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Proteomic analysis of cytoplasmic and surface proteins from yeast cells, hyphae, and biofilms of *Candida albicans*

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Abstract

Candida albicans is a human commensal and opportunistic pathogen that participates in biofilm formation on host surfaces and also on medical devices. We used DIGE analysis to assess the cytoplasmic and non-covalently attached cell surface proteins in biofilm formed on polymethylmethacrylate and planktonic yeast cells and hyphae. Of the 1490 proteins spots from cytoplasmic and 580 protein spots from the surface extracts analyzed, 265 and 108 were differentially abundant respectively (≥1.5 fold, $p<0.05$). Differences of both greater and lesser abundance were found between biofilms and both planktonic conditions as well as between yeast cells and hyphae. The identity of 114 cytoplasmic and 80 surface protein spots determined represented 73 and 25 unique proteins respectively. Analyses showed that yeast cells differed most in cytoplasmic profiling while biofilms differed most in surface profiling. Several processes and functions were significantly affected by the differentially abundant cytoplasmic proteins. Particularly noted were many of the enzymes of respiratory and fermentative pentose and glucose metabolism, folate interconversions and proteins associated with oxidative and stress response functions, host response, and multi-organism interaction. The differential abundance of cytoplasmic and surface proteins demonstrated that sessile and planktonic organisms have a unique profile.

Keywords

biofilm; *Candida albicans*; cytoplasm; cell surface; DIGE

1 Introduction

Candida albicans is a pleomorphic organism that can grow in yeast or hyphal forms and is found as part of mucosal and cutaneous microflora of man. The organism can also cause opportunistic infections on these surfaces when the normal host surface is compromised. It can cause more serious life threatening deep tissue infection most often in the immunocompromised host. As a commensal the organism can be found in biofilms such as tooth plaque. It also colonizes and forms biofilms on various medical devices where it is

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often associated with disease [1]. The presence of dentures on which biofilms may also form is a risk factor for the most common manifestation of oral disease. The prevalence of denture stomatitis is about 50–65% among denture wearers, more frequent in women than men and increases with age [2–4]. The inoculation of the blood stream from catheter biofilms is a major contributor to the position of *Candida* species as the third most common cause of nosocomial blood stream infection [5–7]. In addition to tissue inoculation, a major problem associated with biofilms is reduced susceptibility to antifungal drugs. A biofilm is a community of organisms attached to a biotic or abiotic surface that is surrounded by an extracellular matrix produced by the biofilm organisms. Although *in vitro* studies of growth in a biofilm have appeared for little more than a decade, there is a long history of *in vitro* studies of *C. albicans* propagated in suspension culture as planktonic organisms. Both the yeast and hyphal forms of *C. albicans* are found in biofilms.

Transcriptional analyses have shown that there are differences in the transcriptome between hyphae and yeast cells and between planktonic organisms and biofilms [8–11]. This implies changes in protein abundance. We have employed a proteomic approach to assess global changes in protein abundance in planktonic yeast cells and hyphae and biofilms. The cytoplasm of the cell contains the machinery for converting nutrients into cellular products and energy, organelles that are specialized for various metabolic, energy and transport functions and proteins involved in cell structure and replication. We have previously established a reference map for hyphal cytoplasmic proteins [12]. More recently we applied spectrometric analysis of protein abundance to detect and identify cytoplasmic proteins with changed abundance following *in vitro* macrophage phagocytosis [13]. A similar analysis has been applied to exponential and stationary phase yeast cells [14]. Recently Seneviratne *et al.,* [15] compared difference in silver staining between 2DE separations of cytoplasmic proteins from biofiilms and planktonic organisms. This analysis identified 20 consistently differentially abundant protein spots with all but one more abundant in biofilms. In work reported here, using DIGE proteomic analysis with biological quadruplicate samples prepared from planktonic (yeast and hyphae forms) and biofilm organisms, we quantitated differences and identified proteins that are differentially abundant.

In addition to the cytoplasmic protein analysis, we have examined the surface subproteome of non-covalently attached proteins. Cell surface proteins are of considerable interest in *C. albicans* because of their immediate exposure to and potential interaction with the host. Proteins at the surface function as adhesins, elicit an immune response and alter with morphological state [16,17]. The *C. albicans* cell wall is composed of fibrillar polysaccharides to which are covalently attached two classes of proteins, GPI-anchor proteins and PIR proteins (see reviews [18,19]). There are other proteins that are not covalently attached and found at the cell surface [20]. We have used a proteomic approach to identify surface proteins that are recognized by human sera [21,22]. Several studies have employed proteomic approaches to identify proteins of the cell wall subproteome for both covalent and non-covalent protein species [22–31]. As an initial step to compare proteins extracted from the cell surface of biofilms and planktonic organisms, particularly biofilms formed in a catheter lumen, we have used a one dimensional separation and identified several of the differences by mass spectrometry [31]. Recently among the studies of biofilms have been global approaches to proteomics of abundance of subcellular surface proteins in early [27] and later [29] biofilm development. These studies for the most part compare biofilms to a single planktonic culture.

Thomas et al [29] used Sypro Ruby staining of proteins recovered from the medium and surface extracts obtained in the presence of reducing agent to compare proteins from a 24 h biofilm with those from a planktonic culture. Among the some 300 spots detected, 48 unique proteins were identified of which nine were differentially expressed. Mukherjee et al [27]

examined non-covalent proteins recovered by SDS extraction from the isolated cell walls of a 6 h early development stage of biofilms and similar aged planktonic organisms. DIGE analysis revealed 33 proteins that were found to be less abundant and 38 spots more abundant in biofilms and 24 proteins were identified. The relative abundance of one protein Adh1p was subsequently verified by Western blot analysis. In this study we examined the subproteome of the non-glucan attached proteins of the cell surface from mature 48 h biofilms and both planktonic yeast cells and hyphae. Using the same DIGE proteomic analysis for biological quadruplicate samples, we quantitated differences and identified proteins that are differentially found on the surface of the three populations. The analysis of both cytoplasm and cell surface subproteome revealed patterns of protein abundance unique to each growth condition whether sessile or planktonic.

