

QUANTITATIVE ANALYSES OF *CANDIDA ALBICANS* BIOFILM  
FORMATION

**QUANTITATIVE ANALYSES OF *CANDIDA ALBICANS*  
BIOFILM FORMATION**

By

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## ABSTRACT

Strains of pathogens are typically described as virulent or non-virulent. However, in the majority of pathogens, strains often vary continuously and quantitatively in their virulence and pathogenicity. Biofilm formation is one of the recently recognized virulence factors in many human pathogens and little is known about the variation and evolution of biofilms among natural strains. In this study, I examined quantitative variation of biofilms among natural strains of the human pathogenic yeast *Candida albicans*. A total of 115 natural strains of *C. albicans* from three sources (vaginal, oral and environmental) were quantified by two methods: (i) the XTT tetrazolium reduction assay, and (ii) optical density following staining by crystal violet dye. Mature biofilm was confirmed by observation using confocal laser scanning microscopy. My analyses indicated that strains from each of the three sources varied widely in biofilm formation abilities and that biofilm formation ability was positively correlated to cell surface hydrophobicity (CSH). For each strain, multilocus genotypes were determined by PCR-RFLP, my comparative genotype and biofilm analyses demonstrated that natural clones and clonal lineages of *C. albicans* exhibited extensive quantitative variation for biofilm formation. I also examined potential interactions among strains within *C. albicans* and between different *Candida* species. My preliminary results suggest significant variation and complex patterns of strains or species interaction during *Candida* biofilm development.

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## THESIS FORMAT

This thesis is organized as three chapters. The first chapter is a general introduction that provides a general overview of this study. The second chapter includes one of my projects that has been published in *Microbiology* 2003, 149:353-362. The third chapter includes the other two projects that the majority of the experiments have been done, but they will be finished in the future and also will be submitted to a scientific journal.

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## GENERAL INTRODUCTION

### Human pathogenic fungi

Fungi are simple eukaryotes with rigid cell wall but no chloroplasts. There are three major morphological forms: the filamentous form (called molds), the unicellular form (called yeasts), and the mushrooms. Yeasts are usually spherical to ellipsoidal and vary in diameter from 3 to 15  $\mu\text{m}$ . Most yeasts reproduce by budding. A large number of fungi are parasites of plants. Relatively few fungi are parasitic on animals, including humans. The most common pathogenic fungi are found in the genus *Candida*, including *C. albicans*, *C. parapsilosis*, *C. glabrata*, *C. tropicalis* and *C. krusei*, *C. dubliniensis*. *C. albicans* is the leading cause of invasive fungal disease, especially in those hosts with human immunodeficiency virus infection or other immuno-suppressed conditions.

### *Candida albicans* and its pathogenicity

*C. albicans* is an asexual and diploid yeast and is the most widely researched opportunistic human pathogenic fungus. It is often implicated as the primary cause of candidiasis and the original description of the disease can be traced back to 2 centuries ago (Lynch, 1994). The organism was first described by Berg in 1846 and the current name was established in 1923 by Berkout (Lynch, 1994). The genus *Candida* belongs to class Blastomycetes. *Candida* species colonize mucosal surfaces of most humans often soon after birth. *C. albicans* is typically a benign member of the skin and mucosal flora. When host defenses falter, however, *C. albicans* initiates invasive growth that can lead to severe

disease (Odds, 1988). The pathogenesis of *C. albicans* infection is postulated to involve adhesion to epithelial and endothelial cells, digestion by extracellular proteinases, and morphologic switching from yeast form to various filamentous forms: germ tubes, pseudohyphae and hyphae (Odds, 1988, 1994). Even with current antifungal therapy, mortality of patients with invasive candidiasis can be as high as 40% (Pfaller & Wenzel, 1992; Pfaller et al. 1998; Raad, 1998). The majority of nosocomial candidiasis is usually associated with indwelling medical devices, such as intravascular catheters. Most often, biofilms can be found on these medical devices. (Tunnet, 1996; Hawser & Douglas 1994; Donlan 2001).

## **Biofilm**

Microorganisms can exist in many environmental communities. Broadly speaking, microorganisms are divided into two states: free-living planktonic cells and relatively stable microbial communities. The term biofilm is used to describe matrix-enclosed microbial populations adherent to each other and to inert surfaces, in which microbes are embedded in a matrix principally made of extracellular polymers (Costerton et al., 1985). Compared to planktonic forms, biofilms are highly organized communities of cells that may represent the foundation of multicellular life (O'Toole et al., 2000). Despite its complexity, generally, biofilm formation may be divided into the following two stages: attachment of microbes to surfaces, and the proliferation of the attached cells and associated matrix formations.

Biofilm formation is considered as an important source of infection, particularly in association with medical devices (Tunnet et al., 1996; Hawser & Douglas 1994; Donlan 2001). Biofilm-associated microorganisms are typically more resistant to both host defence mechanisms and to antibiotics than planktonic microbes (Bagge et al., 2000; Baillie & Douglas, 1998; Donlan 2001). The types of organisms that develop biofilms are quite broad and include a number of known pathogenic bacteria and fungi. The molecular mechanism of biofilm formation varies among species with many of the genes and signal transduction pathways still unknown. Some recent works have shown that a number of genes and regulatory systems are activated during biofilm formation (O'Toole & Kolter, 1998; Pratt & Kolter, 1998; Watnik & Kolter, 1999), which are associated with cell-to-surface attachment, cell-to-cell interaction, and the growth and maturation of biofilms with 3-dimensional structure.

### **Factors influencing *C. albicans* biofilm formation**

Many factors can influence adherence and biofilm formation, including temperature, pH, nutrient, cell surface hydrophobicity etc. Douglas and her colleagues found that the structure and amount of biofilm formed on different materials could differ (Hawser & Douglas, 1994; Baillie & Douglas, 1999). Most *C. albicans* biofilms consisted a basal yeast layer and hyphae, as observed using scanning electron microscope (SEM) (Baillie & Douglas, 1999). The basal yeast layer has an important role in anchoring the biofilm to the surface, as hyphae are more easily detached from surface.

## Methods of studying biofilm

Several kinds of methods were designed to study *C. albicans* biofilm formation (Baillie & Douglas, 1999). These methods are divided into two groups: static incubation and gentle shaking. Biofilms formed using static incubation have relatively little matrix while shaking generally induces more matrix material. Biofilms formed by both methods are equally resistant to antifungal drugs (Baillie & Douglas, 2000).

*C. albicans* can exist as yeast form cells, pseudohyphae, or hyphae, which depend on growth conditions. Many liquid media have been used for fungal biofilm formation, such as Sabouroud dextrose broth (SDB) with 8% glucose and RPMI 1640 or Yeast nitrogen base (YNB) with 50mM glucose (Shin et al. 2002; Ramage et al. 2001; Baillie & Douglas et al. 1998). YNB-grown biofilms contained mainly yeast forms and RPMI-grown-biofilms consisted mostly of *C. albicans* filaments (Chandra et al. 2001). No quantitative difference was found between RPMI-grown-biofilms and YNB-grown biofilms (Chandra et al. 2001). However, results obtained from SDB-grown biofilms were not comparable to either RPMI-grown biofilms or to YNB-grown biofilms.

