Published in final edited form as:

Mycopathologia. 2006 January ; 161(1): 3–10. doi:10.1007/s11046-005-0167-2.

Non-glucan attached proteins of *Candida albicans* biofilm formed on various surfaces

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Abstract

Non-glucan attached proteins of the cell surface and extracellular matrix of *Candida albicans* biofilms formed on two catheter surfaces and denture acrylic were examined. The SDS-PAGE protein profiles of these proteins compared with that obtained from planktonic yeast cells and germ tubes were generally similar. This observation suggested that this class of biofilm surface proteins is not composed of a unique set of extracellular proteins or that one or a few proteins dominate the non-glucan attached proteins of biofilm. However, differences were observed in the proteins obtained from biofilm formed on one catheter surface and two proteins, Grp2p and ORF19.822p, identified by mass spectrometry following two-dimensional separation. These proteins have previously been associated with drug resistance and their presence or abundance appeared to be influenced by the surface on which the biofilm was formed.

Keywords

biofilm; *Candida albicans*; cell wall; surface proteins

Introduction

Candida albicans is a human commensal and opportunistic pathogen. In addition to colonizing cutaneous and mucosal surfaces, the organism can colonize and form biofilms on medical devices. Catheters, (intravascular and urinary), dentures, gastrostomy tubes, voice prostheses, and prosthetic heart valves are among the devices that may be colonized [1-3]. Deterioration of devices and infection of host tissue can result from the biofilm formation on the surfaces. Infections associated with colonized devices can range from frequent morbidity to life threatening. Morbidity is found with the some 50% of denture wearers who develop denture stomatitis [4, 5] and some one million Americans with catheter associated urinary tract infections [3]. *C. albicans* is the third most common cause of intravascular catheter related infections and associated with a higher mortality than bacterial infection [6, 7].

Candidal biofilms have been formed in vitro in a number of model systems that include various abiotic supports such as catheter disks, cellulose, polymethylmethacrylate (denture

acrylic), voice prostheses, glass, and with both static and continuous medium replenishment [8, 9]. The conditions, surface supporting biofilm formation and strain my influence the extent of biofilm formation, the morphological forms present and the abundance of extracellular matrix material. From a yeast cell inoculum, *C. albicans* will form a multilayered biofilm on catheter surfaces that consists of yeast cells, germ tubes, hyphae, and pseudohyphae and extracellular matrix material [10-12]. In biofilm formation, adherence of cells to the substrate, formation of yeast cells, hyphae and pseudohyphae, and formation of extracellular matrix are associated with the cell surface and extracellular components. Therefore, the composition of cell surface proteins is a potential target for changes that accompany biofilm formation. Extracellular matrix material reacts with Calcoflour white and concanavalin A suggesting the presence of polysaccharides [13]. The composition of extracellular material obtained from supernatants of disaggregated biofilms formed on catheter disks or from planktonic culture supernatants was primarily carbohydrate in both cases [14]. Compared with the planktonic extract, the material from biofilm contained a higher proportion of glucose. However, about half of the biofilm material was not identified. The aim of the present study was to examine the profile of soluble (non-glucan attached) cell surface and biofilm proteins.

Methods

Organism and culture conditions

C. albicans NCPF 3153 and ATCC 10261 that adheres to salivary components [15] were used in experiments for biofilms formed on catheter material and denture acrylic respectively and the same strain used for comparison of planktonic organisms. The strains were grown in yeast nitrogen base (YNB with amino acids, Difco Laboratories, Detroit, MI) medium containing 50 mM glucose at 37 °C with shaking (150 rpm). Yeast cells from fresh overnight cultures of *C. albicans* resuspended at 2×10^6 cells/ml were used for biofilm formation. To induce hyphal cells, *C. albicans* was grown in rich medium as described below and inoculated (0.1 ml, 2×10^6 cells/ml) into YNB and grown at 37 °C for 18 h with shaking [16]. Nutrient rich liquid medium (yeast extract, peptone, glucose, YEPD) was also used to grow *C. albicans* yeast cells at 37 °C for 18 h with shaking to isolate cell surface proteins.

Biofilm formation

A 30-cm section of silicone-coated catheter was inoculated with 1 ml of suspension (2×10^6 cells/ml) of yeast cells and incubated for 1 h at 37 °C. One end of the catheter tubing was connected to a syringe filter (0.22 μ m) to allow sterile medium into the catheter. The setup was connected to tubing and a peristaltic pump to maintain a constant flow of medium (0.8 ml/min) for 48 h at 37 °C. Catheters were obtained from Argyle (Sherwood Medical, St. Louis, MO) with the first lot manufactured by one subcontractor and the second by another subcontractor after the first subcontract expired.

