Nickerson. 2001. Quorum sensing in the dimorphic fungus *Candida albicans* is mediated by farnesol. Appl. Environ. Microbiol. **67**:2982-2992.

Hudson, D. A., Q. L. Sciascia, R. J. Sanders, G. E. Norris, P. J. B. Edwards, P. A. Sullivan, and P. C. Farley. 2004. Identification of the dialyzable serum inducer of Germ-tube formation in *C. albicans*. Microbiol. **150**:3041-3049.

Imundo, L., J. Barasch, A. Prince, and Q. Al-Awqati. 1995. Cystic fibrosis epithelial cells have a receptor for pathogenic bacteria on their apical surface. Proc. Natl. Acad. Sci. USA. **28**:3019-3023.

Imberty, A., M. Wimmerova, E. P. Mitchell, and N. Gilboa-Garber. 2004. Structures of the lectins from *P. aeruginosa*; insights into the molecular basis for host glycan recognition. Microbes and Infect. **6**:221-228.

Ibrahim, A. S., F. Mirbod, S. G. Filler, Y. Banno, G. T. Cole, Y. Kitajima, J. E. Edwards Jr, Y. Nozawa, and M. A. Ghannoum. 1995. Evidence implicating phospholipase as a virulence factor of *C. albicans*. Infect. Immun. **63**:1993-1998.

Jin, Y., Y. H. S. Samaranayake, H. K. Yip, and L. P. Samaranayake. 2005. Characterization of switch phenotypes in *C. albicans* biofilms. Mycopathologia **160**:191-200.

Joshi, K. R., D. A. Bremner, D. N. Parr, and J. B. Gavin. 1975. The morphological identification of pathogenic yeasts using carbohydrate media. J. Clin. Pathol. **28**:18-24.

Kaleli, I., N. Cevahir, M. Demir, U. Yildirim, and R. Sahin. Anti *Candida*l activity of *Pseudomonas aeruginosa* strains isolated from clinical specimens. Mycoses. **50**: 74-78.

Kerr, J. R. 1994. Suppression of fungal growth exhibited by *P. aeruginosa*. J. Clin. Microbiol. 32:525-527.

Kerr, J. R. 1994. Inhibition of fungal growth by *P. aeruginosa* and *P. cepacia* isolated from patients with cystic fibrosis. J. Infect. **28**:305-310.

Kerr J. R., G. W. Taylor, A. Rutman, N. Høiby, P. J. Cole, and R. Wilson. 1999. *P. aeruginosa* pyocyanin and 1-hydroxyphenazine inhibit fungal growth. J. Clin. Pathol. **52**:385-387.

Kimura, L. H., and N. N. Pearshall. 1980. Relationship between germination of *C. albicans* and increased adherence to human buccal epithelial cells. Infect. Immun. **28**:464-468.

King, R. D., J. C. Lee, and A. L. Morris. 1980. Adherence of *C. albicans* and other *Candida* species to mucosal epithelial cells. Infect. Immun. **27**:667-674.

Lee, K. K., H. B. Sheth, W. Y. Wong, R. Sherburne, W. Paranchych, R. Hodges. 1994. The binding of *Pseudomonas aeruginosa* pili to glycosphingolipids is a tip associated event involving the C-terminal region of the structural pilin subunit. Mol Microbiol. **11**:705-713.

Li, Y., H. Xia, F. Bai, H. Xu, L. Yang, H. Yao, L. Zhang, X. Zhang, Y. Bai, P. E. Saris, T. Tolker-Nielsen, M. Qiao. 2007. Identification of a new gene PA5017 involved in flagella-mediated motility, chemotaxis and biofilm formation in *Pseudomonas aeruginosa*. FEMS Microbiol. Lett. **272**:188-195.

Lightfoot, J., and J. S. Lam. 1991. Molecular cloning of genes involved with expression of A-band lipopolysaccharide, and antigenically conserved form, in *Pseudomonas aeruginosa*. J. Bacteriol. **173**:5624-5630.

Liu, H. 2001. Transcriptional control of dimorphism in *C. albicans*. Curr. Opin. Microbiol. **4**:728-735.

Loris, R., D. Tielker, K. E. Jaeger, L. Wyns. 2003. Structural basis of carbohydrate recognition by the lectin LecB from *P. aeruginosa*. J. Mol. Biol. **331**:861-870.

Lyczak, J. B., C. L. Cannon, and G. B. Pier. 2000. Establishment of *Pseudomonas aeruginosa* infection: lessons from a versatile opportunist. Microbes and Infect. **2**: 1051-1060.

Mateus, C., S. A. Crow, Jr, and D. G. Ahearn. 2004. Adherence of *C. albicans* to silicone induces Immediate enhance tolerance to flucanazole. Antimicrob. Agents and Chemo. **48**:3358-3366.

Nicas, T. I. and B. H. Iglewski. 1985. The contribution of exo products to virulence of *P. aeruginosa*. Can. J. Microbiol. **31**:387-392.

O' Toole, G. A., and R. Kolter. 1998. Flagella and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. Mol. Microbiol. **30**:295-304.

Portez, R. D., and I. J. Goldstein. 1970. An examination of the topography of the saccharide binding sites of concanavalin A and of the forces involved in complexation. Biochemistry **9**:2890-2896.

Ramage, G., K. VandeWalle, B. L. Wickes and J. L. López-Ribot. 2001. Characteristics of biofilm formation by *C. albicans*. Rev. Iberoam. Micol. **18**:163-170.