Many types of materials have been used for growing *Candida* biofilms in vitro, including polystyrene (e. g. San Millan et al. 1996; Ramage et al. 2001; Shin et al. 2002); polyvinyl chloride (Hawser & Douglas 1994); silicone elastomer (Chandra et al. 2001); and polymethylmethacrylate (Chandra et al. 2001). Evaluation of various catheter materials used in indwelling medical devices showed that biofilm formation by *C. albicans* was slightly different (Hawser & Douglas, 1994). Although polystyrene is not a material used in indwelling medical devices, the wide commercial availability of polystyrene plates in

combination with standardized protocols (Ramage et al. 2001) for this material make it a material of choice for my population surveys.

A number of methods and substrate materials have been used to quantify fungal biofilms, and all were adapted from methods reported previously for bacteria (Baillie & Douglas, 1998; Reynolds & Fink 2001; Merritt et al. 2000). First, direct staining method using crystal violet was widely used for measuring bacterial biofilm formation (Merritt et al. 1998, 2000; Vidal et al. 1998; Watnick et al. 1999; Djordjevic et al. 2002). The method of measuring metabolic activities of biofilm-forming cells by using a XTT tetrazolium reduction assay has been widely used by the fungal biofilm research community and is regarded as the standard method for testing *C. albicans* biofilms in vitro (Baillie & Douglas, 1999; Ramage et al. 2001; Chandra et al. 2001). CV method is different from the XTT-reduction assay in that CV can stain both active cells and the extracellular matrices in mature biofilms (Hawser 1996; Ramage et al. 2001).

# Quantitative variation of biofilms among strains in natural populations of *Candida albicans*

## ABSTRACT

This study examined the quantitative variation of biofilm formation and its relationship to multilocus genotypes in 115 strains of the human pathogenic fungus *Candida albicans*. These strains were isolated from three sources: 47 from oral cavities of healthy volunteers, 31 from the environment, and 37 from the vaginas of patients with candidiasis. For each strain, biofilm formation was quantified as the ability to adhere to and grow on polystyrene plastic surfaces. Confocal laser scanning microscopy was used to visualize and confirm biofilm formation. Two methods were used to quantify biofilm formation abilities: (i) the XTT tetrazolium reduction assay, and (ii) optical density following staining by crystal violet dye. Results obtained by the two methods were significantly correlated. Furthermore, biofilm formation ability was positively correlated to cell surface hydrophobicity. My analyses indicated that strains from each of the three sources varied widely in biofilm formation abilities. However, I observed little correlation between biofilm formation and multilocus genotypes as determined by PCR-RFLP at 16 polymorphic loci, regardless of source of strain. Strains with the same or similar multilocus genotypes often showed very different biofilm formation abilities. My results demonstrated that natural clones and clonal lineages of *C. albicans* exhibited extensive quantitative variation in biofilm formation.

## INTRODUCTION

Biofilms are aggregates of unicellular microorganisms forming multicellular structures that adhere to surfaces. Their formation occurs in response to a variety of cues, including high cell density, nutrient deprivation, and physical environmental stresses (O'Toole et al., 2000). Pathogenic bacteria and fungi can form biofilms on inert surfaces of implanted devices, such as catheters, prosthetic heart valves and joint replacements (Tunnet, 1996; Hawser and Douglas 1994; Donlan 2001). Because biofilms are generally more resistant to both host defense mechanisms and to antibiotics than planktonic unicellular microbes, they represent an ongoing source of infection for many patients (Bagge et al., 2000; Baillie & Douglas, 1998; Donlan 2001), and therefore are increasingly recognized as important health problems for patients with microbial infections. Although the majority of these infections are caused by bacteria, fungal infections are becoming increasingly common, especially those caused by species in the genus *Candida*, including *Candida albicans*.

*Candida* infection is particularly prevalent among hosts infected with the human immunodeficiency virus (HIV), and candidiasis is now recognized as one of the most important hospital-acquired infections (Cox & Perfect, 1993; Holmstrup & Samaranayake, 1990; Odds, 1988). The evolution of *Candida* species into important nosocomial pathogens is related to specific risk factors associated with modern medical therapeutics, such as the use of broad-spectrum antibiotics, cancer chemotherapy, immunosuppressive agents following organ transplantation, and implanted medical devices (Cox & Perfect, 1993; Odds, 1988; Kuhn et al. 2002). While biofilm formation by human pathogenic yeasts has been recognized as a potentially important medical problem (Baillie & Douglas, 1999; Chandra et al. 2001),

relatively little is known about the variation and evolution of biofilm formation within populations of these yeasts.

The increasing understanding of the epidemiology of fungal infectious diseases and the expanding collection of *Candida* species allow us to examine the patterns of variation and evolution of pathogenicity traits among natural isolates of these fungal pathogens. Here I refer to natural strains as those isolated from healthy hosts, patients, or the environments but that have not been experimentally manipulated in laboratories. Genetic analyses have identified that natural populations of *C. albicans* were predominantly clonal with some, perhaps cryptic, recombination (Pujol et al. 1993; Xu et al. 1999a, b; Xu and Mitchell 2002). Following traditional terminology, strains with the same multilocus genotype are referred to as the same clone and strains with highly similar genotypes as the same clonal lineage. It has been hypothesized that in clonally evolving species, phenotypes should be constrained, with strains from each clonal lineage possessing phenotypic values more similar to each other than to strains from different clones or clonal lineages (Lynch & Walsh 1998; Xu & Mitchell 2002). This is because in the absence of sexual reproduction and recombination, genes controlling a phenotype cannot be exchanged among strains to create recombinant genotypes with diverse phenotypic values (Xu & Mitchell 2002). Interestingly, previous studies have demonstrated that some strains of *C. albicans* showed tremendous phenotypic plasticity in the laboratory. One of the best-studied examples was colony morphology switching by laboratory strains of *C. albicans* (Soll 1992; 2002). However, the extent of phenotypic diversity and its relation to genotypes are largely unexplored among natural strains of *C. albicans*.



The objective of this study was to test for potential constraints of genotypes on biofilm formation and to examine the extent of phenotypic diversity among natural strains of *C. albicans*. I examined a total of 115 strains of *C. albicans* isolated from three different sources: oral cavities of healthy volunteers, the environment, and the vaginas of female patients with candidiasis. The phenotypic variation was analyzed in the context of sources of isolation and multilocus genotypes as determined by the polymerase chain reaction (PCR)-restriction fragment length polymorphisms (RFLP) at 16 polymorphic loci (Xu et al., 1999b). Polymorphisms at these 16 loci are presumed to reflect the genetic and evolutionary relationships among strains. To test the genetic basis for variation in biofilm formation among strains, I also quantified cell surface hydrophobicity (CSH) for all strains. My results demonstrated significant correlation between biofilm formation abilities and CSH. However, neither biofilm formation nor CSH was correlated to strain genotype as determined by PCR-RFLP. These results suggested extensive diversity in biofilm formation among strains within the same clone or clonal lineage in natural populations of *C. albicans*.