2 Materials and Methods

2.1 Organism and growth conditions

Candida albicans strain SC5314 was maintained on YPD (yeast extract 1%, peptone 2%, dextrose 2%and agar 2%) plates. The organism was grown in suspension culture in Difco™ YNB (Becton Dickenson and Co., Sparsk, MD) with 50 mM dextrose. Planktonic yeast cell cultures were obtained by inoculating 1 L medium in 1 or more 2 L flasks and incubating with shaking at 180 rpm for 48 h at 37 °C. For planktonic hyphae, yeast cells harvested from an early stationary phase culture grown at 25°C were resuspended at 1×10^6 cells/ml in fresh pre-warmed medium at 37°C and incubated with shaking (180 rpm). More than 90% germ tubes as determined microscopically were formed in 90–120 min. Organisms were harvested by centrifugation and protein extracts prepared as described below.

Biofilms were formed on strips (90×20×1.5 mm) of polymethylmethacrylate (denture acrylic) prepared by Dr. Thomas McKinney, Baylor College of Dentistry, Texas A&M University Health Science Center. The strips were placed in slots of a 15×10 cm plexiglass holder connected to a rod that passed through a lid of a box chamber (26 cm). Early stationary phase yeast cells were resuspended in medium at 1×10^6 cells/ml and poured into the chamber and the holder with strips was immersed in the suspension. After 2 h incubation the suspension was drained and a continuous flow of medium at 100 ml/h was initiated and maintained for 48 h. The holder was raised out of the medium every 10 s to simulate air exposure of biofilms in the oral cavity. The device is shown in Supporting Information Fig S1. Biofilms were allowed to develop for 48 hrs at 37°C. For cytoplasmic proteins, the biofilm was scraped from the acrylic strip into phosphate buffered saline and organisms recovered by centrifugation. Cell pellets, about 0.5 ml were maintained frozen until sample preparation. Cells were broken as previously described in a mortar and pestle with glass beads [32]. The powder was resuspended in 5 ml extraction buffer with mixing and incubated for 20 m at 37°C with vortexing at 5 min intervals. The extraction buffer contained 50 mM Tris, pH 7.5, 10% glycerol, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 5 mM ethylenediamine tetraacetic acid, 150 mM NaCl, 50 mM sodium fluoride, 1mM dithiothreitol, 1 mM sodium orthovanadate, 1mM phenylmethylsulfonyl fluoride and 50 µl per gram cells of protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). The mixture was centrifuged at 4°C and the supernatant was sterile filtered (0.2 µm pore) to remove intact cells and residual debris. For surface proteins, biofilms were scraped directly into a tube containing ammonium carbonate and β-mercaptoethanol buffer [33]. Pellets of harvested planktonic organisms were resuspended in the same buffer.

2.2 SEM

Biofilms were formed for 48 h as described above and a portion of a denture acrylic strip with biofilm on it was excised using a sterile blade and was snap frozen by immersing into

the liquid nitrogen slush prepared at 5×10^{-5} mbar. The biofilm was freeze dried in an automated EMS750 freeze drier (Electron Microscopic Sciences, Hatfield, PA) as per the instructions of the manufacturer. The samples were coated with gold particles using a sputter coating/glow discharge attachment EMS 350 (Electron Microscopic Sciences) at 20 mA for 5 min and SEM images were obtained with Hitachi S 500 (Hitachi, Japan).

2.3 DIGE experimental design

Following extraction, interfering components were removed by using 2D-Clean Kit™ (GE Healthcare). The protein was resuspended in 10 mM Tris, 8 M urea, 2 M thiourea and 4% (v/w) CHAPS. The protein concentration was determined using 2D-Quant Kit™ (GE Healthcare).

Each experiment group contained four biological replicates, generating 12 individual samples that were distributed across six DIGE gels with the internal standard pooled sample also present in each separation. Four hundred pmol of Cy dye in 1 µl of anhydrous N,N-dimethylformamide was used per 50 µg of protein for the labeling. After 30 min of incubation on ice in the dark, the reaction was quenched with 10 mM lysine and incubated for 10 min. Samples were combined according to the experimental design and an equal volume of 2 × rehydratation buffer (8 M urea, 2 M thiourea, 4 % w/v CHAPS, 2 % v/w DTT and 4 % pharmalytes pH 3–11) was added for the cup loading.

The 2-DE was performed using GE Healthcare reagents and equipment. For cytoplasmic protein 2-DE the first dimension IEF were performed on 24 cm 3–11 NL pH range IPG strips, previously rehydrated with 7 M urea, 2 M thiourea, 4 % (w/v) CHAPS, 100 mM DeStreak and 2 % pharmalytes pH 3–11. IEF was performed at 20 °C using the following program: 120 V for 2 h, 500 V for 2 h, 500–1000 V for 4 h, 1000–4000 V for 8 h and 4000 V for 12 h.