To develop biofilm on polymethylmethacrylate (acrylic) strips, 8–10 acrylic strips (10 cm $\times$ 1.5–2 mm $\times$ 2 cm (prepared by Dr. Thomas McKinney, Baylor College of Dentistry, Texas A&M University System Health Science Center) were placed in a 60 ml syringe connected to tubing at both ends. A filter was placed in the inlet line and the whole apparatus gas

sterilized. To prepare the strips for biofilm formation, whole saliva was collected from healthy volunteers with informed consent as approved by the Institutional Review Board, pooled, diluted 1:2 with sterile buffer (0.6 M KCl, pH 6.5) sterilized by exposure to UV light for 3 h. Sterility was confirmed by the absence of microbial growth when an aliquot was plated on agar medium. The diluted saliva was added to the syringe and incubated with strips for 1 h at 37 °C. The saliva was removed and a yeast cell suspension added (10^6 cells/ml) and incubation continued for 1 h. The non-adherent yeast cells were removed by washing with phosphate buffered saline. The inlet tubing was connected to a peristaltic pump and a constant medium flow rate of 0.8 ml/min was maintained for 48 h. Temperature was maintained by placing the apparatus in an incubator. For comparison planktonic cells were grown in a flask in similar conditions.

Scanning electron microscopy (SEM)

Essentially, biofilms were fixed and prepared for SEM as described [10]. The catheters or acrylic strips were not suitable for use in the microscope and the biofilms were removed by gentle lifting with a sterile razor blade and placed on a glass coverslip and plunged into a liquid propane: isopentane mixture (2:1, V/V) at -196 °C before freeze-drying under vacuum. Dried samples were coated with gold and viewed under a SEM (Hitachi S-500).

Drug susceptibility

The susceptibility of the biofilm organisms scraped from the catheter and planktonic control cells grown in a flask to amphotericin B was determined as described [12] except that after 1 h treatment the cells were plated on yeast extract peptone glucose agar medium for determining viable count. Plates were incubated for 16 h at 37 °C and cfu were determined. The data were analyzed by analysis of variance (ANOVA) followed by Bonferroni Multiple Comparison Tests with $p = 0.05$.

The sensitivity of the biofilm formed on the saliva-coated denture acrylic strips was determined. Planktonic cells or cells removed from the biofilm were suspended in RPMI medium (10^6 cells/ml) with or without drug and incubated at 37 °C for 15 h. The assay was performed in triplicate. Aliquots of each culture were transferred to a microtiter plate and OD₅₅₀ determined with a plate reader. Sensitivity was determined for amphotericin B and ketoconazole.

Soluble protein extraction and analysis

Biofilm organisms were removed by scraping the catheter tubing or acrylic strips with a sterile blade or needle loop. Biofilm organisms were checked for morphology by light microscopy. Organisms were washed twice with PBS. Washed materials were directly mixed with extraction buffer containing ammonium carbonate (1.89 g/l) and 1% (v/v) β -mercaptoethanol (β ME extract) [17]. Pooled biofilm washes and β ME extracts were dialyzed thoroughly against 5 mM Tris-HCl, pH 7.4 at 4 °C and lyophilized. Protein concentration was determined by the dye binding method of Bradford [18] using gamma globulin as protein standard. Moieties present in the extracts were analyzed by SDS-PAGE (12.5% separating gel, 4% stacking gel, w/v) using slab mini gel format and stained with a commercial silver stain kit (BioRad Laboratories, Hercules, CA) or a double stain performed

by counter staining the silver stained gel with Coomassie brilliant blue [19]. For two dimensional gel electrophoresis (2-DE), the proteins were separated as previously described [19].

For Western blot analysis, proteins separated by SDS-PAGE were transferred to nitrocellulose membranes. Membranes were blocked in Tris buffered saline pH 7.5 (TBS) plus 3% milk, for 1 h at room temperature. The membrane was then incubated with pooled rabbit polyclonal antiserum prepared by combining antiserum prepared to different cell wall extracts of planktonic yeast and germ tubes [20] diluted 1:1000 in TBST (TBS + 0.05 % Tween 20). After washing in TBST, the membrane was incubated with goat anti-rabbit IgG conjugated with horse radish peroxidase diluted 1:1000 in the same buffer with 1% bovine serum albumin for 1 h at room temperature. After washing with TBST four times for ten minutes, the blots were developed with hydrogen peroxide and 4-chloronaphthol substrates.