## **MATERIALS AND METHODS**

**Strains.** The strains used in this study were obtained from three sources: 47 from oral cavities of healthy volunteers, 31 from the environment, and 37 from the vaginas of female patients with clinical candidiasis. For strains from humans, each was isolated from a different host. Environmental isolates were obtained from the effluents of sewage treatment plants. However, these strains are likely transient in the environment because currently, persistent populations of *C. albicans* are not known to exist in any ecological niche other than those in

mammalian hosts and their immediate surrounding environments (Odds, 1988). In this study, since the original source of these environmental strains are not known, I will use the term “environmental isolates” to reflect their current source of isolation.

All strains of *C. albicans* were identified based on the germ tube test, the formation of green colonies on *CHROMagar Candida* (CHROMagar, Paris, France), growth at 45°C; and the fermentation and assimilation profiles on API 20C (BioMérieux, Marcy-Etoile, France). Isolates were maintained in 18% glycerol at -80 °C. For analysis of genotypic and phenotypic traits, strains were first transferred from stock cultures onto YEPD plates (1% yeast extract, 2% Bacto-peptone, 2% D-glucose, 1.5% agar) and incubated at 37 °C. These fresh cultures were further prepared for quantifications of biofilm formation and cell surface hydrophobicity and for DNA extraction.

**Genotype analysis by PCR-RFLP.** DNA extraction and genotyping by PCR-RFLP used methods as described previously by Xu et al. (1999a, b; 2000b). For each strain, the genotype was obtained at all 16 polymorphic restriction sites. These polymorphic sites were distributed among nine DNA fragments amplified by PCR and digested by 4-base cutter restriction enzymes (*DdeI*, *HinfI*, *AluI*, *CfoI*, and *MspI*, GibcoBRL, Maryland, USA) (Xu et al. 1999a, b). Restriction endonuclease digests were performed according to manufacturer’s instructions. PCR products and restriction digests were electrophoresed in 1% agarose in 1 × TAE, stained with ethidium bromide, and viewed and photographed by the image analysis system Fluorochem™-8800 (Alpha InnovTech Cooperation, California, USA). Genotypes at individual restriction sites were scored as previously described (Xu et al. 1999a, b).

**Biofilm formation.** Protocols for inoculum preparation and biofilm growth followed those of Baillie and Douglas (1998, 1999) and Chandra et al. (2001). Briefly, to prepare inoculum, all strains were first streaked onto YEPD agar and incubated at 37 °C for 48 hours. For each strain, a large loop of actively growing cells was transferred to sterile Yeast Nitrogen Base (YNB) broth (Difco Laboratories) containing 0.9% D-glucose. After incubation at 37 °C for 24 hours, the cells were centrifuged and washed twice with 0.5 ml of phosphate-buffered saline (PBS; 0.14 M NaCl, 2.7 mM KCl, 8.5 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8mM KH<sub>2</sub>PO<sub>4</sub>, pH7.4) by vortexing and centrifuged at 5,000 g for 5 min. The washed cells were then re-suspended in 1 ml YNB broth. Optical density of cells was determined for each suspension by spectrophotometer (Ultraspec 2100 Pro, BioChrom) and adjusted to a final OD<sub>600</sub> nm value of 1.0 with YNB broth. These cell suspensions were then used to grow biofilms.

Many types of materials have been used for growing *Candida* biofilms in vitro, including polystyrene (e. g. San Millan et al. 1996; Ramage et al. 2001; Shin et al. 2002; Bachmann et al. 2002); polyvinyl chloride (Hawser & Douglas 1994); silicone elastomer (Chandra et al. 2001); and polymethylmethacrylate (Chandra et al. 2001). Although polystyrene is not a material used in indwelling medical devices, it has been used widely for in vitro diagnostics and shown to be an excellent material for promoting adherence of cells (Merritt et al. 2000). In addition, standardized method for biofilm formation based on polystyrene 96-well plates has been established (Ramage et al. 2001). The wide commercial availability of polystyrene plates in combination with standardized protocols for this material make it a material of choice for population surveys.

For each strain, 100  $\mu$ l of the suspension (OD<sub>600 nm</sub>=1.0) was inoculated into individual wells of polystyrene 96-well plates (Flat-bottom, Nunc). Three repeats were performed for each strain using a randomized block design (Sokal & Rohlf, 1981). YNB broth containing no inoculum was used as a negative control. The plates were incubated at 37 °C for 90 min (adhesion periods). Supernatant including planktonic cells and liquid medium was then discarded and wells were gently washed twice with PBS to get rid of any non-adherent cells. For biofilm growth, 100 $\mu$ l fresh YNB broth was then added to each well. The plates were covered, wrapped with parafilm to prevent evaporation, and incubated at 37 °C for 48 hr. After biofilm formation and growth, planktonic cells were discarded through two rounds of washing with 200  $\mu$ l of sterile PBS buffer. Remaining cells sticking to the plastic surfaces were confirmed as biofilm cells through microscopy and these were quantified using two different methods. Both microscopy and biofilm quantification are described below.

**Microscopy.** Following protocols described in the study by Chandra et al. (2001), confocal laser scanning microscopy (CLSM) was used to confirm that mature biofilms were formed after 48 hr incubation. Because 96-well plates are not conducive for microscopic observations, biofilms for microscopy were prepared on polystyrene petri-dishes. Culture preparation, cell adhesion and biofilm growth were performed exactly as those in 96-well plates. After biofilm formation, 4 ml of PBS solution containing the fluorescent stains FUN-1 (10  $\mu$ M, Molecular Probes, Oregon, US) and Concanavalin A-Alexafluor 488 conjugate (Con A, 25  $\mu$ g/ml; Molecular Probes) were added to stain biofilms for 45 min at 37 °C. FUN-1 (excitation wavelength=543nm, emission =560nm long-pass filter) was taken up by metabolically active

fungal cells and converted from a diffuse yellow color to red. In contrast, Con A (excitation wavelength=488nm, emission =505nm long-pass filter) selectively binds to cell wall polysaccharides to produce green fluorescence. Stained biofilms were observed with a Bio-Rad Radiance 2000 CLSM and images were captured and processed using LaserSharp 2000 software (Bio-Rad Canada).

**Quantifying biofilms.** Two methods were used to quantify biofilm formation abilities: (i) the XTT [2,3-bis (2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide]-reduction assay; and (ii) crystal violet (CV) staining followed by measures of optical density. The XTT-reduction assay was performed according to protocols used by Baillie and Douglas (1999) and Ramage et al. (2001). Briefly, 100  $\mu$ l of mixture of XTT salt solution (1mg/ml) (Sigma) and menadione solution (1  $\mu$ M) (Molecular Probes, Oregon, US, prepared in acetone) were added to each well containing mature biofilms. The cultures were then incubated in the dark at 37  $^{\circ}$ C for 5 hr. Biofilm metabolism would reduce XTT tetrazolium salt to XTT formazan by mitochondrial dehydrogenases and result in a colorimetric change (Hawser 1996; Ramage et al. 2001). The amount of colorimetric change was measured using a microplate reader at OD490 nm (model EL340, Mandel Scientific). Microtiter wells containing only YNB broth but no microbe were used as negative controls.