For surface protein 2-DE, the first dimension IEF were performed on 18 cm 4–7 pH range IPG strips, previously rehydrated with 8 M urea, 2 M thiourea, 4 % (w/v) CHAPS, 100 mM DeStreak and 2 % pharmalytes pH 4–7. IEF was performed at 20 °C using the following program: 120 V for 2 h 500 V for 2 h, 500–1000 V for 4 h, 1000–8000 V for 4 h and 8000 V for 4 h. After this, strips were equilibrated first for 12 min in reducing solution (6 M urea, 50 mM Tris-HCl pH 6.8, 30 % v/v glycerol, 2 % w/v SDS and 2% w/v DTT) and secondly 5 min in alkylating solution (6 M urea, 50 mM Tris-HCl pH 6.8, 30 % v/v glycerol, 2 % w/v SDS and 2.5 % w/v iodoacetamide). Second dimension SDS-PAGE were run on homogeneous 12% T, 2.6 %C (piperazine diacrylamide) polyacrylamide gels cast for cytoplasmic 2-DE and 10% T, 1.6% C for surface 2-DE in low fluorescent glass plates. Electrophoresis was carried out at 20 °C, 2 W/gel for 18 hours, using Ettan-Dalt six unit.

2.4 Image acquisition and DIGE analysis

Proteins were visualized using a Typhoon 9400™ scanner (GE Healthcare) with CyDye filters. For the Cy3, Cy5 and Cy2 image acquisition, the 532-nm/580 nm, 633-nm/670 nm and 488nm/520nm excitation/emission wavelengths were used respectively and 100 µm as pixel size.

Image analysis was carried out with DeCyder™ differential analysis software v6.5 (GE Healthcare). The DIA module was used to assign spot boundaries and to calculate parameters such as normalized spot volumes. Inter-gel variability was corrected by matching and normalization of the internal standard spot maps in the BVA module. The internal standard image gel with the greatest number of spots was used as a master gel. Three comparisons were carried out yeast cells versus biofilms, hyphae *versus* biofilms and yeast cells *versus* hyphae. Average ratio and unpaired Student's t-test were calculated between

groups. In order to reduce the false positive, False Discovery Rate was applied [34]. Protein spots with 1.5 fold as threshold in the average ratio with *p* values less than 0.05 were considered as differentially expressed with statistical significance between the extracts under comparison.

Unsupervised multivariate analysis was performed using the EDA module. Proteins present in 80% of the spot maps for surface protein experiment and 100% for cytoplasmic protein experiment, with a 1.5 fold or more variation in abundance (ANOVA $p<0.05$) were used as a subset for this analysis. PCA was performed following the nonlinear iterative partial least squares method. HCA used the Pearson method and average linkage for distances calculation.

K-means cluster analysis was done using the Spotfire software (Spotfire, Somerville, MA). For the calculation settings, we defined 10 clusters, Euclidean distance was selected as the similarity measure and distances between clusters were defined by cluster centroids.

2.5 Protein identification

Total protein profile was detected by staining the DIGE gels with Colloidal Coomassie blue. Proteins selected for analysis were in-gel digested [35]. Samples were reduced with 10 mM DTT in 25 mM ammonium bicarbonate for 30 min at 56°C and subsequently alkylated with 55 mM iodoacetamide in 25 mM ammonium bicarbonate for 20 min in the dark. Finally, samples were digested with 12,5 ng/μl sequencing grade trypsin (Roche Molecular Biochemicals) in 25 mM ammonium bicarbonate (pH 8.5) overnight at 37°C. After digestion, the supernatant was collected and 1 μl was spotted onto a MALDI target plate and allowed to air-dry at room temperature. Then, 0.4 μl of a 3 mg/ml of α-cyano-4-hydroxy-transcinnamic acid matrix (Sigma) in 50% (v/v) acetonitrile was added to the dried peptide digest spots and allowed again to air-dry at room temperature

MALDI-TOF MS analyses were performed in a MALDI-tandem time-of flight mass spectrometer 4700 Proteomics Analyzer (PerSeptives Biosystems, Framingham, MA). The instrument was operated in reflector mode, with an accelerating voltage of 20000 V. All mass spectra were calibrated externally using a standard peptide mixture (Sigma). Peptides from the auto digestion of the trypsin were used for the internal calibration. MALDI-TOF MS analysis produces peptide mass fingerprints and the peptides observed can be collected and represented as a list of monoisotopic molecular weights with a Signal to Noise greater than 20. The suitable precursors for MS/MS sequencing analyses were selected and fragmentation was carried out using the CID on (atmospheric gas was used) 1 Kv ion reflector mode and precursor mass Windows +/− 10 Da. The plate model & default calibration were optimized for the MS-MS spectra processing.

The search of peptides was performed in batch mode using GPS Explorer v3.5 software with a licensed version 1.9 of MASCOT, using two *Candida* databases: CandidaDB (www.genolist.pasteur.fr/CandidaDB) [36] and CGD (www.candidagenome.org) [37]. The MASCOT search parameters were: (1) species: *C. albicans*; (2) allowed number of missed cleavages: 1; (3) fixed modification: carbamidomethyl cysteine, (4) variable modifications: methionine oxidation; (5) peptide tolerance: ±150 ppm; (6) MS/MS tolerance: ±0.35 Da and (7): peptide charge: +1.

2.6. Biological processes and functions involving differentially abundant cytoplasmic proteins

The relative abundance for each protein with isoforms was determined by summing the isoforms. The differential abundance of the proteins in the three conditions was compared by ANOVA ($p<0.01$) followed by Tukey Multiple Comparison Test ($p<0.05$) using

GraphPad Prism v5.0. Proteins that differed significantly by this analysis were analyzed with the other differentially abundant proteins. The processes, functions, and cellular components associated with the differentially abundant proteins was determined using the Gene Ontology (GO) Term Finder [38] at CGD [39]. For each comparison, biofilms vs hyphae, biofilms vs yeast cells, and hyphae vs yeast cells, the proteins that were more abundant in each comparison were analyzed separate from those that were less abundant. The genes encoding the differentially abundant proteins were analyzed using the default background set (all features in the database) and default settings and returning all hits $p<0.1$. The results of the analyses are in Supporting information Table S7 which shows that most corrected p values were much less than 0.01.

