

Proteomics and Pathway Mapping Analyses Reveal Phase-Dependent Over-Expression of Proteins Associated with Carbohydrate Metabolic Pathways in *Candida albicans* Biofilms

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Abstract: *Candida albicans* is the most commonly isolated fungus associated with biofilms, which are extracellular matrix (ECM)-encased, drug-resistant microbial communities formed on indwelling medical devices. Protein profiles of fungal biofilms have not been investigated in detail, although such profiles are believed to play critical roles in fungal biofilm formation. In this study, we used two-dimensional difference-in-gel electrophoresis (DIGE)-based proteomics to identify differentially expressed proteins in *C. albicans* biofilms grown to early and mature phases, compared to planktonic cells. The resulting proteomic data set was subjected to pathway mapping to reveal phase-specific pathways that were differentially expressed in biofilm cell walls and extracellular matrix (ECM). Our analyses showed 107 proteins to be differentially expressed in ECM, while 44 were differentially expressed in cell walls during biofilm formation, compared to planktonic controls. Furthermore, 95% (102/107) and 68% (30/44) of these differentially expressed proteins were upregulated in ECM and cell walls of biofilms, respectively. These proteins were mapped to cellular pathways, which revealed that these differentially expressed proteins were associated with several metabolic pathways, in a phase-dependent manner. For example, among ECM-associated proteins, proteins within 18 pathways were differentially expressed, with two pathways (glutamate and nitrogen metabolism) unique to early phase, and four pathways (purine, Gly/Ser/Thr, and inositol metabolism, and carbon fixation) unique to mature phase biofilms. Such differences were also observed in cell wall-associated proteins, where proteins associated with 14 specific pathways were differentially regulated. We also found glycolytic enzymes including the key enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were overexpressed in biofilms at both early and mature phases, compared to planktonic controls. Iodoacetate-mediated inhibition of this enzyme completely abrogated the ability of *C. albicans* to form biofilms, indicating the role of glycolysis/gluconeogenesis pathways in biofilm formation. Taken together, we demonstrate that ECM and cell walls of *C. albicans* biofilms express increased levels of specific proteins within pathways in a phase-dependent manner, suggesting that these pathways, especially glycolysis/gluconeogenesis, play critical roles in fungal biofilm formation and maintenance.

INTRODUCTION

Candida albicans is the fourth most common cause of bloodstream infections in the U.S. [1, 2], and has a noticeably high attributable mortality rate (40%-49%) among patients with catheter-related candidemia [3, 4]. *Candida* infections associated with central venous catheters (CVCs) commonly involve formation of biofilms (communities encased within a carbohydrate-rich extracellular matrix, ECM) on surfaces of indwelling medical devices. These biofilms represent a protected niche for fungal cells where they can evade both the host immune system and antimicrobial agents. Current practice guidelines for *Candida*-associated catheter infections recommend removal of catheter for management of catheter-associated infections [5, 6]. Unfortunately, CVCs cannot be removed easily because of the need for continued blood drawing, administration of blood products and antibiotics and continuous infusion of total parenteral nutrition. Therefore, a critical need exists to understand the biology and pathogenesis of *Candida* biofilms, in

order to develop effective strategies to manage such infections.

Candida biofilms form in three distinct developmental phases: early, intermediate, and mature. The morphology and architecture of *Candida* biofilms vary with their developmental phases, with mostly adherent cells and microcolonies observed in the early phase (6 h), and appearance of profuse, carbohydrate-rich matrix in the mature phase (48 h) [7]. In addition, early phase biofilms exhibit reduced susceptibility to antifungals, while mature biofilms are rendered completely resistant to most antifungals [8].

Biofilm-specific proteins are believed to be critical determinants of the morphological and architectural characteristics, and resistance phenotype of fungal biofilms. Technological advances in proteomics have allowed the identification of large-scale changes in protein profiles of biological systems, including microbial biofilms [9-13]. Recently, we performed proteomic analysis of cell walls of mature biofilms formed on catheter discs using two-dimensional difference-in-gel electrophoresis (DIGE), and showed that biofilm cell walls (as compared to planktonic cells) express differential levels of various proteins, including alcohol dehydrogenase (Adh1p) [14]. In a separate study, Thomas *et al.* [15]

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used the traditional two-dimensional gel electrophoresis (2D-GE)-based proteomic approach and identified proteins that were differentially expressed in mature *C. albicans* biofilms formed on surfaces of tissue culture flasks. However, the effect of developmental phases on changes in the proteomic profile of catheter-associated *Candida* biofilms has not been investigated.

The state-of-the-art technique of DIGE allows precise analysis of differences in protein abundance between samples [16], with accurate spot matching among multiple gels. In the current study, we used this robust technique to identify biofilm-associated proteins expressed at early and mature phases of biofilm formation. Specifically we focused on proteins present in cell walls and ECM compartments of *Candida* biofilms, since these proteins may be novel targets that can be exploited to develop diagnostic and/or therapeutic markers.

In this study, we isolated cells walls and ECM from *Candida* biofilms and planktonic cells using SDS- and EDTA-based extraction methods, respectively, and performed DIGE-based proteomic analyses to identify proteins expressed differentially in biofilms. Furthermore, we performed functional mapping of these differentially expressed proteins to pathways to identify pathways that are potentially overexpressed in biofilms. Finally, we determined the effect of an inhibitor associated with one of the overexpressed pathways (glycolysis) on the ability of *C. albicans* to form biofilms. We found that EDTA-treatment was most efficient method to isolate ECM from *Candida* biofilms. Our DIGE analyses showed 151 proteins were differentially expressed in ECM and cell walls of *Candida* biofilms, compared to planktonically grown cells. Pathway mapping analyses revealed that majority of the differentially expressed proteins were associated with metabolic pathways, in a phase-dependent manner. Inhibitor-based assays suggested a role for glyceraldehyde-3-phosphate (GAPDH) in biofilm formation. These results suggested that glycolytic pathway and GAPDH play critical roles in *Candida* biofilm formation.

MATERIALS AND METHODS

Strain

C. albicans SC5314 was used in all experiments described in this study. *Candida* cells were grown overnight at 37°C in yeast nitrogen base medium (YNB, Difco laboratories, Detroit, MI). Cells were harvested, washed with phosphate-buffered saline (PBS) and standardized to 1×10^7 cells/ml.

Biofilm Formation

Biofilms were formed on silicone elastomer (SE) discs using our catheter-based *in vitro* model as described previously [7]. Briefly, SE discs (diameter 12 mm) were pre-coated with fetal bovine serum (FBS), and immersed in 4 ml of a standardized *C. albicans* cell suspension (1×10^7 cells/ml) for 90 min to allow adhesion of fungal cells to the substrate. The catheter discs containing adhered *Candida* cells were then placed in fresh YNB medium in a 12-well tissue culture plate and allowed to form biofilms to early (6 h) or mature (48 h) developmental phases. Biofilms were quantified by a tetrazolium-salt (XTT) – based assay, as described previously [7, 17]. Planktonic cells were grown simi-

larly in tissue culture plates with the difference that no catheter discs were present.

Isolation of Biofilm Matrix

A pre-requisite to determining the protein profile of biofilm ECM is to identify an optimal method that can result in maximum protein yield, which will be suitable for investigating subtle protein-protein interactions involving low-yield proteins in the biofilm matrix. Therefore, we compared the protein yields of ECM isolated using five different methods [18, 19]. The first method (i) was based on centrifugation of scraped biofilms at 10,000 g, followed by collection of supernatant. In the remaining four methods, initially the biofilms were scraped into PBS, and they were then: (ii) incubated with 10 mM EDTA for 2 h at 4°C with gentle shaking, (iii) ultrasonicated for 10 min, (iv) ultrasonicated for 2 min followed by vortexing for 10 min, or (v) vortexed for 10 min (for schematic, see Fig. 1A). After each of these treatments, the sample was centrifuged at $10,000 \times g$. The resulting supernatant was collected, filtered through a 0.22 μ m diameter filter, and stored at -20 °C until further analysis. To determine whether treatment with the five isolation methods induced cell death, biofilms were collected, exposed to the five different methods, and then spread on Sabouraud dextrose agar for quantitative culturing. Agar plates were incubated at 37°C for 24 h and the number of colony forming units (CFUs) counted.

Isolation of Biofilm Cell Walls

Cell walls were isolated from *Candida* biofilms and their labile proteins were solubilized by treatment with SDS as described previously [14, 20]. This method has previously been used by our group and other investigators to separate soluble and insoluble cell wall proteins of *Candida* cells [20-22]. Briefly, biofilms and planktonic cells were harvested, washed with prechilled PBS, and homogenized with glass beads (0.45-mm diameter; Sigma) in buffer A (10 mM sodium acetate, pH 5.0, 1 mM phenylmethylsulfonyl fluoride). The resulting lysate was centrifuged to remove cytoplasmic material, and the cell wall pellet was incubated with buffer B (10 mM Tris-Cl, pH 7.8, 2% SDS, 100 mM EDTA, 40 mM β -mercaptoethanol) for 10 min at 100°C to release SDS-soluble cell wall (CWS) proteins into the mixture. These released CWS proteins were collected after a brief centrifugation step in the supernatant and processed for proteomic analyses.

Determination of Protein, Carbohydrate and DNA Content

Total protein content was determined using BioRad Protein Assay kit (BioRad laboratories) and bovine serum albumin (BSA) as standard, while total carbohydrate content was determined using phenol sulfuric acid method, with glucose as standard [23]. Extracellular DNA (eDNA) content was determined using the diphenylamine reaction [24].

Proteomic Analyses

(i) Two-Dimensional Difference-in-Gel Electrophoresis (DIGE)

The DIGE technique circumvents problems associated with traditional two-dimensional gel electrophoresis (2D-

GE) by allowing simultaneous resolution of two different protein samples (plus an internal standard) in the same gel. We used the DIGE approach to analyze cell wall and ECM proteins isolated from *C. albicans* grown as biofilm or planktonic cells to early (6 h) or mature (48 h) time points (n=4 of each experimental group, total 32 samples), as described previously by our group [14]. Protein concentration was determined using 2D-Quant Kit as described by the manufacturer (GE Healthcare Amersham Biosciences). Briefly, proteins isolated from planktonic or biofilm forms of *C. albicans* were labeled with Cy3 or Cy5 cyanine dyes while an internal standard was generated by pooling an aliquot of all samples which was then labeled with Cy2. Equal amounts of protein were loaded onto the gel for IEF and gel electrophoresis. A dye-swapping scheme was used such that 2 of 4 replicates were labeled with Cy3 and 2 of 4 replicates were labeled with Cy5. Labeling was performed by incubation with 400 pmol of Cy dye per 50 µg of protein for 30 min on ice in the dark, the reaction was then quenched with 10 mM lysine and additionally incubated for 10 min. The three differently labeled samples were mixed together and an additional 200 µg of unlabeled protein pooled from each sample added to increase the amount of protein available for spot picking and identification.

Rehydration of the isoelectric pH gradient (IPG) strips (pH 3-10, 24 cm, GE Healthcare) with protein samples was performed in rehydration buffer containing 8M Urea, 4% CHAPS, bromophenol blue, 1% Phamalyte, and 2 mg/ml DTT using the IPGphor IEF system (GE Healthcare), followed by isoelectric focusing using IPGphor II (GE Healthcare) for a total of 65.5 kVh. The gel strips were then equilibrated for 2 x 15 minutes with gentle shaking in equilibration solution containing 50mM Tris-Cl buffer, 6M urea, 30% glycerol, 2% SDS, and bromophenol blue. DTT (1% w/v) was added to the first, and iodoacetamide (2.5 % w/v) to the second equilibration step to reduce and carbamidomethylate cysteine sulfhydryls. IPG strips were then placed on top of 12.5% homogeneous polyacrylamide gels that were pre-casted with low-fluorescence glass plates. SDS polyacrylamide gel electrophoresis in the second dimension was carried out in the dark at 1W per gel for 2 hours then followed by 2W per gel constant power at 14°C until bromophenol blue dye front runs off. Labeled proteins were visualized using the Typhoon 9410 imager (GE Healthcare). The fluorescence images were individually captured using excitation/emission wavelengths of 488/520 nm, 532/580 nm, and 633/670 nm for cy2, cy3, and cy5 respectively.

(ii) Image Analyses, Spot Detection, and Cluster Analysis

The DeCyder v6.5 2D Differential Analysis Software (GE Healthcare) was used for image analysis including spot detection, background subtraction, matching, and normalization. Briefly, intra-gel spot detection and quantification of individual Cy2, Cy3 and Cy5 images of the same gel was performed in DIA (Differential-In-gel Analysis) module and subsequent inter-gel analysis including matching and statistical analysis was performed in BVA (Biological Variation Analysis). Protein profiles of biofilms grown to early and mature phases were compared with that of planktonic cells grown to the same time points. Differential protein expression between planktonic and biofilm of *C. albicans* grown to 6 h and 48 h were selected based on two-way ANOVA

analyses ($P < 0.05$). Hierarchical clustering was performed using Stanford University software Cluster 2.11 [25]. Normalized volume of protein spots were extracted from DeCyder and transformed to two based log for the cluster analyses. The clustering results were obtained based on average linkage with one minus Pearson correlation distance metrics. Expression of clustered protein spots was visualized using Java Treeview [26].