For protein sequence analysis, protein spots were removed from the 2-DE gel and digested with trypsin (Trypsin Gold, Mass spectrometry grade, Promega, Madison, WI) according to the supplier's protocol. Trypsin digests were submitted to the Texas Tech University Center for Biotechnology Core Facility for LC/MS/MS (Liquid Chromatography-Mass Spectrometry) analysis on a ThermoElectron DECA XPlus. The spectra obtained were compared to *C. albicans* database generated using the ThermoElectron Turbo Sequest software. The predicted molecular weight and the pI values of the proteins were compared to that of the observed data along with the best X correlation values of the matched peptides.

Results

Biofilm formation in catheter tubing and on denture acrylic

To obtain enough material for biochemical analysis of surface proteins, two models were used for biofilm formation in a 30 cm segment of silicone coated elastomer catheter segment and on strips of denture acrylic coated with human saliva. Both biofilms were developed for 48 h with continuous medium flow. Phenotypic properties of the biofilms were examined by susceptibility of biofilm organisms to antifungal drugs and scanning electron microscopy. Similar to the reports for biofilms formed on catheter surfaces [10-12] and in the catheter segment (data not shown), biofilm formed on the surface of the denture acrylic contained multiple layers of organisms present in both yeast cell and hyphal morphologies with the presence of extracellular matrix material (Figure 1a). Much of the extracellular matrix was lost in the recovery and preparation of the biofilm for imaging. As reported by others [12, 14, 21, 22] biofilms from both models showed reduced susceptibility to ketoconazole (Figure 2) and amphotericin B (data not shown).

Biofilm surface proteins

Soluble surface proteins from biofilm organisms obtained from catheter tubing and denture acrylic and planktonic cells were prepared and analyzed by SDS-PAGE. No protein profile difference was found between planktonic yeast cells of *C. albicans* strains 3153 and 10261 grown in YEPD medium. After extraction of biofilm organisms extracellular matrix material was no longer observed (Figure 1b).

The protein profiles obtained in these extracts were compared to determine if the presence of extracellular matrix contributed additional proteins to the soluble component. First, the profiles obtained from biofilm formed in the catheter segment, washes of the biofilm, and planktonic cells were compared and found generally similar. There was no new protein whose abundance dominated the other constituents either within the biofilm extract or in comparison to the extract from yeast cells. However, within this similarity, two proteins, with apparent molecular weights of 33 and 40 kDa were produced more abundantly by biofilm organisms than planktonic cells (Figure 3a). These two proteins were observed in extracts obtained from three biofilms. Amphotericin B affects membrane integrity and we questioned whether this would alter the relationship with the non-glucan attached proteins retained in the cell wall. The biofilm was exposed in situ and composition of soluble proteins was examined. Amphotericin B (1.3 $\mu\text{g/ml}$) was added to the medium and medium flow through the catheter continued for 1 h prior to analysis. The profiles from both the treated biofilm and treated biofilm washes contain similar protein bands to that of unexposed biofilm cells or washes (Figure 3a).

The origin and identity of the 33 and 40 kDa proteins was examined. Since biofilm contained hyphal organisms which were not present in the planktonic control culture, these proteins could be due to the presence of germ tubes and hyphae in the biofilm. Soluble surface proteins of germ tubes formed under planktonic conditions were obtained for comparison. Additional proteins were obtained from yeast cells grown in rich YEPD medium. The 33 and 40 kDa proteins bands were not observed from organisms grown in either condition (data not shown). These two proteins did not react with an antiserum prepared to cell wall proteins of planktonic yeast cells and germ tubes (Figure 3b). However, one reactive 29 kDa protein appeared to be more abundant in extracts from biofilm than from planktonic cells. Two dimensional electrophoresis was performed to obtain better resolution of the 33 and 40 kDa proteins (Figure 4). A single spot was observed for the 40 kDa protein while there were several spots that may be isoforms of the 33 kDa protein. These two spots were removed, trypsin digested, and submitted for LC/MS/MS analysis. With the higher resolution, additional spots that differed between extracts were observed and one of these spots was also removed for sequencing. The sequences of peptides from each spot gave 18–20% coverage of a protein and identified Grp2p (40 kDa protein), hypothetical protein ORF19.822p (33 kDa protein) and Tsa1p (additional protein) (Table 1).