In the method involving staining with crystal violet, 100  $\mu$ l of 1% CV was added to each well and incubated for 20 min at 37  $^{\circ}$ C. Next, 150  $\mu$ l of 95% ethanol were added to dissolve the dyed biofilm cells, and 100  $\mu$ l of each mixture was transferred to a new 96-well microplate. The optical density for each well was determined using a microplate reader at

OD570 nm (model EL340), Mandel Scientific). Similarly, wells containing only YNB broth but no microbe were used as negative controls.

**Cell surface hydrophobicity.** Tests for cell surface hydrophobicity (CSH) followed the protocol of Klotz and co-workers (1985). Briefly, cell suspensions of all strains were first prepared as described above for biofilm formation (OD<sub>600nm</sub>=1.0 in YNB broth). For each strain, 1.2 ml of the suspension was placed in a clean glass tube and overlaid with 0.3 ml of octane. The mixtures were vortexed for 3 min and then the phases were allowed to separate. OD at 600 nm was determined for the aqueous phase soon after the two phases had separated. OD values of strains in YNB broth without octane overlay were used as a negative control. Three repeats were performed for each strain. Following Hazen et al. (1986) and Klotz et al. (1985), the relative hydrophobicity was obtained as:

$$[(\text{OD}_{600} \text{ of the control} - \text{OD}_{600} \text{ after octane overlay}) / \text{OD}_{600} \text{ of the control}] \times 100$$

### **Data Analyses.**

**Biofilm and hydrophobicity data:** To compare variation and correlation within and among *C. albicans* samples, means and standard deviations (SD) of biofilm formation and cell surface hydrophobicity were calculated for each sample. The statistical significance in the difference among samples was compared by the t-test (Sokal & Rohlf, 1981). Pearson's correlation index was used to determine the relationship between hydrophobicity and biofilm formation in my samples and between results obtained from the two different biofilm quantifying methods.

**Relationships among strains:** Similarity coefficients based on multilocus genotypes between pairs of isolates were calculated as the ratio of matches over the total number of alleles scored. An unweighted pair group method with arithmetic mean (UPGMA) phenogram showing the similarities of all strains was generated based on the pairwise similarity coefficient matrix. The calculations of similarity coefficients and the generation of the UPGMA phenogram were done using the software PAUP4.0b5 (Swofford, 2002).

**Genotypic diversities:** For quantitative comparisons of genotype diversities among samples, I used two methods. The first is Stoddart's genotypic diversity (Stoddart & Taylor, 1988),  $G = 1/\sum p_i^2$ , where  $p_i$  is the frequency of the  $i$ th multilocus genotype in the sample. This diversity index has a value that ranges from a minimum of 1, when all isolates are of the same genotype, to a maximum of  $N$  (the sample size), when every isolate has a different genotype. Differences of genotypic diversity were measured using a t-test between the percentages of maximum possible diversity determined for each collection. The formula of calculating variance followed that described by Chen et al. (1994), as follows:

$$\text{Var}(G) = 4G^2 (\sum p_i^3 - 1) / N$$

The second diversity measure was Simpson's unbiased Index of Diversity (Simpson, 1949). Simpson's diversity ( $\lambda$ ) is calculated as  $(1 - \sum p_i^2) N / (N - 1)$  where  $p_i$  represents the frequency of a particular multilocus genotype, and  $N$  is the sample size. This diversity has a value that ranges from a minimum of 0, when all isolates are of the same genotype, to a maximum of 1, when every isolate is a different genotype. The variance of  $\lambda$  was calculated as (Simpson, 1949):

$$2[2(N-2) \sum p_i^3 + \sum p_i^2 - (2N-3)(\sum p_i^2)^2]/N(N-1)$$

Statistical significances of differences in Stoddart's and Simpson's genotypic diversities between samples were then determined by t-tests.

## RESULTS

### Genotype distribution and genotypic diversities

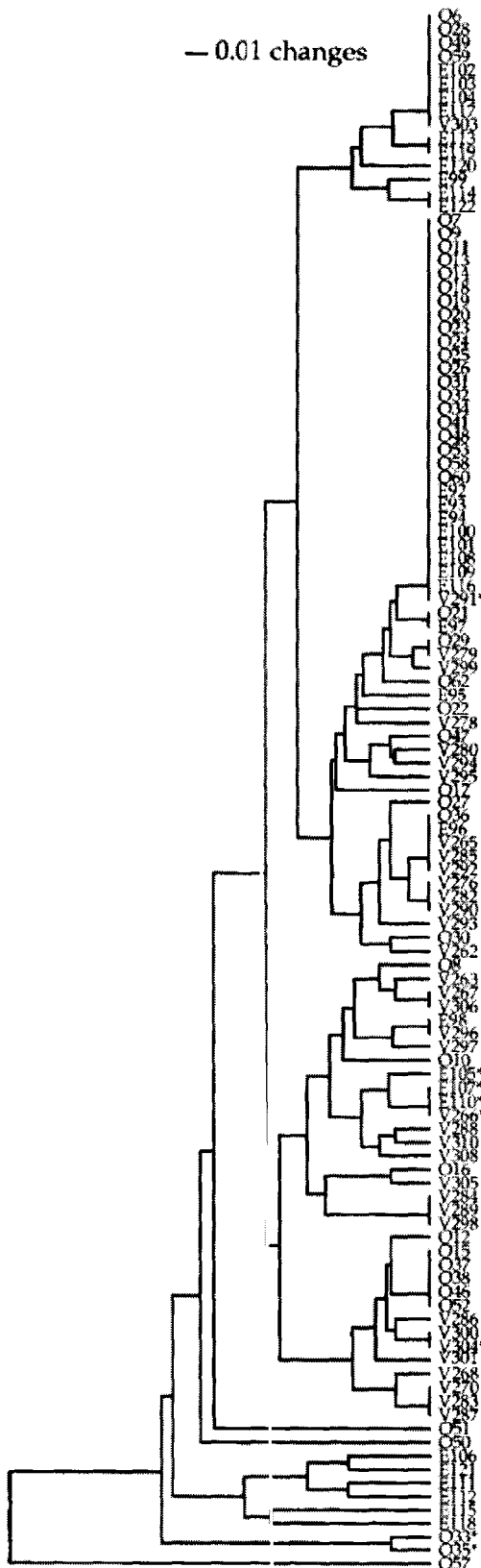
A total of 56 unique multilocus genotypes were identified among the 115 strains. Genetic similarities among these strains and genotypes are presented in Fig. 1. Overall, shared genotypes were dispersed throughout the phenogram, regardless of isolation source. However, there were small clusters of isolation source-specific genotypes or genotype groups (Fig. 1).

The most common genotype included strains from all three sources and accounted for 29 of the 115 isolates (Fig. 1). Two other genotypes also contained strains from all three sources and included 9 and 5 strains, respectively (Fig. 1). Four additional genotypes were shared by strains from two of the three sources and accounted for a total of 9 isolates. Overall, 51% (40/78) of isolates obtained from oral and environmental sources shared genotypes; 34% (23/68) of isolates from environmental and vaginal sources shared genotypes; and 38% (32/84) of isolates from oral and vaginal sources shared genotypes. Among the 56 multilocus genotypes, 15 were shared by more than one isolates, the remaining 41 were represented by only one isolate each (Fig. 1).



Figure 1. UPGMA phenogram describing genetic similarities among 115 strains of *C. albicans*.