3 Results

3.1 *In vitro* biofilm formation

We have previously developed a model for *in vitro* formation of *C. albicans* biofilm in a catheter lumen and on denture acrylic under conditions of continuous medium replenishment [31]. However, those models provided only small quantities of protein that were insufficient for this study. We modified the model for denture acrylic to increase the surface area available for biofilm formation by more than 16 fold. This device, described in Materials and Methods, used continuous medium replenishment with the addition of periods of air exposure further mimicking the environment of biofilms formed in the oral cavity. Acrylic strips were incubated with a yeast cell inoculum and following removal of non-adhered yeast cells the biofilms were developed for 48 h. A robust biofilm was formed that consisted of the typical composition of yeast cells and hyphae and extracellular matrix as shown by SEM (Fig 1).

3.2. Differential protein expression in biofilms

Protein abundance was compared between biofilms and yeast cells, biofilms and hyphae or hyphae and yeast cells. The DIGE approach has a high sensitivity and for this reason we used it for this study. DIGE involves labeling a sample with spectrally resolvable fluorescent CyDyes™ (Cy2, Cy3 and Cy5). Four replicates were prepared for each condition for the experiment.

Samples from yeast cells, hyphae and biofilms were labelled with Cy3 or Cy5 dye. To avoid any possible bias derived from the labelling efficiency, half of the samples of each condition were labeled with Cy3 dye and the other half with Cy5 dye. Cy2 dye was used to label the internal standard sample. The internal standard was formed by mixing aliquots of all individual samples included in the experiment. Two samples from a different replicate (Cy3 and Cy5) and an aliquot of the internal standard pool (Cy2) were separated by 2-DE in each of the 6 gels. A scheme with the experimental design is included as Supporting information Table S1.

The profiling of cytoplasmic proteins showed an average of 1840 spots detected in the three conditions with 1490 spots matched. This is because a maximum number of spots are detected in DIA module from DeCyder and some of them are artifacts. Gel images were analysed and independence pair comparison was done. Differential abundance ratios with a 1.5 fold or more change in abundance ($p < 0.05$) were observed for 365 spots (Fig 2). One hundred seventy-five spots differed in abundance between biofilms and yeast cells with 93 spots more abundant in biofilms and 82 spots less abundant (Table 1). The comparison between biofilms and hyphae revealed 218 differentially abundant spots with 125 more abundant in biofilms than in hyphae. Comparison of planktonic organisms showed 276 differentially abundant proteins with 120 of them more abundant in hyphae. Twenty-three

proteins were not detected in one or more conditions. Fifteen proteins were not found in yeast cell extracts. Three were found only in yeast cell extracts. Five protein species were absent in hyphae and one was only found in hyphae.

We also examined the protein abundance in the non-covalently attached subproteome of the cell surface. The result of the proteomics profiling showed an average of 870 spots detected in the different conditions with 580 spots matched. As with cytoplasmic protein spots, differential abundance was determined and 108 spots identified (Fig 3). Seventy spots showed change in abundance in biofilms versus yeast cells (Table 1). Of these, 38 were over expressed while 32 were under expressed in biofilms. When biofilms and hyphae were compared, 51 spots had differential expression ratios with 14 more abundant in biofilms. Comparison of hyphae and yeast cells showed a change in 40 spots with 27 more abundant on hyphae surfaces. Four protein spots were absent in the surface extract of biofilms.

3.3 Protein identification

A total of 114 protein spots were excised from the cytoplasmic protein gels and 80 protein spots were excised from the surface protein gels. The excised protein was subjected to trypsin digestion and peptide fragments were analyzed by mass spectrometry (MALDI-TOF/TOF). Data from Mascot identifications are found in Supporting information Tables S2 and S3.

The 114 spots identified from the cytoplasmic experiment represented 73 unique proteins. Several of the proteins had multiple species. The numbers on protein species in Fig 2 correspond with the numbers in the Table 2 that summarizes the identified proteins with the average ratio and statistical data from each comparison. These proteins were classified according to their biological function.

Of the 80 proteins identified in the surface experiment, 25 were unique proteins. Table 3 summarizes the total proteins identified. As was observed in the cytoplasm, many of the proteins in the surface subproteome had multiple species. Different protein species numbers in Fig 3 correspond with numbers in Table 3. Some of the proteins identified are involved in metabolism and energy, (e.g., Tpi1, Lys9, Ino1), protein fate (e.g., Ssb1, Ssa4), cell rescue, defense and virulence (Sod1, Tsa1), and biogenesis of cellular component (Tpm2). Some of the proteins were encoded by genes whose functions have not yet been identified, although some have orthologs in *Saccharomyces cerevisiae*. A number of the proteins are present in multiple species such as Fba1, Met6, and Cof1. Pdc11 was particularly notable as 11 species were observed to have differential abundance and an artificial origin of some of them can not be excluded.

3.4. Multivariate analysis

To look for patterns in abundance, multivariate methods were applied to the data from DIGE experiment for cytoplasmic proteins. The 365 protein spots that had a 1.5 fold or greater variation ($p \leq 0.05$) were used for analysis. The PCA reduces the dimensionality of the multidimensional analysis to display the two principal components that distinguish between two largest sources of variation within the dataset. The data sets from the four replicates were very tightly grouped with first component (PC1) showing a clear separation of the yeast spot maps in the upper left quadrant and the second component (PC2) differentiating the hyphal and biofilm spot maps (Fig 4A).

Hierarchical clustering analysis (HCA), based on similarities of abundance patterns in the spot maps, reinforced the PCA view (Fig 4B). When HCA was applied to abundance profile for each analysis, the replicates of biofilms clustered together as did those from hyphae, and yeast cells (Supporting information Fig S2). The clustering showed that protein abundance

in sessile cells some times was similar to either yeast cells or hyphae and sometimes differed from both. The values displayed in an expression matrix (heat maps) are using a standardized log abundance scale ranging from −1 (green) to +1 (red).