Extracts were prepared from two additional biofilms and, in these extracts the 33 and 40 kDa proteins were not present (data not shown). These extracts were obtained from biofilm formed on catheters from a different source, since catheters were no longer available from the original source. These observations suggested that the possibility that the two catheter lots differed in a way that influenced the composition of the soluble proteins of the biofilm. To examine this possibility further with a biofilm on another surface, the profile of soluble proteins extracted from biofilm formed on denture acrylic was examined. The profile of this extract also lacked the 33 and 40 kDa. Analysis of extract from the acrylic biofilm by Western blot with the polyclonal antiserum showed a similar profile to the control planktonic cells.

Discussion

We have used two models for biofilm formation in catheter segments and on denture acrylic that will be useful tools for investigation of *C. albicans* biofilm. These models have two previously described characteristics of biofilms, resistance to antifungal drugs and a multilayered structure of yeast cells and hyphae with extracellular matrix material (Figures 1 and 2). Unlike previous catheter models, both models incorporate continuous flow over the biofilm with removal of eluted organisms, a situation that is analogous to the use of catheters in patients and the flow in the oral cavity. Another advantage of the two models is that it provides more than sufficient material for biochemical and molecular analysis of biofilm formation and components that are produced by biofilm cells.

The profile of non-glucan attached surface proteins extracted from biofilms formed on three different surfaces and from planktonic cells was generally similar (Figure 3). Whether these proteins are only associated with the cell surface or also associated with the extracellular matrix is not shown by this analysis. Since the biofilm was composed of yeast cells and hyphae, the similarity between planktonic organisms and biofilm is not surprising. However, the absence of one or a few proteins that dominated the biofilm extract suggested that the architecture of the biofilm did not require major new proteins associated with the non-glucan attached surface fraction. In addition to the 40 and 33 kDa proteins several other differences were observed in the 2-DE. The failure to detect these other proteins in SDS-PAGE is probably due to relative abundance and resolution of SDS-PAGE. Fluorescent microscopic imaging of extracellular matrix reacted with Calcofluor white or concanavalin A shows that the reagents bind to the surface of cells as well as to extracellular matrix [13]. Baillie and Douglas [14] examined extracellular polymeric material removed from biofilm by sonication and recovered in the supernatant and culture supernatant of planktonic cells. Although the protein component was not further analyzed, total protein and carbohydrate was less from biofilm compared to planktonic cells. The authors suggested that these differences might be due to unique biofilm components.

In their study Baillie and Douglas [14] formed the biofilm on polyvinyl chloride catheter material from two different manufactures. They noted a difference in drug resistance that was dependent on the source of the catheter material and suggested a surface dependent gene expression during biofilm development. The determination of drug resistance of the biofilms in this study was not performed under conditions that permitted an examination of differences in resistance among biofilms. However, the examination of soluble surface proteins among biofilm preparations supported the notion that the surface on which a biofilm forms influences gene expression during biofilm development. In this study, a surface dependent composition of buffer-soluble surface proteins of biofilm was noted (Figures 3 and 4). These 33 and 40 kDa proteins were observed on biofilms formed on one catheter surface but were missing from biofilm formed on a second catheter surface and on denture acrylic. Whether this effect arises from a difference in the contact interaction with the surface that alters the presence or relative abundance or is a result of a diffusible substance that alters the cellular response is unknown. Further investigation is underway to analyze the composition of this fraction of cell wall proteins in more detail, including relative abundance.

In this study, the 40 and 33 kDa proteins (Figure 4) were identified as Grp2p and protein encoded by ORF19.822 respectively (Table 1). The observed size of the Orf19.822p on SDS-PAGE/ 2DE differed from the predicted size of 21.5 kDa. The factor(s) contributing to this anomalous migration were not determined. *GRP2* encodes an oxidoreductase that is upregulated in azole resistant strains by microarray analysis [23–25]. Upregulation correlates with *MDR1* expression [25]. Proteomic analysis of cytoplasmic proteins also showed upregulation between sensitive and resistant strains [26]. Expression of ORF19.822 is upregulated in transcription profiling studies in the presence of the drug ciclopirox olamine [27] and in the absence of adenylate cyclase (*CYR1*) [28] but down regulated when grown on bovine serum albumin in the presence of subinhibitory fluconazole [29]. This appears to be the first verification of this hypothetical protein. The association of these genes with drug resistance is interesting in view of the report that the surface on which a biofilm is formed can influence the extent of drug susceptibility. An additional protein was selected for analysis and identified as Tsa1p (Table 1). Tsa1p has previously been reported to expressed on the cell surface of hyphae but not yeast cells [30, 31] so that finding it among proteins from biofilm is not surprising. The failure to detect Tsa1p and other proteins on SDS-PAGE is probably due to relative abundance and resolution of SDS-PAGE. All three of these proteins lack conventional signals for classical secretion and are among numerous proteins of this type on the surface of *C. albicans*, *S. cerevisiae*, and other microbes (Nombela et al., MS submitted).