Genotypes and genotype groups based on the 16 PCR-RFLP loci were marked for comparison as described in the text. Strain designations reflect the source of isolation, followed by a numerical isolation number. O: strains from oral cavities of healthy volunteers; E: strains from the environment; V: strains from vagina of patients with candidiasis. Clones and clonal lineages are described in the text (see Materials and Methods, and Results). Strains marked by a star sign ‘ \* ‘ are those specifically mentioned in text.



Clone

Clonal lineage

A

I

B

II

C

III

D

IV

Other

The genotype diversities of samples from different sources are presented in Table 1. Analyses with the two diversity measures, Stoddart's genotypic diversity and Simpson's unbiased Index of Diversity, yielded similar results. In both analyses, the vaginal population from patients with vaginal candidiasis had the highest diversity (Table 1). For example, the Stoddart's genotypic diversity ( $\pm$ standard deviation) in the vaginal sample was  $21.2 \pm 11.59$ . The oral sample from healthy volunteers had the lowest genotypic diversity,  $4.6 \pm 1.43$ . The environmental sample had an intermediate genotypic diversity of  $9.2 \pm 7.95$ . Pairwise comparisons revealed that these differences were statistically significant (Table 1;  $P < 0.05$ ).

### **Significant variation in biofilm formation abilities among strains**

After 48 hr incubation at 37°C in YNB broth, mature biofilm formation was confirmed by CLSM (Fig 2). These biofilms contained both fungal cells and extra-cellular matrices.

The amount of biofilm formation was significantly correlated between the two quantifying methods (correlation coefficient = 0.96,  $P < 0.001$ ) (Fig 3). Within each strain, there was very little variation among repeats. The standard deviation among repeats was low, typically 5-10% of the mean by both methods. These results suggest that both quantifying methods were highly reproducible and random errors were small. The summary results obtained by the two methods are described below.

**Results from XTT-reduction assay:** Summary results for biofilms from each source are presented in Table 1. In this assay, the mean OD490 nm value for the 115 strains was 0.073 ( $\pm$  0.038, standard deviation, SD) with a range of 0.049-0.329, a 6.7-fold difference between the highest and lowest

Table 1. Genotype diversities and biofilm formation within samples of *C. albicans* analyzed in this study.

Source	N	No. of Genotypes	No. of unique Genotypes	Genotypic diversity <sup>1</sup>		Biofilm <sup>2</sup>	
				Stoddart's	Simpson's	XTT	Crystal violet
Oral	47	21	16	4.61 ± 1.43	0.801 ± 0.055	0.077 ± 0.052 (0.053-0.329)	0.209 ± 0.424 (0.050-2.179)
Environmental	31	18	12	9.17 ± 7.95	0.923 ± 0.043	0.079 ± 0.025 (0.055-0.158)	0.217 ± 0.246 (0.087-1.032)
Vaginal	37	27	21	21.21 ± 11.59	0.982 ± 0.011	0.063 ± 0.020 (0.049-0.157)	0.143 ± 0.081 (0.043-0.526)

<sup>1</sup>, Data are genotypic diversity ± SD. All of the pairwise differences in genotypic diversities between sources were statistically significant using the methods by Chen et al. (1994) and Simpson (1949).

<sup>2</sup>, Data are mean ± SD (Range). None of the pairwise differences in biofilm formation abilities between sources were statistically significant.

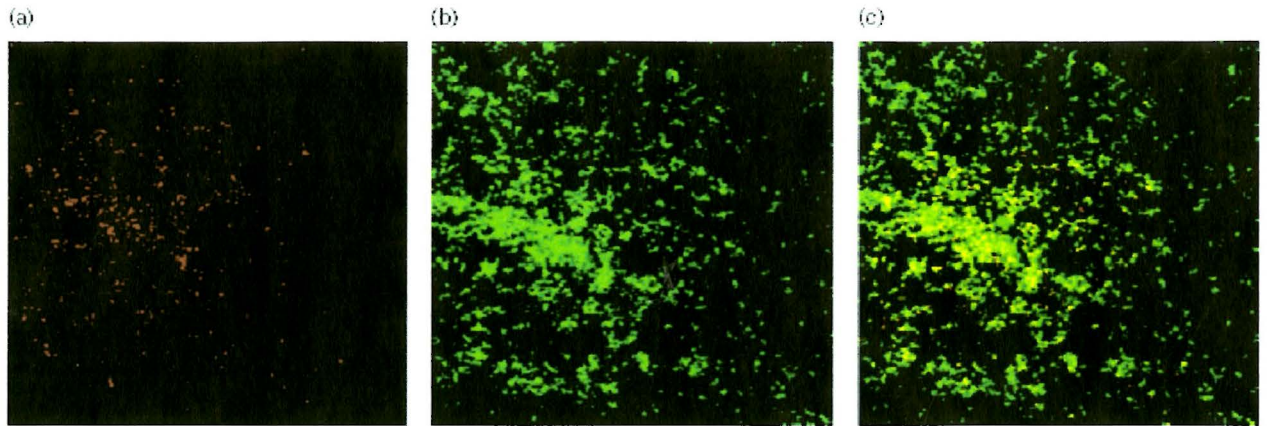


Figure 2. Confirmation of mature biofilm of *Candida albicans* by confocal laser scanning microscopy (CLSM). Metabolically active cells are shown in red (A) and extra-cellular polysaccharides are shown in green (B). Picture C is a composite of A and B. Metabolically active cells with extra-cellular matrices are shown in yellow. Note extensive green staining in both pictures B and C.

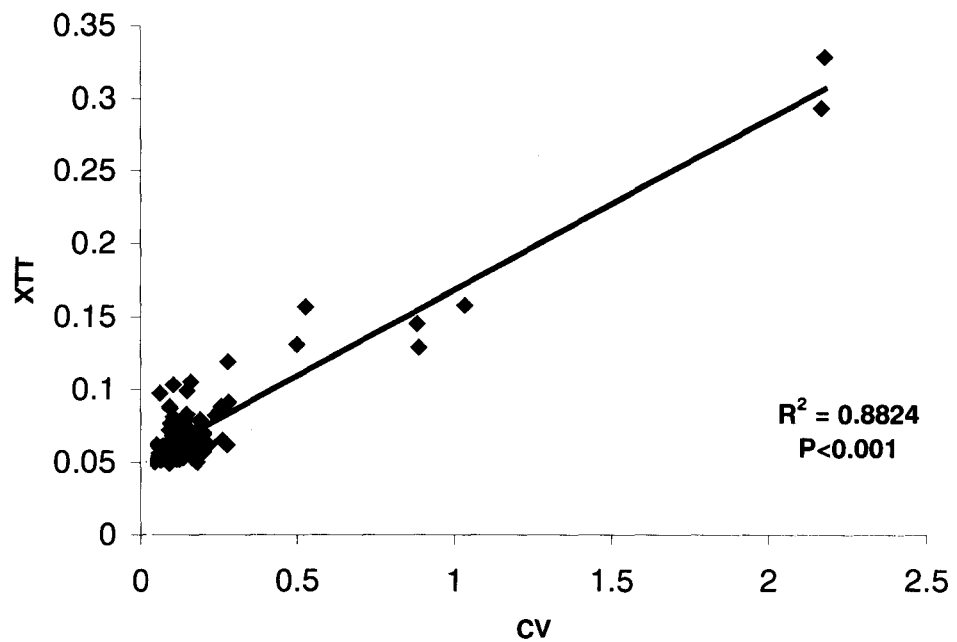


Figure 3. Correlation between XTT-reduction assay and CV assay among 115 natural populations of *C. albicans*. CV assay at OD600nm is shown in X-axis and XTT assay at OD490nm is shown in Y-axis.

biofilm-producing strains. The mean biofilm formation abilities were similar for strains from the three different isolation sources. No statistically significant difference was detected among the three samples ( $P > 0.05$  in all pairwise comparisons) (Table 1). Variations in biofilm production among clones and clonal lineages are presented in Table 2 and discussed in a later section.