Another statistical tool, K-means clustering, showed more clearly the abundance relationship with growth condition (Fig 4C). K-mean cluster analysis was used to place the protein spots with differing abundance for yeast cells, hyphae and biofilms into 10 clusters. The protein spots in each cluster are listed in Supporting information table S4. In clusters 1, 3, 4, 5, 8 and 10 protein abundance in biofilms differed from both yeast cells and hyphae. Clusters 3 and 5 have the protein spots with similar abundance in planktonic conditions but different abundance in biofilms. In clusters 2, 6, 7 and 9 the abundance in biofilms was similar to one planktonic condition but differed from the other.

The same multivariable analysis was done with the 108 spot proteins subset from the cell surface analysis, (≥ 1.5 fold variation, $p<0.05$). In the PCA analysis, PC1 showed a clear separation of the spot maps loaded where the biofilms spot maps were in the left upper quadrant (Fig 5A). PC2 was able to distinguish between hyphal and yeast cell spot maps. PCA distinguished the three cell growth conditions for both cytoplasmic proteins and cell surface proteins, although the quadrants in which each form was found differed with the protein source. This result indicated that yeast cell cytoplasmic profiling is more different than hyphae and biofilms. Otherwise, biofilms were more different than yeast cells and hyphae if we focus on surface profiling.

This grouping assignment was reiterated in the HCA. When HCA was applied to abundance profile for each analysis, the replicates of biofilms clustered together as did those from hyphae and yeast (Supporting information Fig S3). The application of HCA to the three groups is shown on the right in Fig 5B. The biofilms group clustered separately from the other two groups. In the top, proteins with similar profiling of relative expression were clustered.

K-means clustering was also applied to analysis of surface proteins and showed more clearly the abundance relationship with growth condition. The proteins with differing abundance for yeast cells, hyphae and biofilms were placed into 10 clusters. The proteins in each cluster are listed in Supporting information table S5. The abundance of protein spots in biofilms differed from both yeast cells and hyphae in clusters 2, 3, 4, 6, 8 9 and 10. Clusters 2, 3 and 8 included spot proteins that were similar between planktonic cells but differed from biofilms. The abundance in biofilms was similar to one planktonic condition but differed from the other in clusters 5 and 1. Two of the proteins in cluster 8 (spots 749 and 385 Table 3) and in cluster 10 (spots 357 and 358 Table 3) were essentially absent in biofilms. Species of the same protein did not necessarily cluster together. For example, eleven of the species of Pdc11p were least abundant in biofilms. However, for species 11 the abundance was similar in yeast cells and hyphae and only a little less in biofilms (Fig 5, Supporting Table S5). For other species 1, 2, 3, and 7 the abundance was much less than the other two. For species 4 and 5 the abundance in hyphae was less than yeast cells and much less in biofilms On the other hand, spot proteins encoded by IPF8762 were clustered together because they have the same abundance in yeast and hyphae and more abundance in biofilms.

3.5. Functional classification of differentially abundant identified cytoplasmic proteins

Before the biological processes associated with the differentially abundant proteins were analyzed, the relative abundance of proteins with isoforms was determined. All isoforms were assumed to be functional and volumes of the isoforms were added to obtain a single relative volume for the abundance of the protein (Supporting information Table S6). The processes, functions and cellular component significantly affected by or associated with the

differentially abundant proteins were analyzed using the Gene Ontology (GO) Term Finder (1) at CGD (2) and presented in Supporting information Table S7. Of the 80 GO IDs that were associated with differentially abundant proteins in the comparison between biofilms and hyphae, 53 of the terms were associated with proteins more abundant in biofilms. In the comparison of biofilms with yeast cells none of the processes were associated with proteins more abundant in biofilms, although there were 28 processes in which proteins were split between both more abundant in biofilms and less abundant in biofilms. In comparison between hyphae and yeast cells, 67 of the 82 significantly affected processes were associated with proteins less abundant in hyphae and only 10 with proteins more abundant in hyphae. Some of the processes and functions are summarized in Table 4. In addition to metabolic pathways, processes associated with interaction between the host and the organism, interaction with other organisms, response to stimulus and protein folding and refolding were among the processes associated with differentially abundant proteins. A variety of catalytic activities such as ligase activity, oxidoreductase activity, protein binding and unfolded protein binding were among the functions that were impacted by the differentially abundant proteins. Among the cellular components not surprisingly were the cytosol and mitochondrion (Supporting information Table S7). Also proteins were associated with the cell surface as some of these proteins have a dual distribution as observed and discussed elsewhere in this report.

Several of the biological processes were associated with carbohydrate metabolism. Fig 6 shows that enzyme abundance in the glycolytic and gluconeogenesis pathway was altered in the various growth conditions. The identified proteins, Fba1, Tpi1, Tdh3, Pgk1, Gpm1 and Eno1 function in both glycolysis and gluconeogenesis. Cdc19 and Pfk1 that function in glycolysis were the only proteins among those identified that function in only one pathway and both were more abundant in planktonic yeast cells. With the exception of Tdh3, the proteins that function in both pathways were most abundant in yeast cells and least abundant in hyphae with biofilms also being reduced compared to planktonic yeast cell conditions. Some proteins that functioned in pentose metabolism, Gre3, Gnd1, Xyl2, and Tkl1, were also differentially abundant as was Hxk2. These proteins were least abundant in hyphae and generally most abundant in yeast cells.