(iii) Mass Spectrometry Analyses

Prior to protein excision the gel was fixed in 10% methanol and 7% acetic acid for 10 minutes, followed by deep purple staining. This post-stain visualizes ca. 95% of the unlabeled protein and ensures accurate protein excision, as the molecular weight of Cy-dyes can influence protein migration during SDS-PAGE. A Typhoon 9410 variable mode imager with excitation at 532 nm and emission filter of 610BP30 (Molecular Probes) was used to scan the gels. Proteins of interest were excised using an Ettan Spot Picker (GE Healthcare) and then digested with sequencing-grade trypsin (Promega Corp., Madison, WI). Tandem mass spectra of digested peptides were acquired using Fourier Transform LTQ mass spectrometer (FT-LTQ, Thermo Electron Corp., Bremen, Germany) equipped with HPLC system or a LTQ mass spectrometer (Thermo Electron Corp., Bremen, Germany) equipped with Ettan MDLC system (GE Healthcare). The protein digests were loaded onto trapping column (C18, PepMap100, 300 µm × 5 mm, 5 µm particle size, 100 Å, Dionex) to pre-concentrate and to wash salt and then separated with a reverse phase column (C18, 75 µm × 150 mm, 3 µm, 100 Å, Dionex), using mobile phase A (0.1 % formic acid in a water) and B (84 % acetonitrile, 0.1 % formic acid in water) with a linear gradient of 2% per min, starting with 100% of A. Subsequently, the peptides were infused at a flow rate of 300 nL/min and at a voltage of 1.8 kV. MS and tandem MS spectra were acquired in the positive ion mode. A full scan recorded in the FT analyzer at resolution $R=100000$ or LTQ analyzer followed by MS/MS of the eight most-intense peptide ions in the LTQ analyzer. Protein identification was determined by searching against fungi of NCBI database using MASCOT v.2.1.03 [27]. Searches were performed with carbamidomethylation of cysteine, with partial oxidation of methionine, with one missed cleavage allowed, and with mass tolerance of 15 ppm and of 0.8 Da for MS and MS/MS, respectively for FT-LTQ and with mass tolerance of 1.5 Da and of 0.8 Da for MS and MS/MS, respectively for LTQ. Protein identification was determined based on protein hits with at least two matched peptides with peptide ion score exceeding 45.

Pathway Mapping

Next we performed pathway mapping to identify differentially expressed pathways in biofilms compared to planktonic cells. When multiple proteins were matched by the online database to the same spot, the identity with maximum score was used. Differentially expressed pathways were identified using the online "Omics" tool available at <http://pathway.yeastgenome.org:8555/expression.html>, and the Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Coloring Tool (http://www.genome.jp/kegg/tool/color_pathway.html) was used to color differentially expressed enzymes in specific pathways [28]. Overexpressed enzymes

in metabolic pathways were indicated by yellow boxes, while downregulated enzymes were indicated by red boxes.

Effect of GAPDH Inhibitor on *C. albicans* Biofilms

To determine the role of glycolysis and glycolytic enzymes (e.g. GAPDH) in *Candida* biofilm formation, we evaluated the effect of sodium iodoacetate (a specific and irreversible inhibitor of GAPDH) on the ability of *C. albicans* to form biofilms. Biofilm formation was initiated as described above, and adherent cells were incubated in the absence or presence of 300 μ M sodium iodoacetate and allowed to grow under biofilm-inducing conditions for 6 h (early phase biofilms) or 48 h (mature phase biofilms). This concentration of iodoacetate was selected based on previous studies describing the inhibition of GAPDH by this inhibitor [29, 30]. Planktonic *Candida* cells were grown under similar conditions. At the end of the incubation period, biofilms and planktonic cells were scraped into PBS and their dry biomass and metabolic activity were determined using dry weight determination and XTT assay, respectively, as described previously [7, 17, 31].

Confocal Scanning Laser Microscopy (CSLM)

CSLM was used to evaluate the effect of GAPDH inhibitor on the architecture and thickness of *C. albicans* biofilms, as described previously [7]. Biofilms were grown on SE discs in the presence or absence of iodoacetate (300 μ M), and placed on a 35-mm-diameter glass-bottom Petri dish (MatTek Corp., Ashland, Mass.). *Candida* biofilms were stained with FUN1TM and ConA-alexafluor dyes and observed under a Zeiss LSM510 confocal scanning laser microscope equipped with argon and HeNe lasers and mounted on a Zeiss Axiovert100 M microscope (Carl Zeiss, Inc.). The objective used was a water immersion C-apochromat lens (20X). Depth measurements were taken at regular intervals across the width of the device. To determine the effect of inhibitors on the structure of the biofilms, a series of horizontal (*xy*) optical sections, were taken throughout the full length of the biofilm. Confocal images of green (stained with concanavalin A, ConA) and red (stained with metabolic activity indicator FUN1TM dye) fluorescence were conceived simultaneously using a multitrack mode. Planktonically grown *C. albicans* cells were used as comparators in these studies.

Statistical Analyses

Each experiment was performed with at least three replicates. All statistical analyses were performed using StatView (version 4.5; Abacus Concepts Inc., Berkeley, CA). One-way analysis of variance (ANOVA) was performed to compare means of multiple groups, and two-tailed Student's *t*-test was used for analysis of groups. *P* values < 0.05 were considered statistically significant.

RESULTS

EDTA Treatment Results In ECM Isolation With Maximum Protein Yield

Investigation of protein expression of low-yield proteins in the biofilm matrix is dependent on an efficient method of isolation with maximum protein yield. To optimize protein yield, we isolated proteins from biofilms and planktonically

grown *C. albicans* using five different methods (Fig. 1A). Since biofilm ECM are known to contain carbohydrate and eDNA [32, 33], we also determined the levels of these components in the isolated *Candida* biofilm ECM samples. As shown in Fig. (1B), total yield of protein obtained using EDTA treatment was significantly greater than all the other methods (*P*-value < 0.05).

Total carbohydrate yield was lowest for centrifugation method, while no difference in eDNA yields was detected in ECM extracted using the five different methods. The effect of isolation methods on cell viability was checked by scraping biofilms and spreading the resulting suspension on Sabouraud dextrose agar, followed by counting of colony forming units (CFUs). No significant difference was found between the CFUs for any of the five treatment methods (Fig. 1C), indicating that none of the treatment methods affected viability of *C. albicans* grown as biofilms. Since EDTA treatment yielded highest protein content, we used this method to isolate ECM for further experiments described in this study.

DIGE Analyses Revealed Differential Expression Of Proteins In Cell Walls And ECM During *Candida* Biofilm Formation In A Phase-Dependent Manner

A major concern in proteomic studies is variability between different preparations of protein samples. To overcome such potential variability, we performed DIGE analysis of samples obtained from four separate replicates, followed by cluster analyses of the normalized volume of protein spots. Visualization of differentially expressed clusters of protein spots clearly showed that there was minimal variation between the different batches of protein samples (see representative data in Fig. 2).

DIGE-analysis revealed differences in protein profiles of ECM samples isolated from early and mature biofilms compared to planktonic cells (see representative images in Fig. 3). We found that 107 proteins were differentially expressed in biofilm ECM, of which 51 were differentially expressed at early phase, while 56 were differentially expressed at mature phase (Table 1). Among these proteins, 94% (48/51) and 96% (54/56) were overexpressed in ECM isolated from early (6 h) and mature (48 h) phase biofilms, respectively.

Table 1. Number of Differentially Expressed Proteins* in Matrix and Cell Walls of *C. albicans* Biofilms at Early and Mature Phase

Protein Sample	Biofilm Phase	Total	Upregulated	Downregulated
Cell walls	Early	9	6	3
	Mature	35	24	11
Extracellular	Early	51	48	3
	Mature	56	54	2

*with known putative functions.

Similar analysis of cell wall-associated protein samples revealed that 44 proteins were differentially expressed with 9

and 35 differentially expressed at early and mature phase, respectively (Table 1). Furthermore, among these differentially expressed cell wall proteins, 67% (6/9) and 68% (24/35) were overexpressed in cell walls isolated from early (6 h) and mature (48 h) phase biofilms, respectively. These results clearly showed that *C. albicans* cell walls and ECM fractions induce differential expression of proteins in a phase-dependent manner that was independent of batch-to-batch variation.

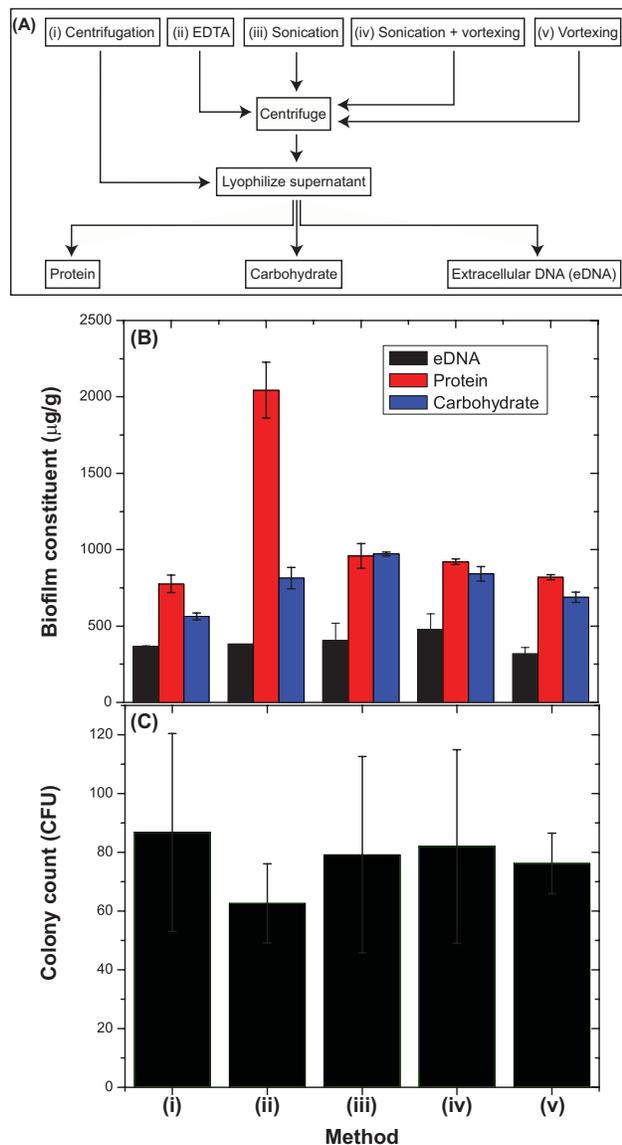


Fig. (1). Isolation of matrix from *Candida* biofilms. (A) Schema showing the five different methods for isolation of extracellular matrix from *C. albicans* grown as biofilm or planktonic cells, (B) total carbohydrate, protein, and extracellular DNA (eDNA) yield in matrix isolated using the five different methods, (C) Effect of different isolation methods on cell viability. Sample in Group (i) was centrifuged at 10,000 x g for 30 min at 4°C, and the supernatant was collected. Samples in Groups (ii), (iii), (iv), and (v) were treated according to the following methods: (ii) incubation with EDTA (10 mM, for 2 h at 4°C), (iii) ultrasonication for 10 min, (iv) ultrasonication for 2 min followed by vortexing for 10 min, and (v) vortexing only, for 10 min.

Mass Spectrometric Identification Revealed Differentially Expressed Proteins in *C. albicans* Biofilms Associated With Metabolic Processes

Next we used mass spectrometry to determine the identities of the differentially expressed proteins. We found that 51% (23/51) and 50% (28/56) of the differentially expressed ECM proteins were identified as metabolic enzymes in early and mature phase biofilms, respectively. In contrast, all the cell wall-associated differentially expressed proteins in early phase were metabolic enzymes (9/9), while 65% (23/35) of cell wall proteins differentially expressed in mature phase were identified as metabolism-related enzymes (Fig. 4). Among the metabolism-associated proteins in biofilm matrix, 100% (23/23) and 96% (27/28) proteins were overexpressed at early and mature phase, respectively. In contrast, 67% (6/9) and 74% (17/23) of the metabolic proteins in biofilm cell walls were overexpressed.

These studies demonstrated that metabolic pathway-associated proteins were upregulated in biofilms compared to planktonic cells, and among biofilms between early and mature phases.

Pathway Mapping Analyses Revealed Metabolic Pathways are Differentially Expressed in Matrix and Cell Walls of Early And Mature *C. albicans* Biofilms

To discern the broader pattern of protein expression and the functional patterns operative among the proteins expressed differentially in *C. albicans* biofilms, we performed pathway mapping of our proteomics data using the online “Omics” pathway analysis tool (<http://pathway.yeastgenome.org:8555/expression.html>) and the KEGG Pathway Coloring Tool (http://www.genome.jp/kegg/tool/color_pathway.html) [28]. Our analyses revealed that among ECM proteins isolated from biofilms, those involved in carbohydrate metabolism, amino acid biosynthesis, and calcium signaling pathways were overexpressed at both early and mature developmental phases (Fig. 5).