**Results from crystal violet staining:** Summary results for biofilms quantified by this method from each source are presented in Table 1. In general, the CV staining method revealed a greater range of variation of biofilm production among strains. The mean biofilms quantified by this method for the 115 strains was  $0.189 (\pm 0.301, \text{SD})$  with a range of 0.043-2.179, a 50.7-fold difference between the highest and lowest biofilm-producing strains. Both XTT-reduction assay and CV staining method detected that the same strain (O33) produced the highest biofilm. However, they detected different lowest ones. Strain V291 was the lowest biofilm producer (0.049) as determined by the XTT-reduction assay while strain V304 the lowest (0.043) by the CV staining method. Their respective values by the other methods were 0.089 by CV staining for V291 and 0.050 by XTT reduction assay for V304. Similar to the results obtained by the XTT-reduction assay, the mean biofilm formation abilities were similar for strains from the three different isolation sources. No statistically significant difference was detected among the three samples ( $P > 0.05$  in all pairwise comparisons) (Table 1). More detailed analyses of variations in biofilm production among clones and clonal lineages are presented in Table 2 and discussed in a later section.

**Positive correlation between biofilm and cell surface hydrophobicity (CSH).**

The CSH of 115 strains of *C. albicans* was measured by the biphasic separation method in YNB medium at 37°C as described in the Materials and Methods. Positive correlations were observed between biofilm formations as quantified by both methods and CSH. The correlation coefficient was 0.801 ( $P < 0.001$ ) between CSH and biofilms determined by XTT-reduction assay. Similarly, a correlation coefficient of 0.765 ( $P < 0.001$ ) was obtained between CSH and biofilms determined by CV staining. Two oral strains (O33 and O35) with the highest CSH (1.001 and 0.745 respectively) were also the biggest biofilm producers. This result suggests that CHS contributes significantly to biofilm formation.

#### **Diversity in biofilm formation among clones and clonal lineages of *C. albicans***

My combined genotypic and phenotypic analyses revealed extensive diversity in biofilm formation among clones and clonal lineages in natural populations of *C. albicans* (Table 2). Here, the diversity for a phenotype is defined as the range of phenotypic values exhibited by a clone or clonal lineage, in relation to phenotypic values from among unrelated clones or clonal lineages. In *C. albicans*, strains with the same multilocus genotype likely belong to a clone or a clonal lineage (Xu & Mitchell 2002). The phenotypic diversity within a clone or a clonal lineage is demonstrated by the following two analyses. First, strains in the same clone often exhibit very different biofilm formation abilities (Table 2). For example, clone A in Fig. 1 had a range of biofilm formation ability from 0.051 (strain V303) to 0.105 (strain E104), a two fold difference, as determined by the XTT-reduction assay (Table 2) (A difference of 4-folds among strains was detected by the CV staining method in clone A).



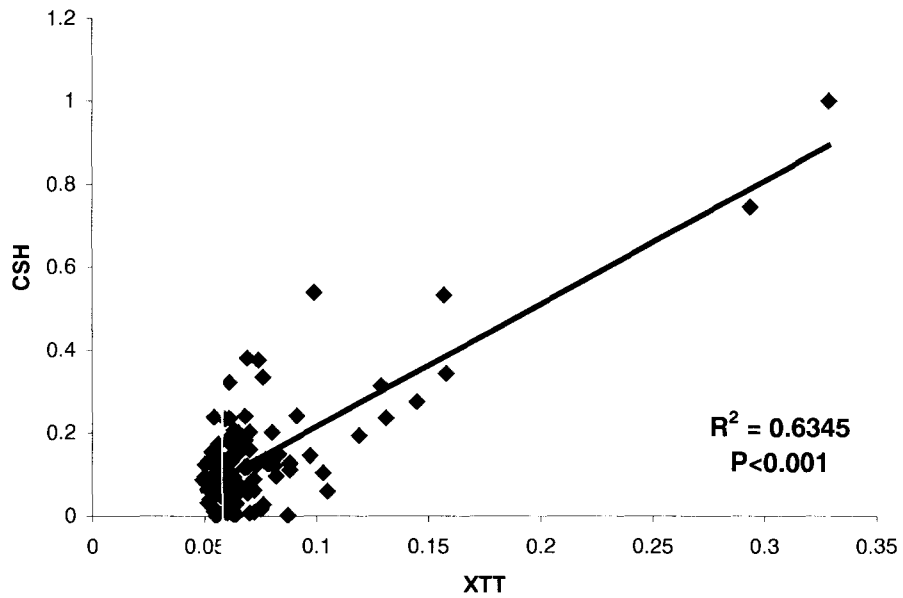


Figure 4. Correlation between XTT-reduction assay and CSH among 115 natural populations of *C. albicans*. XTT assays at OD490nm are shown in X-axis and CSH values are shown in Y-axis.

Strains in clones B, C, and D also showed a wide variation, with biofilms quantified by CV staining showing greater variation than those by the XTT-reduction assay (Table 2).

Second, the lack of constraints by genetic relationships among strains on biofilms could also be demonstrated based on analyses of clonal lineages (Xu & Mitchell 2002). In this analysis, 104 of the 115 strains were grouped into four such lineages (I, II, III, and IV) based on a genetic similarity of 90% among strains within a lineage (Fig. 1). The remaining 11 strains were genetically highly diverse and did not constitute a comparable lineage. These 11 strains were therefore classified as the "Other" group (Fig. 1). My analyses showed no significant differences in biofilm formation ability among these clonal lineages (Table 2;  $P > 0.2$  in all pairwise comparisons). All groups had wide variation in biofilm formation abilities as determined by either method (Table 2). In this analysis, genetic distances separating these lineages were arbitrary. However, analyses based on other *a priori* genetic distances did not change my general conclusion. Overall, there were wide variations in biofilm formation among strains within a lineage but no significant difference among lineages (Table 2). These results further indicated an overall lack of evolutionary constraint on biofilm formation abilities in *C. albicans*.

While my results suggested extensive diversity in biofilm formation within individual clones and clonal lineages, small clusters of strains with relatively uniform biofilm producing abilities can be found. Two specific groups containing strains with high biofilm formation abilities are worth mentioning. One group contained three strains, E105, E107, and E110 (Fig. 1). All three were from the environment and biofilms produced by these strains were 2-3 times the mean of the whole sample as determined by the XTT-reduction assay (a mean of

Table 2. Biofilm production among clones and clonal lineages in *C. albicans*.