Proteins associated with the TCA (tricarboxylic acid) cycle and aerobic metabolisms were also altered. Two of the identified proteins, Cit1 and Kgd1 were more abundant in planktonic yeast cells, 1 protein, Aco1, was more abundant in hyphae while the other proteins, Sdh12, Osm1, and Mdh1 were more abundant in hyphae or biofilms. The relative abundance relationship of the two enzymes that use isocitrate in the TCA cycle, Kgd1, and in the glyoxylate cycle, Icl1 differed with Kgd1 most abundant in yeast cells and least abundant in biofilms and Icl1 most abundant in biofilms and least abundant in planktonic yeast cells. Compared to planktonic yeast cells Kgd1 was about 40% less abundant in biofilms. The synthesis and degradation of many compounds leave or enter the TCA cycle and the proteins for the synthesis of aspartate and asparagine were differentially abundant. However, the abundance relationships were not the same for Aat1 and Asn1/2. Whether this represents a differential need for one of the amino acids is not known.

A switch from aerobic to fermentation would shunt pyruvate to acetaldehyde. Pdc11 was more abundant in planktonic yeast cells than in biofilms or hyphae. The enzymes for metabolism of acetaldehyde to either ethanol, Adh1, or acetate, Ald5, were most abundant in biofilms. However, in both cases there are multiple enzymes that carry out the reactions and thus the potential overall flux through these reactions may be less affected contribution of Adh1 is not known. Two enzymes that catalyze the interconversion between acetate and acetyl-CoA, Acs2 and Ach1, also differed in abundance among planktonic organisms and biofilms. Acs2 was most abundant in hyphae and Ach1 was least abundant in hyphae. This

would suggest that the interconversion favors acetate in hyphae. The abundance in yeast cells and biofilms did not follow the inverse order between Acs2 and Ach1.

Three differentially abundant proteins are involved in one carbon metabolism donated by tetrahydrofolate polyglutamates ($H_4PteGlu_n$) (Figure 7). One carbon metabolism is important in the formation of purines, thymidylate, serine, methionine, formylmethionyl-tRNA and here are serine hydroxymethylases in both the cytoplasm, Shm2, and mitochondrion, Shm1, that catalyze the interconversion of serine and glycine. Cytoplasmic Shm2 was more abundant in hyphae and least abundant in yeast cells. Two other proteins Mis11 and Orf19.7534 are mitochondrial proteins predicted to have several activities and to function as a formate-tetrahydrofolate ligase, methenyltetrahydrofoate cyclhydrolase or methylenetetrahydrofolate dehydrogenase. These proteins were most abundant in hyphae and least abundant in yeast cells. Met6 which transfers a 1 carbon unit from N^5-formyl-tetrahydrofolate to homocysteine results in the formation of methionine. This protein was similar in biofilms and yeast cells and least abundant in hyphae.

C. albicans has 9 proteins annotated as Hsp proteins and 4 of these, Hsp60, Hsp70, Hsp78 and Hsp104 were among the differentially abundant proteins (Table 2). Also the other 4 proteins of the Hsp70 class, Ssa2, Ssb1, Ssc1 and Msi3 were differentially abundant. These proteins are involved in protein folding and refolding. Several of these proteins Hsp60, Ssa2, Ssc1 and Msi3 were least abundant in biofilms and Hsp78 and Hsp104 were of intermediate abundance. Also responsive to stress and temperature and heat were Hsp70, Hsp78 and Hsp104. Other proteins like Sod2p, Tsa1p and Adh1p involved in protection against oxidative stress are more abundant in biofilms and yeast cells than in hyphae.

4. Discussion

Although *C. albicans* participates in biofilm formation as plaque and on mucosal surfaces and medical prostheses and devices, biofilms have been studied *in vitro* for little more than a decade. In addition to the presence of extracellular matrix, another microscopic observation of biofilms is the high cell density (Fig 1). Within a biofilm community the environment may not be homogenous and organisms may respond to the local environment as well as the biofilm structure. We have shown that in biofilms a few yeast cells found on the bottom layer express two proteins associated with stationary phase [40]. Changes in gene expression in early and developed biofilms [8,9,11] also suggest that the protein profile of planktonic and sessile organisms will differ. We have utilized biological replicates and DIGE methodology with internal standard to compare proteins from yeast cells, hyphae, and biofilm organisms. This is the first analysis of cytoplasmic protein abundance that compare biofilms with two planktonic morphologies. One proteomic comparison of biofilms and planktonic cytoplasmic proteins found 20 differences by comparing silver stained protein densities on 2DE gels [15]. In this study a more sensitive detection method has been utilized in addition to the comparison between biofilms and both planktonic yeast cells and hyphae. Protein abundance of the cell surface has been addressed in three limited studies that have examined the profile of non-glucan attached surface proteins obtained from biofilm organisms [27,29,31]. In this study we have expanded the analysis of surface proteins to include planktonic hyphae and used a more robust method with replicates and internal standards to assess the relative abundance of this group of surface proteins.

Overall the abundance profiles for both cytoplasmic and surface extracts were more similar than different among the conditions (Table 1). For about 25% of the cytoplasmic and 19% of surface spots there were differences in abundance among the conditions with greater or lesser abundance found in each comparison between biofilms and planktonic cells and between planktonic yeast cells and hyphae. Twenty-three cytoplasmic protein spots were

essentially absent in one or two conditions (Table 2). There were more differences between biofilms and hyphae (219) than between biofilms and yeast cells (175) (Table 1). While this study was undertaken to compare biofilms with planktonic organisms, analysis of both planktonic yeast cells and hyphae allowed a comparison between planktonic growth forms. The largest number of differences in cytoplasmic proteins (275) was found between planktonic growth forms.