Interestingly, although most pathways were overexpressed at both early and mature phases, the number and/or identity of representative proteins from the pathways differed between early and mature phases. For example, at early phase, the only overexpressed protein involved in glycolysis/gluconeogenesis was glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 9.1 fold) (Tables 2-5). In contrast, at mature phase, we found that three glycolysis/gluconeogenesis-related proteins - GAPDH, phosphoglycerate kinase (PGK), and fructose biphosphate aldolase (FBP) - were overexpressed by 8.6, 11.18, and 15.6 fold, respectively, in biofilms (see representative image, Figs. 6 and 7). Furthermore, while glutamate and nitrogen metabolism pathways were represented only in early phase biofilms, proteins involved in purine metabolism, glycine/serine/threonine metabolism, carbon fixation, and inositol metabolism were overexpressed only in mature phase biofilms (Table 6). Analysis of cell wall proteins revealed that 17 metabolic pathways were overexpressed in cell walls of early phase biofilms. At mature phase, cell walls of biofilms overexpressed proteins involved in 31 metabolic pathways, including 14 that were overexpressed only in biofilms grown to this phase (Table 6). These 14 mature phase-specific pathways included: citrate cycle (TCA cycle), pentose phosphate

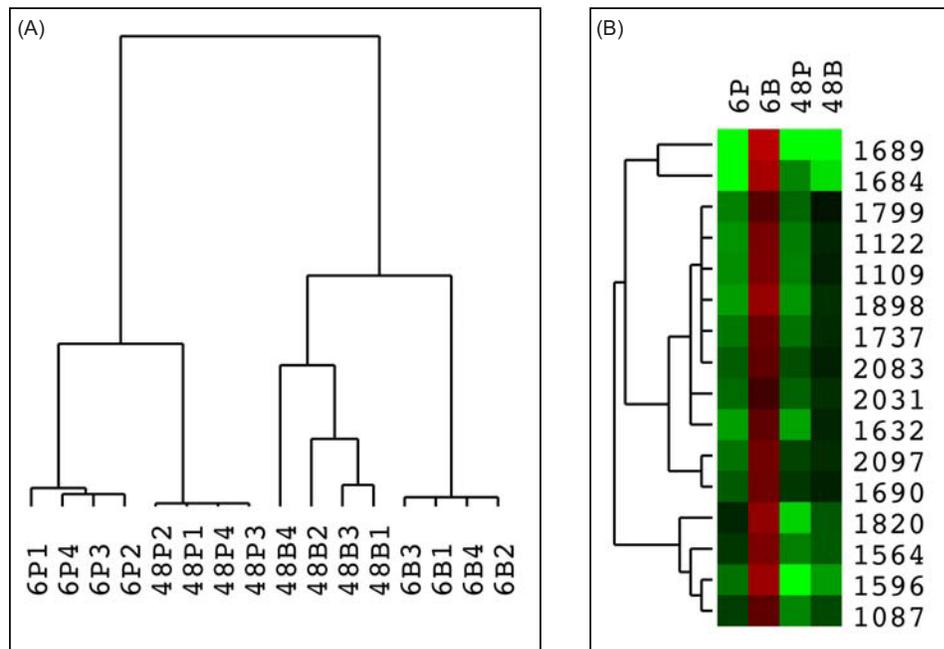


Fig. (2). Cluster analysis with proteins significantly different in matrix isolated from *C. albicans* biofilm compared to planktonic cells, showing (A) overall consistency and (B) changes in average of specific protein spots in each group of gels. Color differences illustrate log-transformed volume of protein spots. Samples from the different experimental groups are indicated on the column labels, and protein spots are displayed on the y-axis. Red color indicates high level expression, green color indicates low expression, and black indicates median expression. 6P: 6 hr Planktonic, 6B: 6hr Biofilm, 48P: 48 hr Planktonic, 48B: 48hr Biofilm.

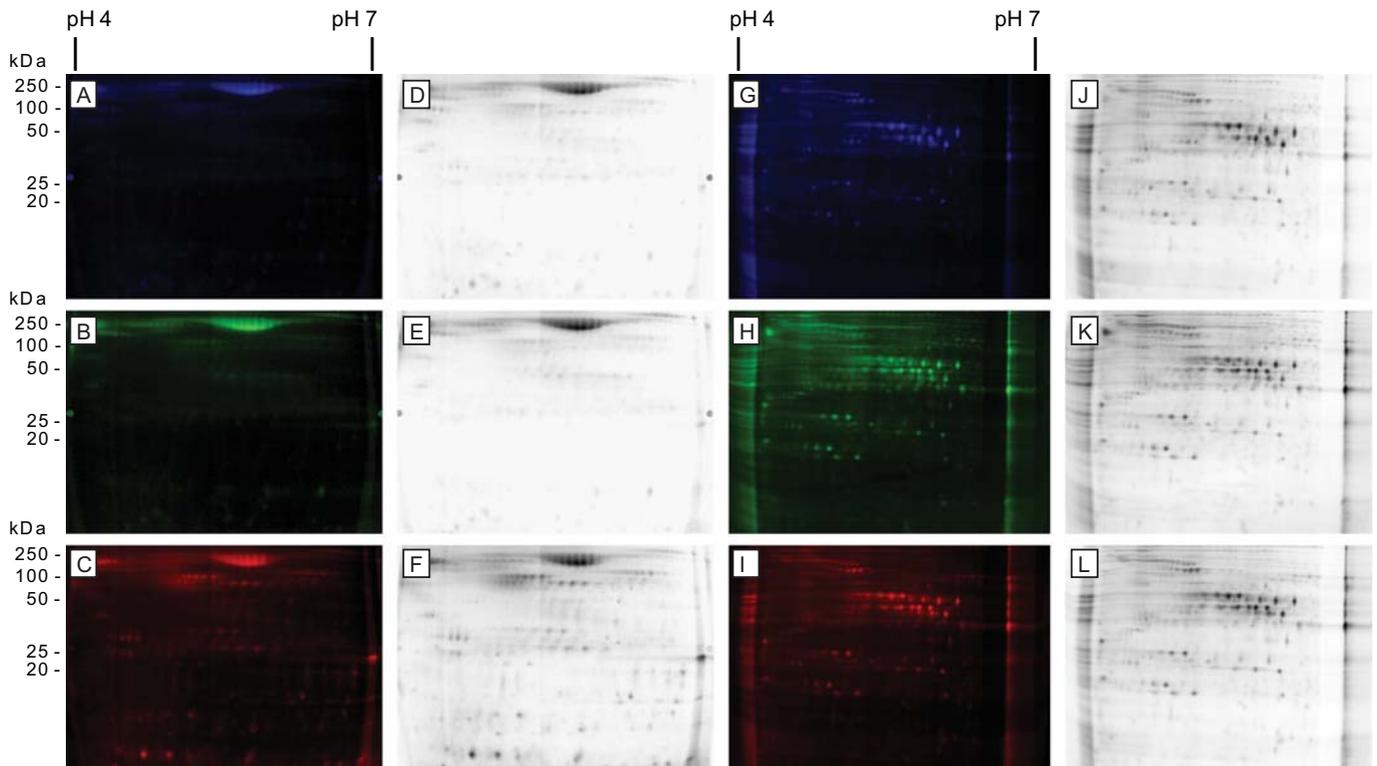


Fig. (3). Two dimensional difference in-gel electrophoresis (2D-DIGE) analyses of extracellular matrix (ECM) proteins isolated from *C. albicans* grown as biofilm or planktonic cells. Panels A-F: ECM samples, panels G-I: cell wall samples; Fluorescent scan images of gels are shown in panels A-C and G-I, while the corresponding post-stained purple gels are shown in panels D-F and J-L. Protein samples were stained with Cy3 (green), or Cy5 (red). Internal standard samples were stained with Cy2 (blue). Panels A,D,G,J: internal standard; Panels B,E,I,L: biofilm samples, and Panels C,F,H,K: planktonic samples. pI range: pH 4-7, molecular weight range: 20-250 kDa, indicated on the left of gel images.

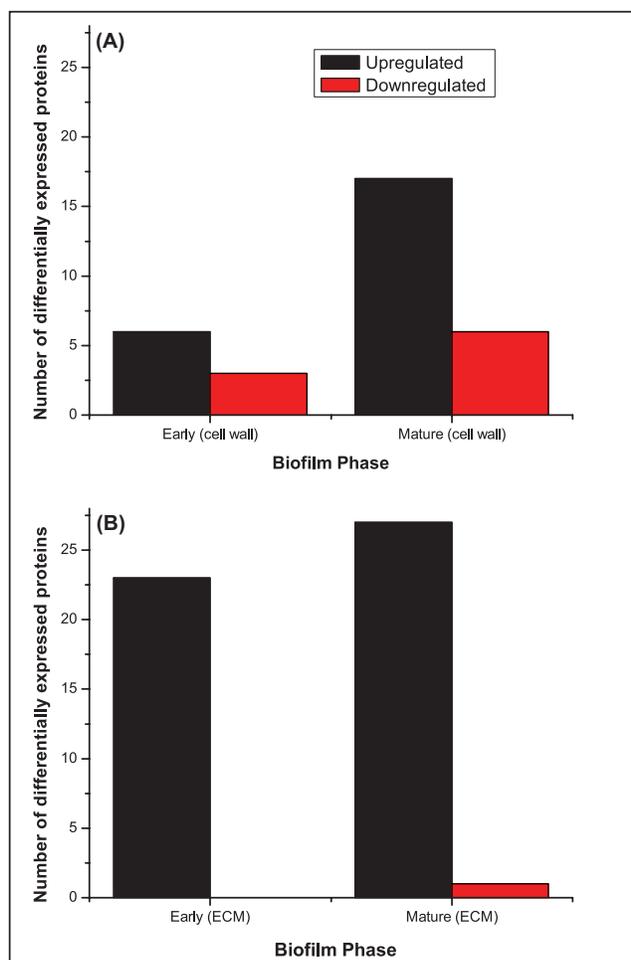


Fig. (4). Differentially expressed metabolic enzymes in (A) cell walls and (B) extracellular matrix (ECM) of *C. albicans* biofilms grown to early or mature phases. Majority of metabolic enzymes were upregulated in biofilms at both developmental phases.

pathway, pyruvate metabolism, amino acid metabolism, alpha-linolenic acid metabolism, polyunsaturated fatty acid biosynthesis, aminoacyl-tRNA biosynthesis, PPAR signaling pathway, and calcium signaling pathway. Phase-dependent differences in the number and identity of pathway associated proteins were also observed among cell wall proteins. In this regard, four glycolytic proteins – alcohol dehydrogenase, enolase, triose phosphate isomerase, and phosphoglycerate mutase were overexpressed in early phase biofilms, while eight glycolytic proteins – alcohol dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, pyruvate decarboxylase, fructose-bisphosphate aldolase, phosphopyruvate hydratase, enolase, triose-phosphate isomerase, and phosphoglycerate mutase were overexpressed at mature phase (Table 4). When comparing the levels of ADH1 between the two phases of biofilms (early vs. mature), we found the levels of Adh1p decreased by 1.59 fold, in agreement with our previously published study which showed that Adh1p level is decreased in mature biofilms. Taken together, these analyses revealed that metabolic pathways (especially glycolysis/gluconeogenesis) were differentially expressed in matrix and cell walls of early and mature *C. albicans* biofilms.

The GAPDH Inhibitor Sodium Iodoacetate Inhibits Biofilm Formation

Since glycolysis was one of the metabolic pathways that was overexpressed in both matrix and cell walls isolated from early and mature *C. albicans* biofilms, and GAPDH was the most highly overexpressed glycolytic enzyme at both developmental phases, we hypothesized that inhibition of this enzyme will attenuate the ability of *C. albicans* to form biofilms. To test this hypothesis, we determined the effect of sodium iodoacetate, which specifically and irreversibly inhibits GAPDH [29], on the ability of *C. albicans* to form biofilms. As shown in Fig. (8), the ability of *C. albicans* to form early and mature phase biofilms was significantly reduced in the presence of this inhibitor, as measured by dry weight determination (Fig. 8A-B; $P \leq 0.001$ for both).

Similar effect of sodium iodoacetate was observed on the metabolic activity (determined by XTT assay) of *C. albicans* biofilms (data not shown). Next, we used confocal microscopy to determine the effect of sodium iodoacetate on the morphology and architecture of *C. albicans* biofilms. We found that *C. albicans*, when exposed to sodium iodoacetate, grew only as sparse, adherent blastospores and did not form robust biofilms (Fig. 8D). As expected, in absence of sodium iodoacetate, *C. albicans* formed typical, hyphae-rich biofilms (Fig. 8C). These results clearly demonstrated that glycolytic pathway, and specifically GAPDH, plays a critical role in biofilm formation.

DISCUSSION AND CONCLUSIONS

In this study, we performed proteomic analyses of ECM and cell wall proteins isolated from *C. albicans* biofilms and demonstrated that the protein profiles in these two compartment of biofilms expressed differential levels of proteins in a phase-dependent manner. Additionally, we mapped these differentially expressed proteins to cellular pathways and identified metabolic pathways that are differentially expressed in *Candida* biofilm. These results demonstrate the value of pathway mapping in interpreting the “omic” data on biofilms.