Genotype group <sup>1</sup>	N	Biofilm <sup>2</sup>	
		XTT	CV
Clone A	9	0.068±0.018 (0.051-0.105)	0.127±0.058 (0.062-0.257)
Clone B	29	0.068±0.013 (0.049-0.101)	0.117±0.044 (0.061-0.281)
Clone C	5	0.059±0.004 (0.052-0.063)	0.131±0.043 (0.087-0.198)
Clone D	5	0.061±0.006 (0.054-0.068)	0.085±0.020 (0.050-0.100)
Clonal lineage I	15	0.070±0.017 (0.051-0.105)	0.134±0.061 (0.062-0.260)
Clonal lineage II	55	0.068±0.018 (0.049-0.157)	0.135±0.072 (0.061-0.526)
Clonal lineage III	20	0.073±0.031 (0.050-0.158)	0.250±0.299 (0.080-1.032)
Clonal lineage IV	14	0.060±0.007 (0.050-0.076)	0.109±0.045 (0.043-0.198)
Other	11	0.117±0.098 (0.055-0.329)	0.537±0.817 (0.088-2.179)

<sup>1</sup>, Genotype groupings correspond to those described in Figure 1 and in the text.

<sup>2</sup>, Biofilm values are presented as mean ± standard deviation (range). Wide variation in biofilm production was observed in most clones and clonal lineages. No statistically significant differences in biofilm formation were observed among these genotype groupings.

0.144 for the three strains vs. the mean of 0.073 for the whole sample of 115 strains). When measured by the CV staining method, the differences increased (0.933 vs. 0.189).

Interestingly, a vaginal strain (V266) with the same multilocus genotype as E107 and E110 (Fig. 1) had a much lower biofilm formation ability of 0.062. The second group consisted of two strains, O33 and O35. Among the 115 strains, these two formed by far the most biofilms, over four times of the population mean as determined by the XTT-reduction method (0.312 vs. 0.073); and over 10 times by the CV staining method (2.174 vs. 0.189).

## DISCUSSION

In this study, I applied population genetics to analyze quantitative variation in biofilm formation in the human pathogenic yeast *C. albicans*. I found significant differences in genotype diversity among samples from the three different sources: oral, vaginal and environmental. Strains from each of the three sources exhibited wide variations in biofilm formation. Results from my two different biofilm quantifying methods were comparable and significantly correlated. The positive correlation observed between biofilm formation and cell surface hydrophobicity (CSH) suggested that CSH played a major role in biofilm formation in *C. albicans*. This result is consistent and extended previous observations of positive correlation between adhesion to plastic surfaces and CSH among *Candida* species (el-Azizi & Khardori, 1999; Klotz et al. 1985). Furthermore, the combined analyses of multilocus genotypes and biofilm formation indicated extensive phenotypic diversity in biofilm formation among clones and clonal lineages in natural populations of *C. albicans*.

Among the three samples, the vaginal sample had the highest genotypic diversity, followed by the environmental sample and the oral sample. While the mechanisms for these differences are not presently known, there are a couple of possibilities. The first is that the observed differences were purely a sampling effect. Samples derived from different groups of hosts, body sites, or other physical environments may have patterns of genetic diversities different from the ones observed here. Indeed, subtle differences in genotypic diversity have been observed in oral yeast samples isolated from geographically or ecologically diverse groups of people (Xu et al. 1999a, b, 2000b; Xu, unpublished data). The second possibility is that ecological factors that differed among the three sampling sources did play a role in

generating and maintaining genotypic diversity of *C. albicans*. The three isolation sources represented very diverse ecological niches and differ in many biotic and abiotic factors, e.g. other microbial species within the community, host factors (or lack of), temperature, nutrient levels, generation times, and accessibility to external sources of yeast populations. Whether and how these and other potential factors contribute to the generation and maintenance of *C. albicans* genotypic diversity awaits further investigations.

In this study, I used polystyrene plates to grow biofilms. Because polystyrene is not a material used in indwelling medical devices, the results I obtained here may not correspond exactly to clinical biofilm formation abilities. It is possible that using other materials might produce results different from these based on polystyrene plates. Indeed, Baillie and Douglas (2000) found that *C. albicans* biofilms grown on two different polyvinyl chloride (PVC) catheters (supplied by different manufacturers) showed significant differences in susceptibility to Amphotericin B. However, for several reasons, I believe my results obtained from polystyrene materials are relevant and important for understanding fungal biofilms. First, polystyrene has been used widely for in vitro diagnostics in a variety of microorganisms and shown to be an excellent material for promoting adherence of cells (for a review see Merritt et al. 2000). Second, many studies of *C. albicans* biofilms used polystyrene (e. g. San Millan et al. 1996; Ramage et al. 2001; Shin et al. 2002; Bachmann et al. 2002), therefore, my results can be compared to these and other future studies. Third, an efficient method for biofilm formation in *C. albicans* based on polystyrene 96-well plates has been established and standardized (Ramage et al. 2001; Bachmann et al. 2002), and unlike many other materials, polystyrene plates are commercially available and relatively inexpensive. Fourth, the

significant correlation between biofilm formation and cell surface hydrophobicity (CSH) observed here suggests that adhesion to and growth on polystyrene surfaces reflected intrinsic structural and biochemical differences among strains of *C. albicans*.

My results indicated an overall lack of difference in biofilm production among samples from the three examined sources. However, this result does not exclude the possibility that strains from other samples might show statistically different amount of biofilms. Indeed, a recent study by Kuhn et al. (2002) showed that invasive isolates of *C. albicans* produced more biofilms than non-invasive strains when measured by dry weight. However, such a pattern was not observed when biofilms were measured by the XTT-reduction assay (Kuhn et al. 2002). The authors attributed the discordance of their results to several potential factors, including the presence/absence of a blastospore layer, differences in metabolic rates and the amount of extracellular matrices (Kuhn et al. 2002). While biofilm dry weight was not determined for my strains, results from the two methods examined in my study were highly correlated. It should be noted that Kuhn and co-workers (2002) only analyzed a total of ten strains of *C. albicans*. These ten strains were from seven different body sites, with three strains from blood, two from catheters, and one each from denture, urine, vagina, skin and bronchia. Therefore, the conclusion that invasive isolates produced more biofilms needs to be confirmed using a larger sample from each of the body sites. Such a study could be done in a large medical center where such collections are available. At present, my samples are limited, with most strains originated from oral cavities of healthy hosts.