The predominance of non-differentially expressed surface proteins with most spots consistently observed in each condition is in agreement with our previous suggestion of similar profiles of biofilms, yeast and hyphal organisms in a 1D analysis. It also concurs with the analysis of Thomas et al [29] for biofilms and planktonic yeast cells who observed about 300 spots in a 2-DE analysis. In the present study the abundance of 580 spots was analyzed (Fig3) which is a larger number than previously reported [27,29]. There were differences in abundance for 105 spots between conditions with some proteins increased in abundance and some decreased in abundance between the various forms (Table 1). Four proteins were essentially absent from the biofilm surface (Table 3). The largest number of differences was between biofilms and yeast cells (70) followed by biofilms and hyphae (51) (Table 1). Contrary to the observation with cytoplasmic proteins, the least difference for surface proteins was between planktonic yeast cells and hyphae (40 protein spots) with greater abundance (27 protein spots) on hyphal surfaces. A similar finding of more proteins with increased abundance on hyphal surfaces compared to the yeast surface was also observed with proteins obtained by a different extraction procedure from isolated cell walls [26].

When the abundance patterns for the protein species of both cytoplasmic and surface extracts were analyzed by PCA (Fig 4A, Fig 5A) the conditions differed from each other and the replicates were tightly clustered. The HCA (Fig 4B, Fig 5B, Fig S2 and Fig S3) analysis shows more graphically that each of the conditions had a unique profile of protein abundance. The additional multivariate analysis, K-means clustering, highlighted the abundance relationships among the species and conditions (Fig 4C and Fig 5C). The clusters showed that both in the cytoplasm and on the cell surface that relationships differed both between conditions and in the extent of the differences.

When 114 differentially abundant protein spots were identified from cytoplasmic extracts (Table 2 and S2) and 80 from surface extracts (Table 3 and S3) some spots were species of the same protein. From cytoplasmic extracts, 41 proteins were identified once while the other proteins were represented by 2 or more species with 6 protein species identified as Pdc11p. In some cases species of the same protein clustered in the same group and in other cases species of the same protein clustered in different groups (Supporting information Table S4). The analysis of Seneviratne *et al* [15] reported that of the 20 differentially abundant spots, 4 were isoforms of Ahp1. Among the 20 differentially abundant spots were six proteins, Bmh1, Sti1, Tal1, Mcr1, Egd1 and Cyp1 not identified in the study reported here. However, as less than one-third of the 365 differentially abundant proteins detected in this study were identified, these six proteins may be in the unidentified group.

In the surface extracts, spots that differed in abundance represented 25 unique proteins. Multiple species of a protein have been observed in several analyses of the non-glucan attached subproteome of the yeast cell wall by us and others [21,26–29,41]. Since not all protein spots were identified in this or other studies, a complete comparison between differentially abundant proteins is not possible. Among those proteins with reported multiple species are Pdc11, Met6, Fba1, Adh1, and Eno1 (Table 3, [27,29]). Interestingly, there were differences in the abundance of species among surface proteins of biofilms, yeast cells and hyphae (Table 3, Fig 3). For example, like Pdc11 species in the cytoplasm that were found

in different clusters, the species of this protein were found in clusters 2, 7, 8, and 10 (Supporting information Table S5).

The differentially abundant cytoplasmic proteins were analyzed for the processes, functions, and cellular component associated with them (Table 4 and Supporting information Table S7). Many of the proteins were associated with carbohydrate metabolism. When glucose is abundant *C. albicans* prefers to grow aerobically and metabolize available glucose by respiration rather than fermentation [42]. In YNB medium with 50 mM glucose used here we have shown that maximum culture density is reached earlier than in YPD medium with 2% glucose [40,43]. We showed that planktonic yeast cells were grown in YPD medium containing 2% glucose that the glucose is exhausted by about 22 hrs with ethanol appearing in the medium at about 19 hr and disappearing by 45 h [43]. This supported the preference for respiration when glucose is present. After both ethanol and glucose were utilized and there is a decrease in expression of some but not all genes encoding glycolytic proteins while genes such as *MAE1* encoding an enzyme of gluconeogenesis is increased [43]. The genes with reduced expression include *ENO1, FBA1, CDC19, PFK1*. Together these observations predict that the growth medium of planktonic yeast cells should be depleted of glucose while the medium of biofilms which is replenished and that of hyphae is not. Secondly, the observations predict that the Eno1, Fba1, Cdc19 and Pfk1 should be less abundant in the planktonic yeast cells than in hyphae and biofilms. However, this prediction was not borne out by the analysis shown in Fig 6. With the exception of Tdh3, the proteins that function in both pathways were most abundant in yeast cells and least abundant in hyphae with biofilms also being reduced compared to planktonic yeast cell conditions. Another prediction that organisms growing in the presence of glucose would have a similar abundance profile was also not observed. Biofilms and hyphae did not share an abundance profile despite the presence of glucose. These observations suggest that the relationship between transcription and protein abundance for at least some of the proteins in glycolysis and gluconeogenesis may be influenced by factors other than glucose availability. A limitation of proteomic analysis is that the abundance of enzymes is used to infer the flux through enzymes and pathways. With enzymes functioning in both glycolysis and gluconeogensis and aerobic and fermentative metabolism, the direction of flux through these pathways is unclear. To partially address questions of metabolic changes in response to these changes in conditions, we are undertaking a study to determine the abundance of various metabolites.

Also showing alterations in protein abundance were proteins associated with the TCA cycle and aerobic metabolism. These changes in abundance were consistent with increased use of the glyoxylate cycle in biofilms. However, Mdh1 and Mdh1-1 but not Mdh1-3 which is part of the glyoxylate cycle were the proteins using malate that were altered. Interestingly for most of these proteins there is also greater abundance in planktonic hyphae compared to yeast cells. The increased use of the glyoxylate cycle by organisms ingested into the nutrient poor macrophage was first shown by transcriptional profiling [44,45]. More recently we showed by proteomic analysis that proteins associated with use of the glyoxylate cycle were more abundant in phagocytosed *C. albicans* yeast cells than uningested cells [13]. If the glyoxylate cycle is used more in biofilms than planktonic organisms despite the presence of glucose, this may suggest that within the biofilms there are niches that are nutrient poor and thus respond with use of the glyoxylate cycle.