To identify the optimal method for isolation of biofilm matrix, we compared the protein, DNA, and carbohydrate yield in matrix isolated from *C. albicans* biofilms using five different methods – centrifugation, EDTA-treatment, ultrasonication with or without vortexing, and vortexing alone. Our studies revealed that the protein content was significantly higher in matrix isolated using EDTA extraction than in samples isolated using the other four methods tested. In a previous study, investigators reported using ultrasonication to isolate *Candida* biofilm matrix [15, 34] and treatment with beta-mercaptoethanol to isolate cell surface components [15]. Previously, Zhang *et al.* [35] compared similar methods to extract extracellular polymeric substances (EPS) from biofilms formed by bacteria, and showed that EDTA-based method was the most efficient. Since our goal in the current study was to evaluate the protein profile of biofilm matrix and cell walls, we used the EDTA-based method to isolate extracellular matrix from biofilms and planktonic cells. The higher yield of EDTA method renders it especially suitable for investigating subtle protein-protein interactions involving low-yield proteins in the biofilm matrix. In this regard, our

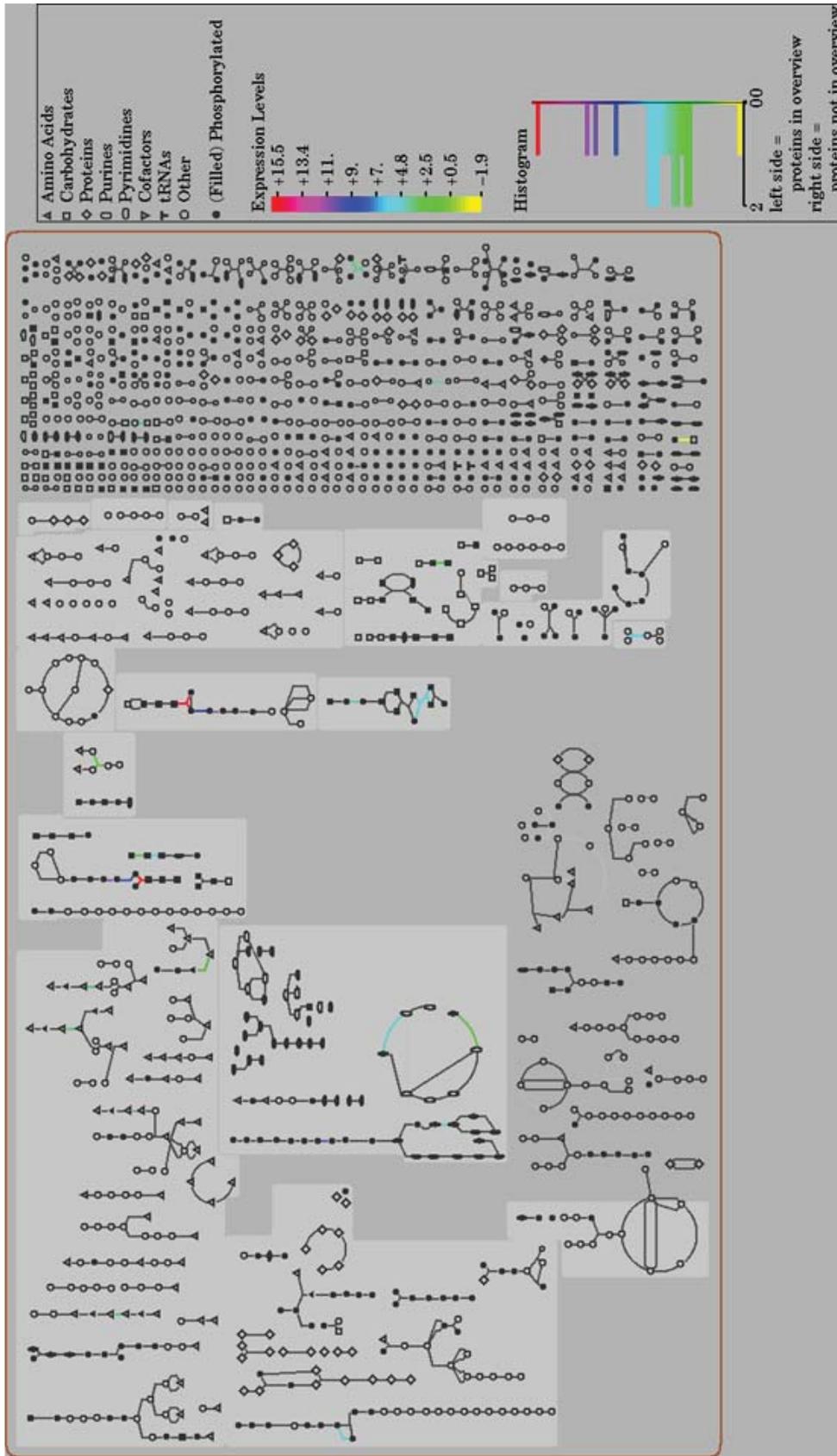


Fig. (5). Pathway mapping of matrix proteins isolated from mature phase *C. albicans* biofilms. This diagram provides a schematic of all pathways of *S. cerevisiae* metabolism in the yeast genome database available online at <http://www.yeastgenome.org/>. Nodes represent metabolites, with shape indicating class of metabolite (see key); lines represent reactions. Differentially expressed pathways are indicated in color. Analysis was based on comparison with the online yeast genome database using *S. cerevisiae* S288C as the comparator.

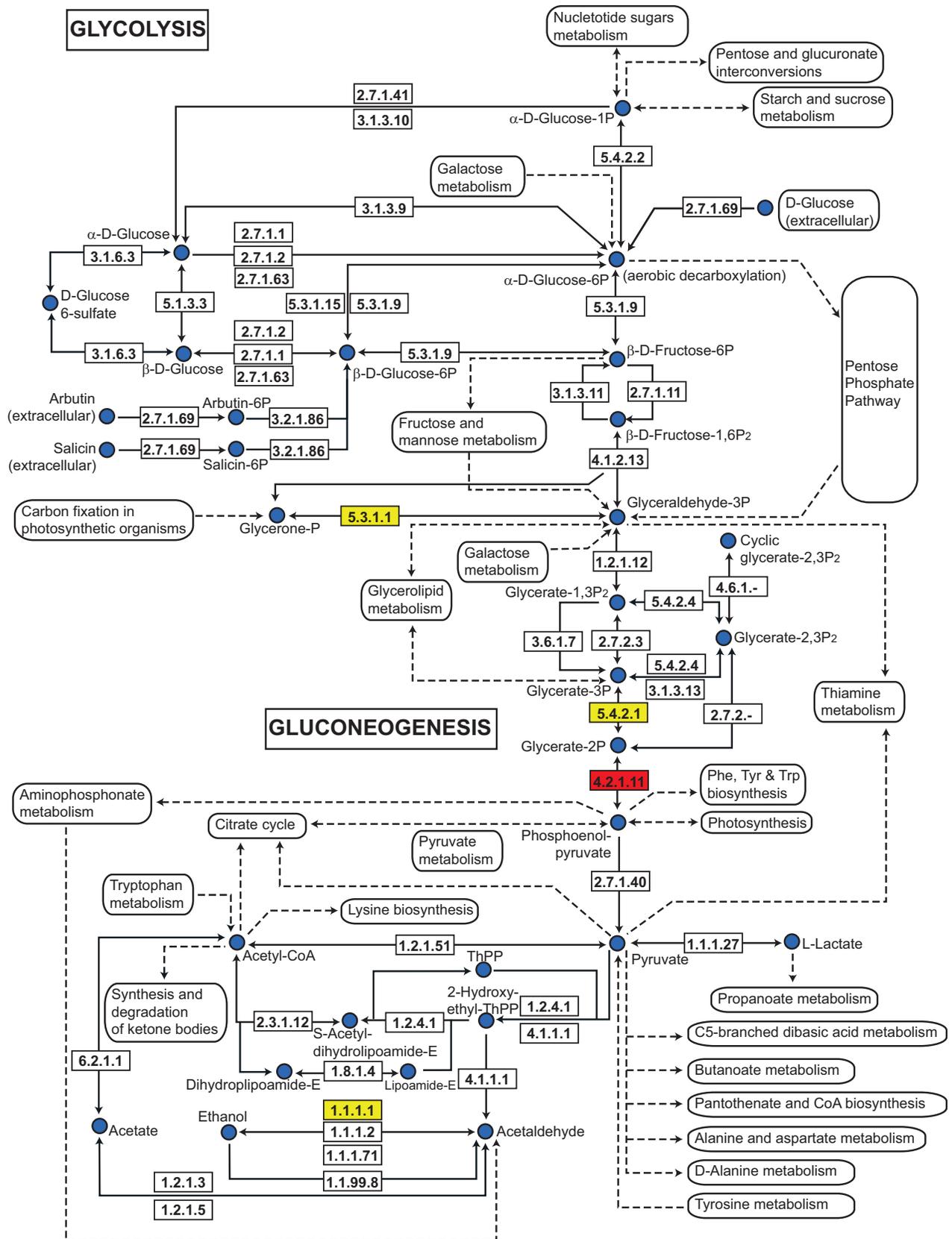


Fig. (6). Representative image showing differentially expressed proteins in the glycolysis/gluconeogenesis pathways in early phase *C. albicans* biofilms. The KEGG Pathway coloring tool was used to identify enzymes whose levels in biofilms were upregulated (yellow) or downregulated (red).

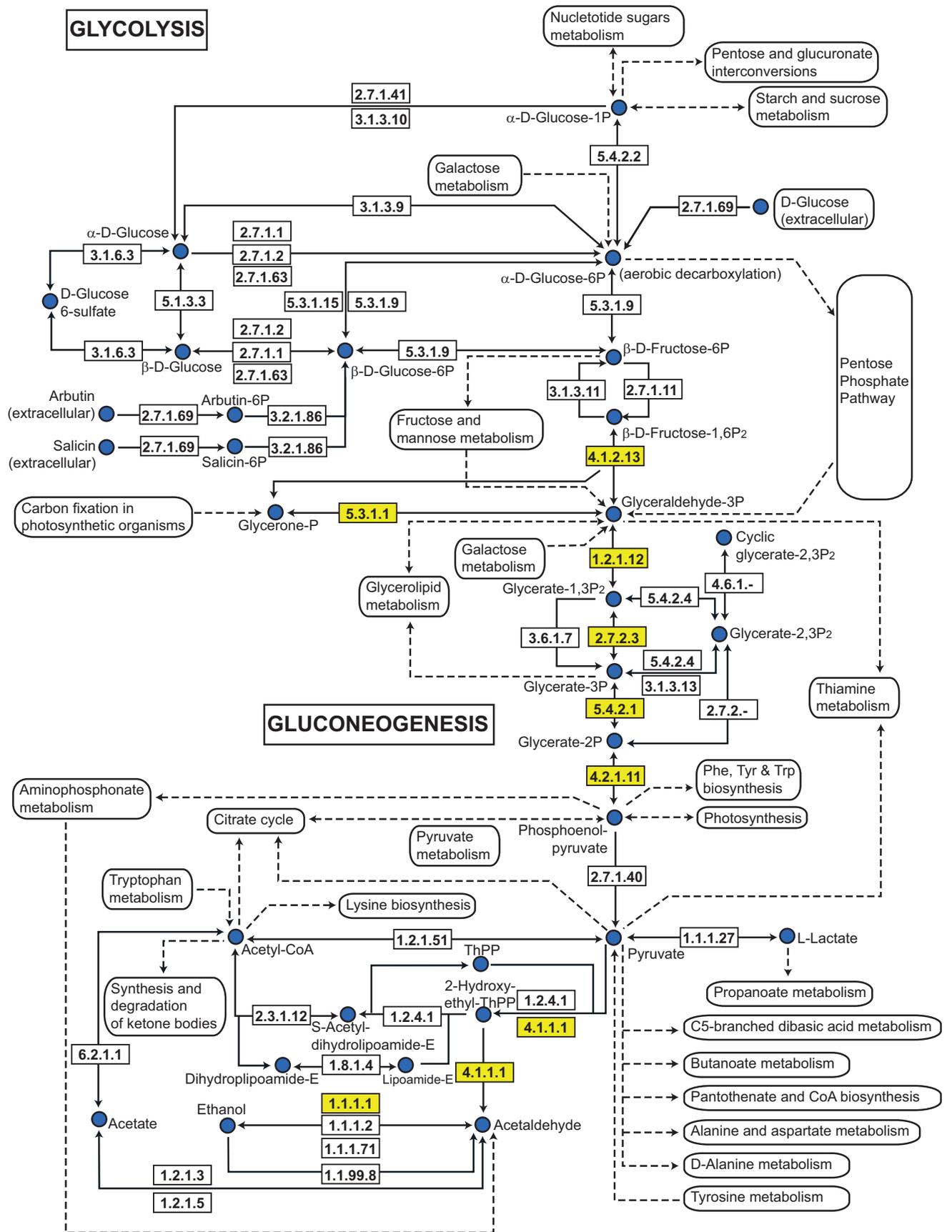


Fig. (7). Representative image showing differentially expressed proteins in the glycolysis/gluconeogenesis pathways in mature phase *C. albicans* biofilms. The KEGG Pathway coloring tool was used to identify differentially expressed enzymes in biofilms. Yellow boxes - up-regulated enzymes, red boxes - down-regulated enzymes.