In this study we have developed two models that provide sufficient material for biochemical and molecular analysis of *C. albicans* biofilms and utilized these models to examine the soluble surface proteins. The analysis of the soluble surface proteins suggested that *C. albicans* cells in contact with other *C. albicans* cells in the community of a biofilm do not require major new proteins for this interaction compared to the soluble proteins of cells growing individually in suspension culture. However, there may be differences that reflect the support surface. This is in agreement with the report of Baillie and Douglas that the support surface affected the extent of drug resistance [14].

Acknowledgments

The assistance of Mark Grimson, Department of Biology, Texas Tech University and Mary Catherine Hastert, with SEM is gratefully acknowledged. Partial support from Public Health Service award 1R01 DE14029 to WLC is acknowledged.

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Figure 1. Scanning electron microscopy of *C. albicans* biofilm developed on denture acrylic. Biofilm organisms were processed for SEM before (a) and after extraction (b) of soluble cell surface proteins by β ME-ammonium carbonate buffer. Note the absence of extracellular material on the cell surface after extraction (b). Arrows indicate extracellular materials (a). Bar markers indicate 5 μ m.

Figure 2.

Ketoconazole resistance of biofilm formed on acrylic surface. Growth of planktonic yeast cells and dissociated biofilm organisms was determined in the presence of different concentrations of ketoconazole (Mean $\pm$ SEM). Significant differences ($P < 0.05$) are indicated by *.

Figure 3.

Analysis of soluble surface proteins from biofilm organisms and from planktonic organisms. Surface proteins were extracted from biofilm organisms (48 h old) formed in catheter sections or planktonic organisms. Extracts (15 μg of protein, Panel (a) or 50 μg panel (b)) were separated by SDS-PAGE and the gels were double stained (Panel (a)) or transferred to nitrocellulose for Western blotting with polyclonal antiserum to yeast and hyphal cell wall proteins (panel (b)) as described in Methods. Lane 1, planktonic yeast cells grown in YNB; lane 2, biofilm formed in a catheter section (lot 1); lane 3: biofilm organisms exposed to amphotericin B. Arrows in Panel (a) indicate abundant proteins in biofilm. Symbols for Panel (b): <, location of proteins indicated by arrows in panel (a) that were not reactive with antiserum; #, 29 kDa band with greater reactivity in biofilm extract. Observations were based on analyses from three different biofilms. Broad-range SDS-PAGE standards (lane M) shown on the left were top to bottom (M in kDa): myosin (200), B-galactosidase (116), phosphorylase b (97.4), BSA (66.2), ovalbumin (45), carbonic anhydrase (31) and trypsin inhibitor (21).

Figure 4.

Two dimensional gel electrophoresis (2-DE) of buffer-soluble surface proteins from biofilm and planktonic yeast cells. Surface proteins (50 μ g) from biofilm formed in a catheter (lot 1) (upper panel) and planktonic yeast organisms (lower panel) were analyzed by 2-DE and stained as described in Methods. A single arrow indicates the 40 kDa protein and two arrows indicate two similar size and possible isoforms of the 33 kDa protein. Rectangles indicate additional proteins found in biofilm and the protein marked by open arrow was LC/MS/MS analyzed. Broad range SDS-PAGE protein markers run in parallel are shown; (a) and (b) indicate the acid (pH 3) and basic (pH 10) ends of the pH gradient.

Table 1

Identification of proteins

Protein	Molecular weight (kDa)		No. peptides matched	% amino acids covered
	Experimental [*]	Predicted ^{**}		
Grp2p	40	37.6	7	22.29
Orf19.822p	33	21.5	5	21.16
Tsa1p	23	21.8	3	17.86

^{*} Determined from SDS-PAGE and 2DE.

^{**} Predicted from amino acid composition.