To compare biofilm formation at the population level, it is critical to have an efficient and highly reproducible method for quantification. A number of methods and substrate

materials have been used to quantify fungal biofilms, and all were adapted from methods reported previously for bacteria (Baillie & Douglas, 1998; Reynolds & Fink 2001; Merritt et al. 2000). In this study, I used and compared two methods and both were highly reproducible with little random error. The first method measured metabolic activities of biofilm-forming cells by using a XTT tetrazolium reduction assay. This method has been widely used by the fungal biofilm research community and is regarded as the standard method for testing *C. albicans* biofilms in vitro (Baillie & Douglas, 1999; Ramage et al. 2001; Chandra et al. 2001). Here I adopted the microtiter format of the XTT-reduction assay as applied by Ramage et al. (2001). In addition, I also used direct staining method using crystal violet. Crystal violet (CV) is a basic dye. It binds to negatively-charged extracellular molecules, including cell surface molecules and polysaccharides in the extracellular matrices in mature biofilms. The CV method is different from the XTT-reduction assay in that CV can stain both active cells and the extracellular matrices in mature biofilms (Hawser 1996; Ramage et al. 2001). The CV staining method is widely used for measuring biofilms in bacteria (Merritt et al. 1998, 2000; Vidal et al. 1998; Watnick et al. 1999; Djordjevic et al. 2002). My analyses showed that results from these two methods were significantly correlated, with a correlation coefficient of 0.96 ( $P < 0.001$ ). This correlation indicated that higher biofilm metabolic activities (due to either higher density of cells and/or higher metabolic activity per cell) likely generate higher amount of extracellular matrices. Compared to the XTT-reduction assay, the crystal violet staining method was cheaper and faster.

A typical laboratory fungal model of biofilm formation involves two operational steps: adhesion, and biofilm growth and maturation (Baillie & Douglas, 1999; Chandra et al. 2001).



Using different species of *Candida*, previous researchers observed that fungal adherence to plastic surfaces were correlated with CSH (Hazen et al. 1986; Klotz et al., 1985; Silva et al. 1995; Samaranayake et al. 1995). In my study, I identified significant positive correlation between CSH and biofilm formation in a large sample of natural strains of *C. albicans*. While the relative contributions of hydrophobicity to the different steps of biofilm formation in *C. albicans* is not known, my results were consistent with previous findings that hydrophobicity was a major determinant of biofilm formation in *C. albicans*. The importance of cell surface hydrophobicity for adhesion to inert surfaces has also been demonstrated experimentally through gene knockout experiments in the Baker's yeast *Saccharomyces cerevisiae* (Reynolds & Fink, 2001).

My comparative analyses demonstrated abundant diversity in biofilm formation for clones and clonal lineages of *C. albicans*. Within most clones and clonal lineages, I found a wide variation in biofilm formation among strains. This pattern of genotype and phenotype relationship has been observed for other traits in *C. albicans*, including susceptibility to antifungal drugs (Cowen et al. 1999; Xu et al. 2000a); colony morphology (Hellstein et al. 1993); and ecological distributions (Xu et al. 1999a, b; 2000, see also Fig. 1 in this study). I would like to stress that, though the 16 PCR-RFLP markers could help define potentially divergent and robust evolutionary lineages in *C. albicans*, the objective here was not to use these genetic markers to define such evolutionary lineages. Instead, groupings as defined in my analyses served only to provide a simple way to visualize, analyze, and present the quantitative diversities of biofilms in *C. albicans*. Delineating robust evolutionary lineages in *C. albicans* could be better achieved using DNA sequence polymorphisms at multiple nuclear

and mitochondrial genomic regions (e. g. as that done for the pathogenic basidiomycete yeast *Cryptococcus neoformans*, Xu et al. 2000a).

In the last twenty years, significant progress has been made to address the long-standing evolutionary question of why a predominantly clonally reproducing species such as *C. albicans* could survive and persist in human populations and cause diseases (e.g. Soll 2002). The results from this and other studies suggest that in the absence of sexual reproduction and recombination, populations of *C. albicans* could adapt to and successfully colonize human populations through its extraordinary phenotypic diversity (for a recent review, see Soll 2002). Phenotypic diversity in pathogenic microbes could have significant practical implications as well. For example, if genotypes based on neutral markers can 't be used as reliable predictors of medically important traits, targeted treatment strategies based on strain genotypes are less likely to succeed for pathogens exhibiting greater phenotypic diversities.

It should be noted that environmental and clinical biofilms rarely, if ever, consist only of cells from a single species. Indeed, earlier observations found that almost all yeast biofilms from medical grade silicone rubber contained bacteria (Neu et al., 1994). Co-aggregation and extensive interactions of *Candida* spp. with other bacteria and yeast could promote colonization and biofilm formation of yeast cells on host tissues and plastic surfaces (el-Azizi & Khardori, 1999; Adam et al., 2002). Additional studies are needed to determine whether genotypes and evolutionary relationships play any role in biofilm formation involving multiple species or strains.

## **Perspectives and preliminary experiments on patterns of strain and species interactions during biofilm development**

Natural biofilms often contain a mixture of cells and interactions among these cells are potentially important for the stability and function of these biofilms (James et al. 1995). In human hosts, different strains of the same yeast species or different species are often found in the same ecological niches (Bisno & Waldvogel, 1989; Costerton et al. 1985), suggesting that potential interactions among strains and species might exist in human hosts. In this section, I will present two preliminary studies on patterns of interactions, one between strains within *C. albicans* and the second between *C. albicans* and *C. parapsilosis*, *C. tropicalis*, *C. krusei* and *C. glabrata*. Preliminary results are summarized below.

### **3.1. Patterns of interactions among strains within *C. albicans*.**

Genetically marked strains of *Candida albicans* were used to examine patterns of interactions during adhesion and biofilm growth. The effect of treatment by the common antifungal drug fluconazole was also included. My preliminary results found that during adhesion, all except one mixed-culture showed neutral interactions, regardless of fluconazole treatment. During biofilm growth and maturation, strain pair-specific interactions were observed, including neutral, positive and negative interactions. With

fluconazole treatment, I observed two distinct patterns: neutral interactions between fluconazole-sensitive strains and positive interactions between a fluconazole-resistant strain and five sensitive strains. This result suggests that mixed cultures with fluconazole resistant and sensitive strains enhanced biofilm formation in the presence of fluconazole. Additional assays were used to determine cell numbers of individual strains in mixed biofilms. I observed that wild type cells had competitive advantages over auxotrophic mutants in both adhesion and biofilm growth phases with or without fluconazole treatment. PCR method was used to distinguish the relative proportions in the mixed TW1 (fluconazole-sensitive) and TW16 (fluconazole-resistant) using relative band intensities at the ERG16 gene as an indicator. The proportion of TW1 increased from 46% to 75% in adhesion but decreased from 60% to 9% in mature biofilms when the concentration of fluconazole increased from 0 to 64ug/ml. Overall, my analyses indicated that the presence of fluconazole enhances adhesion but retards biofilm growth and maturation.

### **3.2 Interactions between *Candida albicans* and non-*albicans* *Candida* species during biofilm development**

In this preliminary investigation, three isolates of *Candida albicans* with different biofilm formation ability were selected to examine potential ecological interactions with four other *Candida* species *C. parapsilosis*, *C. glabrata*, *C. tropicalis* and *C. krusei*. Three strains were included from each of the four species. Similar to the patterns observed between strains within *C. albicans*, I observed species and strain specific pattern of interactions.

Most interactions were neutral, however, positive and negative interactions were also observed. Furthermore, because *C. albicans* can be distinguished from other *Candida* species using an indicator medium CHROMAgar, I assayed the relative proportions of individual species after the formation of biofilms. Overall, I found a significantly higher proportion of *C. albicans* than other species in mixed mature biofilms. These results suggest a potential explanation for the prevalence of *C. albicans* in human hosts.

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