Table 2. Differentially Expressed Proteins in Matrix Isolated from *C. albicans* Biofilms Grown to Early Phase and Planktonic Cells Grown to the Same Time Point

Spot Number	Ratio	GI Number	EC Number	Proteins	Score	MW
1564	3.68	68484087	1.1.1.14	oxidoreductase [Candida albicans SC5314]	502	33017
1358	8.7	68468783	1.1.1.3	homoserine dehydrogenase [Candida albicans SC5314]	1294	38812
1357	8.83	68484035	1.1.1.86	mitochondrial ketol-acid reductoisomerase [Candida albicans SC5314]	3666	44821
1839	5.36	68477997	1.11.1.15	thioredoxin peroxidase [Candida albicans SC5314]	368	27425
2058	6.3	68471010	1.11.1.15	alkyl hydroperoxide reductase [Candida albicans SC5314]	49	19246
2169	8.6	6094316	1.15.1.1	Superoxide dismutase [Cu-Zn]	239	16136
1518	9.13	68472227	1.2.1.12	glyceraldehyde-3-phosphate dehydrogenase [Candida albicans SC5314]	1865	35811
1081	5.66	68491798	1.4.1.4	NADP-specific glutamate dehydrogenase [Candida albicans SC5314]	764	49546
1429	6.02	68481811	2.2.1.2	transaldolase [Candida albicans SC5314]	2443	35650
1820	3.77	68481466	2.4.2.8	hypoxanthine guanine phosphoribosyltransferase [Candida albicans SC5314]	238	24246
1632	6.41	68482416	2.5.1.16	spermidine synthase [Candida albicans SC5314]	117	33878
1332	6.13	68473424	2.7.1.20	adenosine kinase [Candida albicans SC5314]	1456	41792
2051	5.96	68489972	2.7.4.8	guanylate kinase [Candida albicans SC5314]	187	27998
1809	6.12	68488739	3.1.1.31	6-phosphogluconolactonase [Candida albicans SC5314]	743	28467
1973	9.19	68470360	3.1.1.5	phospholipase/carboxylesterase [Candida albicans SC5314]	113	32700
1689	35.39	68476319	3.1.3.21	DL-glycerol-3-phosphatase [Candida albicans SC5314]	340	28116
1596	6.94	68481478	3.1.3.3	phosphoserine phosphohydrolase [Candida albicans SC5314]	704	33346
2170	7.76	68469052	5.2.1.8	cyclophilin type peptidyl-prolyl cis-trans isomerase [Candida albicans SC5314]	60	17564
1109	6.87	68482860	5.3.1.8	mannose-6-phosphate isomerase [Candida albicans SC5314]	1157	48952
1572	5.82	68471036	5.3.3.2	isopentenyl diphosphate isomerase [Candida albicans SC5314]	706	32313
1889	5.39	68468755	5.4.2.8	phosphomannomutase [Candida albicans SC5314]	729	29001
1443	6.46	68472217	5.4.2.8	phosphomutase-like protein [Candida albicans SC5314]	677	37268
1736	11.86	1172759	6.3.2.6	Phosphoribosylaminoimidazole-succinocarboxamide synthase (SAICAR synthetase)	1558	32889
1878	-3.03	68472093		hypothetical protein CaO19_6745 [Candida albicans SC5314]	54	26595
1740	-1.46	68469633		hypothetical protein CaO19_8442 [Candida albicans SC5314]	293	21482
1834	-1.04	68469783		hypothetical protein CaO19_8522 [Candida albicans SC5314]	1170	27437
1883	1.05	68469783		hypothetical protein CaO19_8522 [Candida albicans SC5314]	1269	27437
1870	1.09	68469783		hypothetical protein CaO19_8522 [Candida albicans SC5314]	741	27437
1867	1.48	68469783		hypothetical protein CaO19_8522 [Candida albicans SC5314]	977	27437
2064	1.59	68485595		putative zinc-binding dehydrogenase [Candida albicans SC5314]	47	38494
1675	1.6	7271036		hypothetical protein [Candida albicans]	203	26250
1965	2.02	68476035		hypothetical protein CaO19_3268 [Candida albicans SC5314]	661	18546
1179	2.41	68488457		hypothetical protein CaO19.8025 [Candida albicans SC5314]	6378	47203
1618	4.15	68468907		hypothetical protein CaO19_3014 [Candida albicans SC5314]	1653	29462
2005	4.41	68482868		hypothetical protein CaO19.8972 [Candida albicans SC5314]	1245	18738
1298	4.42	68467815		hypothetical protein CaO19.11480 [Candida albicans SC5314]	3834	46173
1303	4.63	68467815		hypothetical protein CaO19.11480 [Candida albicans SC5314]	3345	46173

(Table 2) contd....

Spot Number	Ratio	GI Number	EC Number	Proteins	Score	MW
1627	4.68	68468907		hypothetical protein CaO19_3014 [Candida albicans SC5314]	1149	29462
1980	5.21	68466605		putative salvage phosphoribosyl transferase [Candida albicans SC5314]	367	20864
1187	5.27	68471189		hypothetical protein CaO19.7080 [Candida albicans SC5314]	1497	40061
1567	5.42	68469216		hypothetical protein CaO19.6559 [Candida albicans SC5314]	300	34584
1223	5.51	68475061		putative translation initiation factor eIF4A subunit [Candida albicans SC5314]	1291	44571
1724	5.82	68481819		hypothetical protein CaO19.4375 [Candida albicans SC5314]	223	30410
1784	6.64	68475757		hypothetical protein CaO19_5749 [Candida albicans SC5314]	359	23899
1778	7.13	68475892		hypothetical protein CaO19_13172 [Candida albicans SC5314]	505	23856
2106	7.58	68468985		hypothetical protein CaO19_3053 [Candida albicans SC5314]	58	20310
1566	8.43	68469216		hypothetical protein CaO19.6559 [Candida albicans SC5314]	1579	34584
1424	8.79	68467815		hypothetical protein CaO19.11480 [Candida albicans SC5314]	1430	46173
1898	9.31	68488079		putative ubiquitin-conjugating enzyme Ubc1p	435	25441
2104	11.83	68482868		hypothetical protein CaO19.8972 [Candida albicans SC5314]	350	18738
1816	12	68469783		hypothetical protein CaO19_8522 [Candida albicans SC5314]	284	27437

Table 3. Differentially Expressed Proteins in Matrix Isolated from *C. albicans* Biofilms Grown to Mature Phase and Planktonic Cells Grown to the Same Time Point

Spot Number	Ratio	GI Number	EC Number	Proteins	Score	MW
1447	3.17	68470480	1.1.1.14	oxidoreductase [Candida albicans SC5314]	457	37611
1312	3	68468783	1.1.1.3	homoserine dehydrogenase [Candida albicans SC5314]	740	38812
1358	3.15	68468783	1.1.1.3	homoserine dehydrogenase [Candida albicans SC5314]	1294	38812
1357	7.71	68484035	1.1.1.86	mitochondrial ketol-acid reductoisomerase [Candida albicans SC5314]	3666	44821
1343	9.92	68484035	1.1.1.86	mitochondrial ketol-acid reductoisomerase [Candida albicans SC5314]	531	44821
1839	5.46	68477997	1.11.1.15	thioredoxin peroxidase [Candida albicans SC5314]	368	27425
2058	8.14	68471010	1.11.1.15	alkyl hydroperoxide reductase [Candida albicans SC5314]	49	19246
2023	10.39	68477997	1.11.1.15	thioredoxin peroxidase [Candida albicans SC5314]	135	27425
2169	5.77	6094316	1.15.1.1	Superoxide dismutase [Cu-Zn]	239	16136
1518	8.6	68472227	1.2.1.12	glyceraldehyde-3-phosphate dehydrogenase [Candida albicans SC5314]	1865	35811
1429	5.31	68481811	2.2.1.2	transaldolase [Candida albicans SC5314]	2443	35650
1820	2.41	68481466	2.4.2.8	hypoxanthine guanine phosphoribosyltransferase [Candida albicans SC5314]	238	24246
1632	2.51	68482416	2.5.1.16	spermidine synthase [Candida albicans SC5314]	117	33878
1332	3.75	68473424	2.7.1.20	adenosine kinase [Candida albicans SC5314]	1456	41792
1330	4.79	68473424	2.7.1.20	adenosine kinase [Candida albicans SC5314]	1920	41792
1242	11.18	68489602	2.7.2.3	3-phosphoglycerate kinase [Candida albicans SC5314]	2484	45152
2051	5.54	68489972	2.7.4.8	guanylate kinase [Candida albicans SC5314]	187	27998
1809	3.82	68488739	3.1.1.31	6-phosphogluconolactonase [Candida albicans SC5314]	743	28467
1973	3.78	68470360	3.1.1.5	phospholipase/carboxylesterase [Candida albicans SC5314]	113	32700

(Table 3) contd....

Spot Number	Ratio	GI Number	EC Number	Proteins	Score	MW
1684	-1.9	68476319	3.1.3.21	DL-glycerol-3-phosphatase [Candida albicans SC5314]	284	28116
1596	2.03	68481478	3.1.3.3	phosphoserine phosphohydrolase [Candida albicans SC5314]	704	33346
1380	15.6	68466123	4.1.2.13	fructose-bisphosphate aldolase [Candida albicans SC5314]	1657	39191
2170	4.42	68469052	5.2.1.8	cyclophilin type peptidyl-prolyl cis-trans isomerase [Candida albicans SC5314]	60	17564
1109	2.03	68482860	5.3.1.8	mannose-6-phosphate isomerase [Candida albicans SC5314]	1157	48952
1572	5.09	68471036	5.3.3.2	isopentenyl diphosphate isomerase [Candida albicans SC5314]	706	32313
1889	5.18	68468755	5.4.2.8	phosphomannomutase [Candida albicans SC5314]	729	29001
1443	5.65	68472217	5.4.2.8	phosphomutase-like protein [Candida albicans SC5314]	677	37268
1744	10.25	1172759	6.3.2.6	Phosphoribosylaminoimidazole-succinocarboxamide synthase (SAICAR synthetase)	641	32889
2104	-3.17	68482868		hypothetical protein CaO19.8972 [Candida albicans SC5314]	350	18738
1965	1.69	68476035		hypothetical protein CaO19_3268 [Candida albicans SC5314]	661	18546
1980	1.91	68466605		putative salvage phosphoribosyl transferase [Candida albicans SC5314]	367	20864
1898	2.09	68488079		putative ubiquitin-conjugating enzyme Ubc1p [Candida albicans SC5314]	435	25441
1834	2.4	68469783		hypothetical protein CaO19_8522 [Candida albicans SC5314]	1170	27437
1223	2.52	68475061		putative translation initiation factor eIF4A subunit [Candida albicans SC5314]	1291	44571
2005	3.02	68482868		hypothetical protein CaO19.8972 [Candida albicans SC5314]	1245	18738
1778	3.4	68475892		hypothetical protein CaO19_13172 [Candida albicans SC5314]	505	23856
1724	3.68	68481819		hypothetical protein CaO19.4375 [Candida albicans SC5314]	223	30410
1784	3.78	68475757		hypothetical protein CaO19_5749 [Candida albicans SC5314]	359	23899
1567	4.16	68469216		hypothetical protein CaO19.6559 [Candida albicans SC5314]	300	34584
2106	4.42	68468985		hypothetical protein CaO19_3053 [Candida albicans SC5314]	58	20310
1566	4.91	68469216		hypothetical protein CaO19.6559 [Candida albicans SC5314]	1579	34584
1187	4.94	68471189		hypothetical protein CaO19.7080 [Candida albicans SC5314]	1497	40061
1424	5.68	68467815		hypothetical protein CaO19.11480 [Candida albicans SC5314]	1430	46173
1816	8.09	68469783		hypothetical protein CaO19_8522 [Candida albicans SC5314]	284	27437
1675	8.93	7271036		hypothetical protein [Candida albicans]	203	26250
2064	8.97	68485595		putative zinc-binding dehydrogenase [Candida albicans SC5314]	47	38494
1179	9.25	68488457		hypothetical protein CaO19.8025 [Candida albicans SC5314]	6378	47203
1618	9.53	68468907		hypothetical protein CaO19_3014 [Candida albicans SC5314]	1653	29462
1870	9.64	68469783		hypothetical protein CaO19_8522 [Candida albicans SC5314]	741	27437
1298	10.04	68467815		hypothetical protein CaO19.11480 [Candida albicans SC5314]	3834	46173
1627	10.28	68468907		hypothetical protein CaO19_3014 [Candida albicans SC5314]	1149	29462
1303	11.17	68467815		hypothetical protein CaO19.11480 [Candida albicans SC5314]	3345	46173
1878	11.34	68472093		hypothetical protein CaO19_6745 [Candida albicans SC5314]	54	26595
1740	13.27	68469633		hypothetical protein CaO19_8442 [Candida albicans SC5314]	293	21482
1883	13.54	68469783		hypothetical protein CaO19_8522 [Candida albicans SC5314]	1269	27437
1867	20.39	68469783		hypothetical protein CaO19_8522 [Candida albicans SC5314]	977	27437

Table 4. Differentially Expressed Proteins in Cell Walls Isolated from *C. albicans* Biofilms Grown to Early Phase and Planktonic Cells Grown to the Same Time Point