Ramage, G., P. S Saville, D. P. Thomas and L. J. Lopez-Ribot. 2005. *Candida* Biofilms: an Update. Eukaryot. Cell **4**:633-638.

Rodrigues, A. G., P. A. Mardh, C. Pina-Vaz, J. Martinez-de-oliveira, and A. F. Fonseca. 1999. Germ tube formation changes surface hydrophobicitiy of *Candida* cells. Infect. Disea. Obstet. and Gynecol. **7**:222-226.

Rozen, S., and H. J. Skaletsky. 2000. Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds) Bioinformatics Methods and Protocols: Methods in Molecular Biology. Humana Press, Totowa, NJ, 365-386.

Ruiz-Herrera, J., M. V. Elorza, E. Valentin, and R. Sentandreu. 2006. Molecular organization of the cell wall of *Candida albicans* and its relation to pathogenicity FEMS Yeast Res. **6**:14-29.

Saad, N., E. Jozefowicz, C. Béatrice, B. Sendid, C. D. Pompeo, F. Dewavrin, R. Favory, M. Roussel-Delvallez, and A. Durocher. 2007. Impact of antifungal treatment on *Candida–Pseudomonas* interaction: a preliminary retrospective case–control study. Intensive Care Med. **33**:137-142.

Sambrook, J., E. F. Fritsch, T. Maniatis. 1989. Molecular cloning. A Laboratory Manual. Second Edition. Cold Spring Harbor Laboratory press.

Schirm, M., S. K. Arora, A. Verma, E. Vinogradov, P. Thibault, R. Ramphal, S. M. Logan. 2004. Structural and genetic characterization of glycosylation of type A flagellin in *Pseudomonas aeruginosa*. J. Bacteriol. **186**:2523-2531.

Senet, J. M. 1998. *Candida* adherence phenomena, from commensalism to pathogenicity. Int. Microbiol. **1**:117-122.

Senet, J. M. 1997. Risk factors and physiopathology of Candidiasis. Rev. Ibrroam Micol. **14**:6-13.

Shephered, M. G. 1987. Cell envelope of *C. albicans*. Crit. Rev. Microbiol. **15**:7-25.

Shibata, N., M. Arai, E. Haga, T. Kikuchi, M. Najima, T. Satoh, H. Kobayashi, and S. Suzuki. 1992. Structural identification of an epitope of antigenic factor 5 in mannans of *Candida albicans* NIH B-792 (serotype B) and J-1012 serotype A) as beta-1,2-linked oligomannosyl residues. Infect. Immun. **60**:4100-4110.

Shibata, N., A. Suzuki, H. Kobayashi, and Y. Okawa. 2007. Chemical structure of the cell-wall mannan of *Candida albicans* serotype A and its difference in yeast and hyphal forms. Biochem. J. **404**:365-372.

Slifkin, M. R., and J. Doyle. 1990. Lectins and their application to clinical microbiology. Clin. Micro. Rev. **3**:197-218.

Sonawane, A., J. Jyot, and R. Ramphal. 2006. *Pseudomonas aeruginosa* LecB is involved in pilus biogenesis and protease IV activity but not in adhesion to respiratory mucins. Infect. Immun. **74**:7035-7039.

Stover, C. K., X. Q. Pham, A. L. Erwin, S. D. Mizoguchi, P. Warrener, M. J. Hickey, F. S. L. Brinkman, W. O. Hufnagle, D. J. Kowalik, M. Lagrou, R. L. Garber, L. Goltry, E. Tolentino, S. Westbrock-Wadman, Y. Yuan, L. L. Brody, S. N. Coulter, K. R. Folger, A. Kas, K. Larbig, R. Lim, K. Smith, D. Spencer, G. K. S. Wong, Z. Wu, I. T. Paulsen, J. Reizer, M. H. Saier, R. E. W. Hancock, S. Lory and M. V. Olson. 2000. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. Nature **406**:959-964.

Sudbery, P., N. Gow, and J. Berman. 2004.The distinct morphogenetic states of *C. albicans*. Trends Microbiol. **12**:317-324.

Tart, A. H. and D. J. Wozniak. 2008. Shifting paradigms in *Pseudomonas aeruginosa* biofilm research. Curr. Top. Microbiol. Immunol. **322**:193-206.

Tielker, D., S. Hacker, R. Loris, M. Strathmann, J. Wingender, S. Wilhelm, F. Rosenau, and K. E. Jaeger. 2005. *P. aeruginosa* lectin LecB is located in the outer membrane and is involved in biofilm formation. Microbiology **151**:1313-1323.

Uhlenbruck, G. 1987. Bacterial Lectins: Mediators of Adhesion. Zbl. Bakt. Hyg. **263**:497-508.

Van Delden, C., and B. H. Iglewski. 1998. Cell-to-Cell Signaling and *P. aeruginosa* Infections. Emerg. Infect. Disease **4**:551-560.

Wentworth, J. S., F. E. Austin, N. Garber, and N Gilboa-Garber. 1991. Cytoplasmic lectins contribute to the adhesion of *P. aeruginosa*. Biofouling **4**:99-104.

Wixon, J. 2000. Pathogen special: *Vibrio cholera*, *Pseudomonas aeruginosa* and *Xylella fastidiosa*. Yeast **17**:307-313.

Yu, L., K. K. Lee, R. S. Hodges, W. Paranchych, and R.T. Irvin.1994. Adherence of *P. aeruginosa* and *C. albicans* to glycosphingolipid (Asialo-GM1) receptors is achieved by a conserved receptor-binding domain present on their adhesins. Infect. Immun. **62**:5213-5219.