Spot No.	Average Ratio*	P - Spot Vol.	Accession	Protein	Protein Probability	Coverage	pI	Molecular Weight
			68479353	gi 68479353 ref XP_716208.1 hypothetical protein CaO19_6367 [Candida albicans SC5314]	1.60E-11	17.8	5.1	66408.6
			607055	gi 607055 emb CAA82929.1 heat shock protein 70 [Candida albicans]	1.50E-10	23.2	4.9	70281.2
219	-1.8	3019435	68485837	gi 68485837 ref XP_713199.1 mitochondrial import protein [Candida albicans SC5314]	1.87E-09	22.1	5.3	69705.5
			68485837	gi 68485837 ref XP_713199.1 mitochondrial import protein [Candida albicans SC5314]	1.20E-10	29.5	5.3	69705.5
			68468638	gi 68468638 ref XP_721638.1 putative vacuolar ATPase V1 complex subunit A [Candida albicans SC5314]	1.83E-09	19.4	5.1	63250
233	-1.73	4619734	607055	gi 607055 emb CAA82929.1 heat shock protein 70 [Candida albicans]	2.15E-09	29.3	4.9	70281.2
			68479353	gi 68479353 ref XP_716208.1 hypothetical protein CaO19_6367 [Candida albicans SC5314]	3.33E-15	29.4	5.1	66408.6
287	-1.07	8757535	68485837	gi 68485837 ref XP_713199.1 mitochondrial import protein [Candida albicans SC5314]	6.35E-11	21.5	5.3	69705.5
315	-1.2	689448	68468218	gi 68468218 ref XP_721948.1 dihydroxyacid dehydratase [Candida albicans SC5314]	5.78E-12	20.7	6.2	62824.9
329	-1.26	2738890	68485963	gi 68485963 ref XP_713100.1 heat shock protein 60 [Candida albicans SC5314]	1.76E-12	39.6	5.1	60088.7
			68485963	gi 68485963 ref XP_713100.1 heat shock protein 60 [Candida albicans SC5314]	1.30E-11	32.2	5.1	60088.7
338	-1.21	1946380	68477333	gi 68477333 ref XP_717293.1 hypothetical protein CaO19_11293 [Candida albicans SC5314]	6.69E-08	13.9	4.6	58494.5
348	-1.19	15085306	68480872	gi 68480872 ref XP_715589.1 pyruvate decarboxylase [Candida albicans SC5314]	5.68E-13	37.4	5.3	62402
382	1.12	3956254	68480872	gi 68480872 ref XP_715589.1 pyruvate decarboxylase [Candida albicans SC5314]	3.82E-12	30.3	5.3	62402
405	-1.22	10257758	68480872	gi 68480872 ref XP_715589.1 pyruvate decarboxylase [Candida albicans SC5314]	1.47E-12	39.2	5.3	62402
407	-1.08	11359439	68480872	gi 68480872 ref XP_715589.1 pyruvate decarboxylase [Candida albicans SC5314]	6.44E-12	35.8	5.3	62402
410	-1.07	8195618	68480872	gi 68480872 ref XP_715589.1 pyruvate decarboxylase [Candida albicans SC5314]	4.31E-13	45.1	5.3	62402
			68480872	gi 68480872 ref XP_715589.1 pyruvate decarboxylase [Candida albicans SC5314]	2.27E-11	36.9	5.3	62402
459	-1.51	2993086	68472545	gi 68472545 ref XP_719591.1 hypothetical protein CaO19_9565 [Candida albicans SC5314]	2.86E-09	17.9	5.2	50275.4
520	1.02	196838	68480872	gi 68480872 ref XP_715589.1 pyruvate decarboxylase [Candida albicans SC5314]	7.37E-12	22.6	5.3	62402
			68487498	gi 68487498 ref XP_712370.1 putative mitochondrial matrix dihydroliipoamide dehydrogenase Lpd1p [Candida albicans SC5314]	2.23E-10	24.2	6.4	52823.8
584	1	2185488	68465104	gi 68465104 ref XP_723251.1 putative glucose-6-phosphate dehydrogenase [Candida albicans SC5314]	2.09E-08	10.7	6.2	58253.1
			68488457	gi 68488457 ref XP_711912.1 hypothetical protein CaO19_8025 [Candida albicans SC5314]	8.24E-13	26.6	5.4	47202.6
659	-2.31	1196912	68479882	gi 68479882 ref XP_716109.1 saccharopine dehydrogenase [Candida albicans SC5314]	1.51E-10	28.8	5.3	49185.6
744	1.1	1026949	68467359	gi 68467359 ref XP_722341.1 hypothetical protein CaO19_12491 [Candida albicans SC5314] similar to gi 2309076 dbj BAA21690.1 6-phosphogluconate dehydrogenase [Candida albicans]	3.26E-10	18.6	6.1	56874.2
881	-1.13	7305742	68489602	gi 68489602 ref XP_711371.1 3-phosphoglycerate kinase [Candida albicans SC5314]	1.32E-11	30.2	6.1	45151.8
923	-2.83	10252634	68467815	gi 68467815 ref XP_722066.1 hypothetical protein CaO19_11480 [Candida albicans SC5314] similar to gi 608690 emb CAA57342.1 alcohol dehydrogenase [Candida albicans]	4.73E-11	48	8.2	46172.7
			608690	gi 608690 emb CAA57342.1 alcohol dehydrogenase [Candida albicans]	7.81E-12	37.7	6	36856
954	-1.23	5423859	68475148	gi 68475148 ref XP_718292.1 homoisocitrate dehydrogenase [Candida albicans SC5314]	5.56E-11	27.7	5.5	40643.1
1021	1.61	4255656	68484035	gi 68484035 ref XP_714020.1 putative mitochondrial ketol-acid reductoisomerase [Candida albicans SC5314]	2.42E-12	42.8	6.2	44820.5
1029	1.32	7946847	68484035	gi 68484035 ref XP_714020.1 putative mitochondrial ketol-acid reductoisomerase [Candida albicans SC5314]	1.31E-10	33	6.2	44820.5

(Table 4) contd....

Spot No.	Average Ratio*	P - Spot Vol.	Accession	Protein	Protein Probability	Coverage	pI	Molecular Weight
1034	1.01	7790567	68484035	gi 68484035 ref XP_714020.1 putative mitochondrial ketol-acid reductoisomerase [Candida albicans SC5314]	4.63E-10	29.8	6.2	44820.5
			68472462	gi 68472462 ref XP_719792.1 glyceraldehyde-3-phosphate dehydrogenase [Candida albicans SC5314]	2.04E-08	14.6	6.7	35810.7
1058	1.12	1366267	68489602	gi 68489602 ref XP_711371.1 3-phosphoglycerate kinase [Candida albicans SC5314]	2.81E-08	19.4	6.1	45151.8
			68473257	gi 68473257 ref XP_719434.1 hypothetical protein CaO19_7676 [Candida albicans SC5314]	4.87E-11	19.7	5.6	38746.7
1060	-1.63	6537573	68481267	gi 68481267 ref XP_715408.1 putative anthranilate phosphoribosyl transferase [Candida albicans SC5314]	1.04E-09	18.7	5.7	39456.6
			68466123	gi 68466123 ref XP_722836.1 fructose-bisphosphate aldolase [Candida albicans SC5314]	4.79E-12	27.3	5.7	39190.8
1084	1.31	2388312	68472462	gi 68472462 ref XP_719792.1 glyceraldehyde-3-phosphate dehydrogenase [Candida albicans SC5314]	3.73E-10	28.7	6.7	35810.7
1330	1.22	1311176	68466123	gi 68466123 ref XP_722836.1 fructose-bisphosphate aldolase [Candida albicans SC5314]	1.87E-08	20.6	5.7	39190.8
1335	1.42	2172392	68476319	gi 68476319 ref XP_717809.1 putative DL-glycerol-3-phosphatase [Candida albicans SC5314]	2.48E-10	22	5.4	28116.1
1367	1.37	2059619	68491391	gi 68491391 ref XP_710493.1 NAD-dependent D-arabinitol dehydrogenase [Candida albicans SC5314]	8.26E-07	14.9	5.5	30653.2
1408	-1.29	7767708	68469633	gi 68469633 ref XP_721068.1 hypothetical protein CaO19_8442 [Candida albicans SC5314]	7.02E-06	11.6	5	21481.5
1413	-2.76	20017893	608690	gi 608690 emb CAA57342.1 alcohol dehydrogenase [Candida albicans]	1.76E-07	20.9	6	36856
1414	-4.71	22228723	608690	gi 608690 emb CAA57342.1 alcohol dehydrogenase [Candida albicans]	2.14E-12	23.7	6	36856
1434	-1.29	1478393	608690	gi 608690 emb CAA57342.1 alcohol dehydrogenase [Candida albicans]	1.75E-08	36.3	6	36856
1450	1.63	5957463	68488457	gi 68488457 ref XP_711912.1 hypothetical protein CaO19.8025 [Candida albicans SC5314] (N-terminal of it)	7.33E-14	27.5	5.4	47202.6
1484	-1.86	5708876	37548637	gi 37548637 gb AAN11300.1 29 kDa IgE-binding protein [Candida albicans]	3.16E-08	26.3	4.6	25818
1495	-1.8	5083411	68482097	gi 68482097 ref XP_715066.1 putative polyamine N-acetyl transferase [Candida albicans SC5314]	5.52E-05	22.3	4.6	24773.4
			68472462	gi 68472462 ref XP_719792.1 glyceraldehyde-3-phosphate dehydrogenase [Candida albicans SC5314]	2.14E-08	26.3	6.7	35810.7
1507	1.15	1947515	1628589	gi 1628589 gb AAC49800.1 glyceraldehyde-3-phosphate dehydrogenase [Candida albicans]	9.41E-08	15.7	6.4	35394.4
			3850151	gi 3850151 emb CAA21951.1 rehydrin-like protein [Candida albicans]	9.58E-10	21.4	7.5	27458.3
1510	1.37	3122796	68468755	gi 68468755 ref XP_721436.1 phosphomannomutase [Candida albicans SC5314]	1.43E-06	14.3	5.4	29000.6
			68468755	gi 68468755 ref XP_721436.1 phosphomannomutase [Candida albicans SC5314]	4.36E-09	19.8	5.4	29000.6
1528	1.4	4811576	68469783	gi 68469783 ref XP_721143.1 hypothetical protein CaO19_8522 [Candida albicans SC5314]	7.79E-09	23	5.8	27437.5
			68468755	gi 68468755 ref XP_721436.1 phosphomannomutase [Candida albicans SC5314]	5.38E-12	39.7	5.4	29000.6
1539	1.41	6184612	68469783	gi 68469783 ref XP_721143.1 hypothetical protein CaO19_8522 [Candida albicans SC5314]	8.34E-08	31	5.8	27437.5
1547	1.81	7292565	68469783	gi 68469783 ref XP_721143.1 hypothetical protein CaO19_8522 [Candida albicans SC5314]	1.22E-09	36.7	5.8	27437.5
1565	1.38	1579898	68472685	gi 68472685 ref XP_719661.1 putative mitochondrial complex I [Candida albicans SC5314]	1.16E-05	16	7.9	27284.8
1586	1.88	1040836	7270988	gi 7270988 emb CAB77631.1 triosephosphate isomerase [Candida albicans]	1.61E-07	23.8	5.7	26608.8
1593	1.42	763906	68482810	gi 68482810 ref XP_714631.1 hypothetical protein CaO19.8941 [Candida albicans SC5314]	2.99E-10	17.9	5.5	23113.8
1596	1.36	3094722	68470996	gi 68470996 ref XP_720505.1 hypothetical protein CaO19_2755 [Candida albicans SC5314]	0.000197	15	5.5	14112.1
1627	1.65	1407978	68472462	gi 68472462 ref XP_719792.1 glyceraldehyde-3-phosphate dehydrogenase [Candida albicans SC5314] N-terminal	1.64E-08	20	6.7	35810.7
			68479826	gi 68479826 ref XP_716082.1 putative thioredoxin peroxidase [Candida albicans SC5314]	3.88E-11	40.8	4.8	21846.3

(Table 4) contd....

Spot No.	Average Ratio*	P - Spot Vol.	Accession	Protein	Protein Probability	Coverage	pI	Molecular Weight
1666	1	16242598	68482234	gi 68482234 ref XP_715001.1 hypothetical protein CaO19.11063 [Candida albicans SC5314]	4.59E-08	37.3	6	25812.7
1693	1.11	2011633	68479826	gi 68479826 ref XP_716082.1 putative thioredoxin peroxidase [Candida albicans SC5314]	5.31E-12	25.5	4.8	21846.3
			68472462	gi 68472462 ref XP_719792.1 glyceraldehyde-3-phosphate dehydrogenase [Candida albicans SC5314] N-terminal	2.61E-09	19.7	6.7	35810.7
1700	-1.13	6273637	68482775	gi 68482775 ref XP_714697.1 hypothetical protein CaO19.6991 [Candida albicans SC5314]	2.79E-08	27.6	6.5	23286.5
			68478409	gi 68478409 ref XP_716706.1 putative heat shock protein regulator [Candida albicans SC5314]	1.81E-12	17.7	4.8	17099.5
1705	-1.02	4023501	68468985	gi 68468985 ref XP_721551.1 hypothetical protein CaO19_3053 [Candida albicans SC5314]	2.56E-09	29.8	5	20310.4
1709	-1.06	10567480	68468985	gi 68468985 ref XP_721551.1 hypothetical protein CaO19_3053 [Candida albicans SC5314]	4.76E-11	29.8	5	20310.4
1717	-1.12	11609830	68466444	gi 68466444 ref XP_722704.1 putative cytosolic ribosomal protein L20 [Candida albicans SC5314]	2.32E-05	11.6	10.6	20325
1741	1.03	4316714	68483469	gi 68483469 ref XP_714295.1 putative glutathione peroxidase/redox transducer [Candida albicans SC5314]	1.30E-07	33.5	6.9	18091.3
1794	1.04	5507615	68470049	gi 68470049 ref XP_720786.1 FKBP-type peptidyl-prolyl cis-trans isomerase Rbp1p [Candida albicans SC5314]	3.30E-07	18.5	7.1	13258.9
1803	-1.31	15541205	68470079	gi 68470079 ref XP_720801.1 cyclophilin type peptidyl-prolyl cis-trans isomerase [Candida albicans SC5314]	5.15E-06	25.3	8.2	17563.8

*Ratio of biofilm:planktonic spot intensities

Table 5. Differentially Expressed Proteins in Cell Walls Isolated from *C. albicans* Biofilms Grown to Mature Phase and Planktonic Cells Grown to the Same Time Point

Spot No.	Average Ratio*	P - Spot Vol.	Accession GI	Protein	Protein Probability	Coverage	pI	Molecular Weight
116	-1.58	589363	68468339	gi 68468339 ref XP_721729.1 putative fatty-acyl coenzyme A oxidase [Candida albicans SC5314]	5.08E-09	7.1	5.6	78913.3
120	-2.3	709673	68468339	gi 68468339 ref XP_721729.1 putative fatty-acyl coenzyme A oxidase [Candida albicans SC5314]	3.33E-07	5.5	5.6	78913.3
126	-2.26	434430	68488040	gi 68488040 ref XP_712128.1 hypothetical protein CaO19.13993 [Candida albicans SC5314]	1.00E-05	4.3	5.7	77290.6
187	-1.59	705455	68484803	gi 68484803 ref XP_713713.1 putative acetyl CoA synthetase Acs2p [Candida albicans SC5314]	3.92E-07	16.8	5.7	73815.5
195	-1.99	1316446	68484803	gi 68484803 ref XP_713713.1 putative acetyl CoA synthetase Acs2p [Candida albicans SC5314]	3.33E-07	6.9	5.7	73815.5
			68485837	gi 68485837 ref XP_713199.1 mitochondrial import protein [Candida albicans SC5314]	2.73E-10	12.7	5.3	69705.5
209	-2.51	2070709	607055	gi 607055 emb CAA82929.1 heat shock protein 70 [Candida albicans]	2.75E-10	18.3	4.9	70281.2
210	-1.79	551108	68479353	gi 68479353 ref XP_716208.1 hypothetical protein CaO19_6367 [Candida albicans SC5314]	2.00E-08	9	5.1	66408.6
234	-3.35	2171433	68485837	gi 68485837 ref XP_713199.1 mitochondrial import protein [Candida albicans SC5314]	1.40E-07	13.1	5.3	69705.5
			68478453	gi 68478453 ref XP_716728.1 hypothetical protein CaO19_3419 [Candida albicans SC5314]	1.95E-11	13.2	5.7	69700.6
			2213423	gi 2213423 emb CAA66308.1 heat shock protein 70 [Candida albicans]	6.34E-09	5.9	5	66391.6
239	-4.84	3024764	3859677	gi 3859677 emb CAA22014.1 phenylalanyl-tRNA synthetase [Candida albicans]	1.21E-08	8.1	5.1	66779.4
241	-5.93	1792761	68478453	gi 68478453 ref XP_716728.1 hypothetical protein CaO19_3419 [Candida albicans SC5314]	1.31E-05	5.2	5.7	69700.6
242	-5.65	2837226	2213423	gi 2213423 emb CAA66308.1 heat shock protein 70 [Candida albicans]	1.97E-10	6.4	5	66391.6
			68478453	gi 68478453 ref XP_716728.1 hypothetical protein CaO19_3419 [Candida albicans SC5314]	3.22E-10	10.2	5.7	69700.6

(Table 5) contd....

Spot No.	Average Ratio*	P - Spot Vol.	Accession GI	Protein	Protein Probability	Coverage	pI	Molecular Weight
			68479353	gij68479353 ref XP_716208.1 hypothetical protein CaO19_6367 [Candida albicans SC5314]	1.38E-08	5.5	5.1	66408.6
244	-5.47	1226083	68480862	gij68480862 ref XP_715584.1 hypothetical protein CaO19_10389 [Candida albicans SC5314]	5.16E-08	11.7	6	70131.6
			68478453	gij68478453 ref XP_716728.1 hypothetical protein CaO19_3419 [Candida albicans SC5314]	8.28E-11	4.8	5.7	69700.6
245	-7.89	2422010	68480862	gij68480862 ref XP_715584.1 hypothetical protein CaO19_10389 [Candida albicans SC5314]	3.35E-08	6.1	6	70131.6
			68478796	gij68478796 ref XP_716560.1 hypothetical protein CaO19_1252 [Candida albicans SC5314]	1.67E-09	6.4	7	75824
250	-5.15	941176	68480862	gij68480862 ref XP_715584.1 hypothetical protein CaO19_10389 [Candida albicans SC5314]	7.47E-07	7.6	6	70131.6
262	-4.29	710345	68480862	gij68480862 ref XP_715584.1 hypothetical protein CaO19_10389 [Candida albicans SC5314]	1.30E-09	13.7	6	70131.6
269	-3.36	1547885	68480862	gij68480862 ref XP_715584.1 hypothetical protein CaO19_10389 [Candida albicans SC5314]	1.46E-06	7.8	6	70131.6
342	-1.88	4374340	68485963	gij68485963 ref XP_713100.1 heat shock protein 60 [Candida albicans SC5314]	1.47E-11	29.9	5.1	60088.7
358	2.05	20379960	68480872	gij68480872 ref XP_715589.1 pyruvate decarboxylase [Candida albicans SC5314]	2.58E-11	32.3	5.3	62402
408	1.65	15058075	68480872	gij68480872 ref XP_715589.1 pyruvate decarboxylase [Candida albicans SC5314]	3.40E-13	34.6	5.3	62402
			68488457	gij68488457 ref XP_711912.1 hypothetical protein CaO19.8025 [Candida albicans SC5314]	1.00E-30	36.6	5.4	47202.6
782	1.71	5625470	68485391	gij68485391 ref XP_713373.1 O-acetylhomoserine O-acetylserine sulphhydrylase [Candida albicans SC5314]	6.44E-14	20.9	5.5	47958.6
			68488457	gij68488457 ref XP_711912.1 hypothetical protein CaO19.8025 [Candida albicans SC5314]	4.44E-14	36.8	5.4	47202.6
784	1.64	13767138	68485391	gij68485391 ref XP_713373.1 O-acetylhomoserine O-acetylserine sulphhydrylase [Candida albicans SC5314]	6.27E-13	21.8	5.5	47958.6
791	1.81	15371336	68488457	gij68488457 ref XP_711912.1 hypothetical protein CaO19.8025 [Candida albicans SC5314]	5.66E-11	38.6	5.4	47202.6
799	1.81	11009924	68488457	gij68488457 ref XP_711912.1 hypothetical protein CaO19.8025 [Candida albicans SC5314]	1.00E-30	41.8	5.4	47202.6
803	1.72	46851281	68488457	gij68488457 ref XP_711912.1 hypothetical protein CaO19.8025 [Candida albicans SC5314]	1.00E-30	44.1	5.4	47202.6
861	1.64	2083832	68489602	gij68489602 ref XP_711371.1 3-phosphoglycerate kinase [Candida albicans SC5314]	1.83E-11	28.1	6.1	45151.8
			68489602	gij68489602 ref XP_711371.1 3-phosphoglycerate kinase [Candida albicans SC5314]	4.40E-11	44.4	6.1	45151.8
862	1.73	3353727	68466811	gij68466811 ref XP_722601.1 aspartic-semi-aldehyde dehydrogenase [Candida albicans SC5314]	9.26E-10	19.5	5.6	39200.4
876	1.85	2345118	68489602	gij68489602 ref XP_711371.1 3-phosphoglycerate kinase [Candida albicans SC5314]	1.47E-11	30.9	6.1	45151.8
880	1.64	1594386	68489602	gij68489602 ref XP_711371.1 3-phosphoglycerate kinase [Candida albicans SC5314]	1.50E-12	14.4	6.1	45151.8
			68488457	gij68488457 ref XP_711912.1 hypothetical protein CaO19.8025 [Candida albicans SC5314]	2.55E-14	37.5	5.4	47202.6
885	2.49	6943451	68467327	gij68467327 ref XP_722325.1 actin [Candida albicans SC5314]	1.24E-11	16.4	5.8	40154.2
1005	1.64	4250638	68473257	gij68473257 ref XP_719434.1 hypothetical protein CaO19_7676 [Candida albicans SC5314]	1.91E-12	23.1	5.6	38746.7
			68466123	gij68466123 ref XP_722836.1 fructose-bisphosphate aldolase [Candida albicans SC5314]	1.32E-12	27.9	5.7	39190.8
			68473257	gij68473257 ref XP_719434.1 hypothetical protein CaO19_7676 [Candida albicans SC5314]	6.08E-12	19.2	5.6	38746.7
1026	1.68	3688268	68476713	gij68476713 ref XP_717649.1 alcohol dehydrogenase 2 [Candida albicans SC5314]	3.45E-07	19.5	6.3	36784.1
			608690	gij608690 emb CAA57342.1 alcohol dehydrogenase [Candida albicans]	8.81E-12	36.6	6	36856
			68473257	gij68473257 ref XP_719434.1 hypothetical protein CaO19_7676 [Candida albicans SC5314]	2.04E-11	20.6	5.6	38746.7

(Table 5) contd....

Spot No.	Average Ratio*	P - Spot Vol.	Accession GI	Protein	Protein Probability	Coverage	pI	Molecular Weight
			68466123	gi 68466123 ref XP_722836.1 fructose-bisphosphate aldolase [Candida albicans SC5314]	2.55E-11	28.1	5.7	39190.8
1030	2.09	1520348	68482781	gi 68482781 ref XP_714700.1 branched-chain amino acid transaminase [Candida albicans SC5314]	3.17E-10	37.4	5.8	40778
			68473257	gi 68473257 ref XP_719434.1 hypothetical protein CaO19_7676 [Candida albicans SC5314]	1.44E-12	26.7	5.6	38746.7
			68466123	gi 68466123 ref XP_722836.1 fructose-bisphosphate aldolase [Candida albicans SC5314]	1.64E-12	32.6	5.7	39190.8
			68482781	gi 68482781 ref XP_714700.1 branched-chain amino acid transaminase [Candida albicans SC5314]	2.86E-12	40.9	5.8	40778
1032	1.51	2505270	608690	gi 608690 emb CAA57342.1 alcohol dehydrogenase [Candida albicans]	7.38E-10	43.1	6	36856
			608690	gi 608690 emb CAA57342.1 alcohol dehydrogenase [Candida albicans]	1.32E-11	32.9	6	36856
			68466123	gi 68466123 ref XP_722836.1 fructose-bisphosphate aldolase [Candida albicans SC5314]	4.20E-11	14.8	5.7	39190.8
1037	1.58	1417496	68482781	gi 68482781 ref XP_714700.1 branched-chain amino acid transaminase [Candida albicans SC5314]	8.85E-09	32.8	5.8	40778
			68466123	gi 68466123 ref XP_722836.1 fructose-bisphosphate aldolase [Candida albicans SC5314]	1.05E-11	28.1	5.7	39190.8
1056	1.61	3049164	608690	gi 608690 emb CAA57342.1 alcohol dehydrogenase [Candida albicans]	3.07E-11	29.4	6	36856
1204	1.8	2163527	68472462	gi 68472462 ref XP_719792.1 glyceraldehyde-3-phosphate dehydrogenase [Candida albicans SC5314]	6.40E-09	23.3	6.7	35810.7
1262	-1.82	3239065	68468907	gi 68468907 ref XP_721512.1 hypothetical protein CaO19_3014 [Candida albicans SC5314]	3.33E-15	45.1	4.6	29461.8
1436	2.71	1287288	68472462	gi 68472462 ref XP_719792.1 glyceraldehyde-3-phosphate dehydrogenase [Candida albicans SC5314]	4.08E-11	26.9	6.7	35810.7
1463	2.98	843777	68472462	gi 1628589 gb AAC49800.1 glyceraldehyde-3-phosphate dehydrogenase [Candida albicans]	4.08E-11	20.2	6.4	35394.4
1499	2.06	1935113	68469783	gi 68469783 ref XP_721143.1 hypothetical protein CaO19_8522 [Candida albicans SC5314]	0.000222	5.2	5.8	27437.5
1520	1.53	1271010	68471743	gi 68471743 ref XP_720178.1 hypothetical protein CaO19_7983 [Candida albicans SC5314]	2.09E-09	19.9	4.9	27790.9
			68475280	gi 68475280 ref XP_718358.1 vacuolar ATPase V1 domain subunit E [Candida albicans SC5314]	2.15E-10	45.8	5.3	23825.9
1535	1.84	4392263	68469783	gi 68469783 ref XP_721143.1 hypothetical protein CaO19_8522 [Candida albicans SC5314]	1.74E-08	35.1	5.8	27437.5
			68469783	gi 68469783 ref XP_721143.1 hypothetical protein CaO19_8522 [Candida albicans SC5314]	3.43E-09	24.2	5.8	27437.5
			68466105	gi 68466105 ref XP_722827.1 hypothetical protein CaO19_12079 [Candida albicans SC5314]	1.92E-08	18.9	5.6	26661.9
1546	1.94	3180876	7270988	gi 7270988 emb CAB77631.1 triosephosphate isomerase [Candida albicans]	2.63E-08	25	5.7	26608.8
			68469783	gi 68469783 ref XP_721143.1 hypothetical protein CaO19_8522 [Candida albicans SC5314]	2.34E-09	31.9	5.8	27437.5
			68468755	gi 68468755 ref XP_721436.1 phosphomannomutase [Candida albicans SC5314]	1.84E-08	26.6	5.4	29000.6
1551	1.71	10404306	68475280	gi 68475280 ref XP_718358.1 vacuolar ATPase V1 domain subunit E [Candida albicans SC5314]	5.59E-06	25.5	5.3	23825.9
1561	1.74	8831269	68469783	gi 68469783 ref XP_721143.1 hypothetical protein CaO19_8522 [Candida albicans SC5314]	2.03E-10	36.3	5.8	27437.5
1563	2.18	1438165	68469783	gi 68469783 ref XP_721143.1 hypothetical protein CaO19_8522 [Candida albicans SC5314]	3.05E-08	34.7	5.8	27437.5
1564	2.2	1031779	68469783	gi 68469783 ref XP_721143.1 hypothetical protein CaO19_8522 [Candida albicans SC5314]	2.73E-10	35.1	5.8	27437.5
1655	1.58	1271290	68480453	gi 68480453 ref XP_715825.1 Ras family GTP-binding protein Rho1p [Candida albicans SC5314]	3.84E-08	15.2	5.4	21985.4
1668	1.71	1039839	68470310	gi 68470310 ref XP_720659.1 hypothetical protein CaO19_11696 [Candida albicans SC5314]	9.43E-08	15.3	5.9	20820.5

(Table 5) contd....

Spot No.	Average Ratio*	P - Spot Vol.	Accession GI	Protein	Protein Probability	Coverage	pI	Molecular Weight
1703	2.41	3503757	68479834	gi 68479834 ref XP_716086.1 hypothetical protein CaO19_7421 [Candida albicans SC5314]	2.33E-14	12.7	6.3	25341.8
1704	1.55	2852610	68482775	gi 68482775 ref XP_714697.1 hypothetical protein CaO19.6991 [Candida albicans SC5314]	8.61E-07	11.7	6.5	23286.5
1710	1.85	11010304	68491679	gi 68491679 ref XP_710366.1 hypothetical protein CaO19.5285 [Candida albicans SC5314]	7.88E-05	10.1	5.8	21160.9
1776	1.63	9594945	68473729	gi 68473729 ref XP_719189.1 ubiquinol cytochrome-c reductase subunit 7 [Candida albicans SC5314]	6.74E-06	25.2	5.7	14414.6

Table 6. Differentially Expressed Pathways in Matrix and Cell Walls Isolated from *C. albicans* Biofilms Grown to Early and Mature Developmental Phases

Protein Sample	Early Phase (6 h)	Mature Phase (48 h)
Matrix	Purine metabolism Fructose and mannose metabolism Pentose phosphate pathway Glycine, serine and threonine metabolism Glycolysis / Gluconeogenesis Biosynthesis of steroids Urea cycle and metabolism of amino groups Glutamate metabolism* Methionine metabolism Valine, leucine and isoleucine biosynthesis Lysine biosynthesis beta-Alanine metabolism Glycerolipid metabolism Glycerophospholipid metabolism Pantothenate and CoA biosynthesis Terpenoid biosynthesis Nitrogen metabolism* Calcium signaling pathway	Fructose and mannose metabolism Purine metabolism* Glycolysis / Gluconeogenesis Pentose phosphate pathway Glycine, serine and threonine metabolism* Carbon fixation* Inositol metabolism* Biosynthesis of steroids Urea cycle and metabolism of amino groups Methionine metabolism Valine, leucine and isoleucine biosynthesis Lysine biosynthesis beta-Alanine metabolism Glycerolipid metabolism Glycerophospholipid metabolism Pantothenate and CoA biosynthesis Terpenoid biosynthesis Calcium signaling pathway
Cell walls	Glycolysis / Gluconeogenesis Fructose and mannose metabolism Inositol metabolism Fatty acid metabolism Bile acid biosynthesis Oxidative phosphorylation Valine, leucine and isoleucine degradation Valine, leucine and isoleucine biosynthesis Lysine biosynthesis Lysine degradation Tyrosine metabolism Phenylalanine, tyrosine and tryptophan biosynthesis Glycerolipid metabolism 1- and 2-Methylnaphthalene degradation 3-Chloroacrylic acid degradation Carbon fixation Pantothenate and CoA biosynthesis	Glycolysis / Gluconeogenesis Fructose and mannose metabolism Fatty acid metabolism Oxidative phosphorylation Carbon fixation Inositol metabolism Aminoacyl-tRNA biosynthesis* PPAR signaling pathway* Citrate cycle (TCA cycle)* Pentose phosphate pathway* Bile acid biosynthesis Glycine, serine and threonine metabolism* Methionine metabolism* Cysteine metabolism* Valine, leucine and isoleucine degradation Valine, leucine and isoleucine biosynthesis Lysine biosynthesis Arginine and proline metabolism* Tyrosine metabolism Phenylalanine, tyrosine and tryptophan biosynthesis Glycerolipid metabolism alpha-Linolenic acid metabolism* Pyruvate metabolism* 1- and 2-Methylnaphthalene degradation 3-Chloroacrylic acid degradation Pantothenate and CoA biosynthesis Metabolism of xenobiotics by cyt-P450* Polyunsaturated fatty acid biosynthesis* Proteasome* Type III secretion system* Calcium signaling pathway*

*Phase-specific pathways overexpressed specifically in matrix or cell walls of early or mature biofilms.

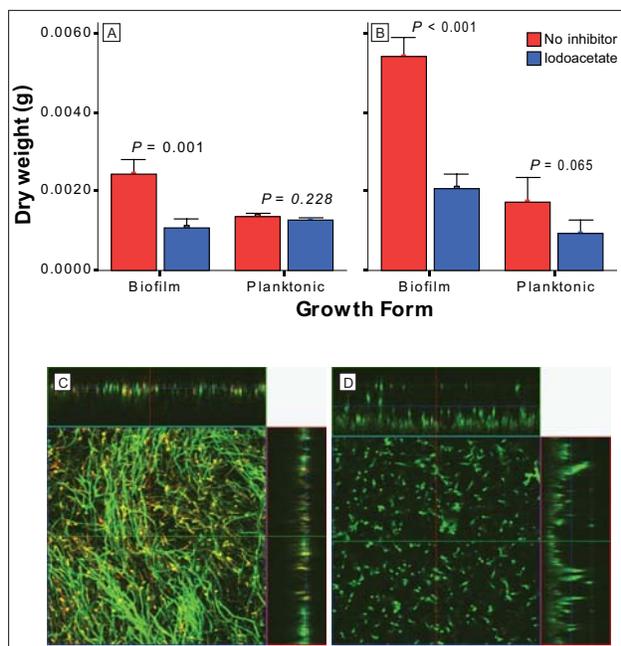


Fig. (8). Effect of GAPDH inhibitor sodium iodoacetate on the ability of *C. albicans* to form biofilms. (A-B) Dry weight determination of biofilm grown to (A) early or (B) mature phase. (C-D) Confocal microscopy images of mature biofilms grown in the (C) absence or (D) presence of sodium iodoacetate. Panels C and D show top-down images obtained after three-dimensional reconstruction of all the z-stacks resulting from the confocal analyses.

analyses revealed the presence of proteins like transcription factors (e.g. translation initiation factor eIF4A subunit), DNA binding proteins (e.g. HSP70, PDR, BMH1), NTPase activity (RHO1), and kinases (e.g. BCY1) with regulatory functions, which are expected to be present at low levels.

Traditional 2D-GE approach has been earlier used to analyze protein profile of *Candida* cells. In one such study, Kusch *et al.* [36] performed 2D-GE proteomic analysis of planktonically grown *C. albicans* yeast cells to exponential and stationary growth phases, and used similar tools to identify several differentially expressed pathways in a growth phase dependent manner. In a separate study, Thomas *et al.* [15] reported the use of conventional 2D-GE-based proteomics for the comparative analysis of the protein profiles of surface-associated and secreted proteins obtained from mature biofilm and planktonic cultures of *C. albicans*. These investigators reported that 9 surface-associated and 34 secreted proteins were differentially expressed in biofilms. In contrast, our DIGE analyses revealed much higher number of differentially expressed proteins in mature *C. albicans* biofilms (35 cell wall-associated and 56 extracellular matrix-associated proteins were differentially expressed, of which 24 and 54 were upregulated, respectively) and early phase biofilms.

The higher number of differentially expressed proteins detected in our studies compared to those reported by Thomas *et al.* [15] may be attributed to the fact that the methods used by these investigators for protein extraction and gel electrophoresis were different from those used in our study. In this regard, Rathsam *et al.* [9] showed that compared with 2D-GE proteome analysis, DIGE analysis resulted in a 3-

fold increase in the number of identified protein spots that were significantly altered in their level of expression in biofilms formed by *Streptococcus mutans*. Additionally, we used an EDTA-based extraction method which resulted in higher protein yield than the vortexing method, which also likely contributed to the differences in protein profiles between the two studies.

Another probable reason for the difference in protein profile observed in our study compared to that reported by Thomas *et al.* [15] is that in our study, biofilms were formed on serum-pre-coated catheter discs, while these investigators analyzed biofilms grown in tissue culture flasks. In this regard, it is notable that different surface types induce distinct differences in biofilm morphology and architecture [31], and could also account for the observed differences in protein profiles between the two studies.

A common concern with systems biology “omics” approaches (e.g. proteomics, genomics, metabolomics) is an abundance of data points which are not mined properly to arrive at an over-arching global perspective of cellular functions [37, 38]. To gain insight into cellular functions involved in *Candida* biofilms, we used online Omics (based on a *S. cerevisiae* database) and KEGG pathway analysis tools (incorporating a pre-existing, online *C. albicans* dataset) [28] to map the differentially expressed proteins to specific pathways. These analyses revealed that proteins associated with several pathways were differentially expressed in *C. albicans* biofilms at early and mature phases, and suggested a general trend of increased expression of metabolic pathway proteins during biofilm formation. We also found that although the same pathways (e.g. glycolysis) may be differentially expressed at both early and mature phases in biofilms, the number and identity of the actual proteins involved in these pathways may differ considerably between the two phases. Such changes in the number and identity of differentially expressed proteins involved in carbohydrate and amino acid metabolism, and in the calcium and PPAR signaling pathways may account for the dramatic increase observed in metabolic activity and matrix production in mature biofilms. This increase is not surprising since it is expected that different functional processes may be operative at different biofilm developmental phases. Such phase-specific expression of pathways may be critical for increased production of cell walls, extracellular matrix, and the observed antifungal resistance associated with mature biofilms. While a majority of the differentially expressed proteins identified in the current study are involved in metabolic pathways, it is also possible that these proteins may play non-metabolic roles in biofilm formation. In this regard, several enzymatic proteins have been shown to play diverse unrelated functions like control of gene expression, organelle assembly, and modification of the activity of metabolic pathways (reviewed in [39]). The role played by differentially expressed proteins in biofilm formation by *C. albicans* remains to be investigated.

We found that glycolysis/gluconeogenesis was the most common pathway that was differentially expressed at both early and mature phases of biofilm development. Other pathways that were differentially expressed (mostly up-regulated) during biofilm formation included pentose phosphate pathway, tricarboxylic acid (TCA) cycle, amino acid metabolism, and sterol biosynthesis. Since several of the up-

regulated pathways are involved in carbohydrate metabolism, it is possible that such up-regulation is associated with increased cell wall and matrix synthesis – two functional processes known to occur during *C. albicans* biofilm formation. Previously, several microarray studies described changes in gene expression during early phase of biofilm development [40-43]. These studies demonstrated that genes involved in carbohydrate assimilation, amino acid metabolism, and intracellular transport mechanisms were overexpressed during the early phase of biofilm formation. Our proteomic data are in agreement with the overall trend seen in these microarray studies, although the specific genes and proteins did not always correlate. Interestingly, in one of the microarray studies reported by our group [40] it was shown that at mature phase (48 h), few genes were differentially expressed, suggesting a relative lack of initiation of new metabolic activity at the transcriptional level. However, in the current study we observed active metabolic processes occurring at the mature phase. The difference between the microarray data and our proteomic analyses may be due to the fact that cellular processes in *Candida* cells are known to be regulated differently at the transcriptional and translational levels, and similar differential regulation of pathways involved in cell wall biogenesis, general metabolism and signaling events is likely to occur in mature *Candida* biofilms. Another reason for the difference between previous microarray results and our current proteomic results could be the fact that mRNAs and proteins have different stabilities [44, 45]. In this regard, Belle *et al.* [45] suggested that transient changes in the abundance of mRNAs coding for stable proteins may take significant time to induce comparable changes in protein abundance. Taken together, our results demonstrate that *C. albicans* biofilm formation is associated with changes in protein profiles belonging to different yet interconnected metabolic pathways. Our proteomic analyses also demonstrated that information obtained using proteomics complements that generated from transcriptional profiling, and can be used to glean comprehensive insight into the phase-dependent processes operative during the biofilm formation. Moreover, these results show that biofilm formation is a dynamic process, with changes at both mRNA and protein levels occurring at different developmental phases.

Our studies demonstrated that glycolysis was one of the pathways overexpressed in biofilms at both early and mature phases, and that GAPDH was the most overexpressed glycolytic enzyme in biofilms at both developmental phases. Moreover, inhibition of GAPDH using iodoacetate (a specific and irreversible inhibitor) resulted in abrogation of the ability of *C. albicans* to form biofilms. Confocal analyses confirmed such effect of this inhibitor on biofilm formation. Interestingly, iodoacetate specifically inhibited *C. albicans* biofilms grown to early or mature phases but had not significant effect on planktonic cells grown to similar time points. These results suggest that GAPDH may play a specific role in biofilm formation, unrelated to normal fungal growth. In this regard, Solem *et al.* [30] previously showed that GAPDH has no control over glycolytic flux in growing cells of *Lactococcus lactis*. It is possible that a similar situation exists in planktonically grown *C. albicans*, where inhibition of GAPDH may be compensated by other enzymes of the pathway. However, in biofilm mode of growth, GAPDH

may exert its inhibition of glycolysis, thereby also reducing biofilm formation. The role of GAPDH in biofilm formation needs to be further confirmed by targeted disruption of the gene encoding this protein. The mechanism/s by which phase-associated pathways (e.g. glycolysis/gluconeogenesis) and enzymes like GAPDH regulate *C. albicans* biofilm formation is unknown and is currently under investigation.

Overall, our studies revealed that specific metabolic pathways are differentially expressed in *C. albicans* biofilms in a phase-dependent manner. These pathways and associated proteins may play critical roles in *Candida* biofilm formation, and represent potential targets for designing anti-biofilm drugs, as well as for early diagnosis of infections associated with *Candida* biofilms.

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