Under conditions where YNB medium is not replenished during biofilm formation, Adh1 decreases in a fraction enriched for cell surface protein compared to planktonic cell abundance [27]. In this study with YNB replenished, the abundance of Adh1 on the cell surface was greater in biofilms than hyphae but similar in biofilms and yeast cells (Table 3). Whether this difference relates to medium replenishment or differences in studies is not

clear. In the cytoplasm, we observed that Adh1 was greater in biofilms than the planktonic growth conditions (Fig 6,Supporting information Table S7). So on both the surface and in the cytoplasm Adh1 abundance was greatest in biofilms. Also involved in 2-carbon metabolism were the differentially abundant Acs1 and Ach2. There are two ACS genes. While loss of *ACS1* has no phenotype, loss of *ACS2* results in loss of viability on most carbon sources including ethanol and glucose [46]. It is this apparently more important protein that responds to the changes in conditions. One of the sources of acetyl-CoA from a non-fermentable carbon source is β-oxidation. In YPD after three days there is increased expression of genes whose products function in β-oxidation [43]. In this study only one protein, among those identified was involved with β-oxidation, Pxp2, and that protein was part of a spot containing another protein and thus its relative abundance could not be determined.

Single carbon metabolism though folate compounds was also a process affected by differentially abundant proteins (Fig 7). The proteins associated with interconversions were more abundant in hyphae. These observations suggest that single carbon groups are needed in greater quantity in hyphae than in the other two conditions. Transcriptional analysis of biofilms reported that Orf19.7534 was increased in biofilms compared to the planktonic conditions used [8,11] as was *MET6* [8]. The difference between the transcriptional analysis and the proteomic analysis here may represent a difference in conditions or a difference in transcription and protein abundance. Increased expression of *MET6* has been reported in biofilms compared to planktonic organisms [8]. In this case the proteomic analysis (Fig 7) also found Met6 to be more abundant in biofilms than hyphae but equally abundant with yeast cells.

Other than metabolism, proteins related to response to stimulus, response to stress, response to temperature stimulus or heat were noted. Proteins associated with these classes were most abundant in yeast cells. It is important to note that Met6, Ahp1, Lsp1, Sod2, Gre3, Hsp70, Gnd1, Hsp104, Tps1, Tsa1, Hsp78, Hxk2, orf19.6757 were more abundant in biofilms than in hyphae (Table 2 and Supporting information Table 6). Three of these proteins Ahp1 (putative alkyl hydroperoxide reductase), Tsa1 (Thiol-specific antioxidant like protein) and Lsp1 (possible sphingolipid chain-base sensory protein that possesses an echinocandin binding domain) have been recently seen over-expressed in biofilms (Seneviratne *et al.,* 2008). In addition to Ahp1 and Tsa1 we have identified Sod2 (managanase-superoxide dismutase) another protein with antioxidant properties. In addition two of these proteins (Ahp1 1nd Tsa1) were identified in surface extracts where they were highly expressed in biofilms (see below).

Transcriptional profiling on biofilms and planktonic organisms in various conditions shows differences in gene expression [8,9,11]. Among the functional categories that were differentially affected were amino acid metabolism with particular note for involving sulfur metabolism, nucleotide metabolism, energy, carbohydrate, C-compound metabolism, lipid metabolism, transcription, and protein synthesis. Perhaps due to differences in conditions and time of biofilm development, when comparing biofilms and planktonic organisms within these functional categories, many genes were found in only one study and only a few genes in all three studies. The conditions for this study were not identical to the other studies. However, many differentially abundant proteins (Table 4 and Table 2,) were involved in the same processes of amino acid metabolism, energy, carbohydrate, and C-compound metabolism. Although proteins with differential abundance were found that are involved in other processes identified by transcriptional analysis, they were not sufficient in number to be significant. It is perhaps not surprising given the differences in the transcriptional studies, that comparison of the differentially abundant proteins observed in this study with differentially expressed genes that there was limited agreement beyond the

processes. Post-transcriptional regulation may also affect the relationship between expression and protein abundance.

The proteins that were identified on the surface (Table 3) belong to a group of proteins frequently termed moonlighting because they were first identified in other cellular compartments with functions that are likely to be different than in the cell wall. This class of proteins and most of those identified in this study have been reported to be present on the surface of yeast cells and bacteria in numerous studies [20]. These proteins lack signal sequences for export by the endoplasmic reticulum Golgi pathway [20]. However, in addition to presence in extracts such as used in this study, proteins of this class have been demonstrated by non-disruptive and genetic methods to be present within and on the cell surface [25,47–53]. Among those found in this study, several of them are associated with carbohydrate metabolism, e.g. Pdc11, Eno1, Fba1, Pgk1, and Tpi1 (Table 3). Others are chaperones, e.g. Hsp70, Ssb1, and several involved with core and oxidative stress, e.g. Tsa1, Ahp1, Grp2 (similar to *S. cerevisiae* methylglyoxal reductase), Tpm2p and Sod1p. Surface activities for planktonic cells have been described for some of these proteins. For example, Adh1, Tsa1, and Pgm1 are major contributors to plasminogen binding while Fba1 and Pgkp are less important [54]. Although identified in the cytoplasm but not among the proteins identified as differentially abundant surface proteins, Gpm1p also binds plasminogen and deletion of *GPM1* reduces surface plasminogen binding [55]. Hsp70 has been identified as the receptor for human peptides histatin 5 and Beta-defensins [56,57]. Cell surface proteins such as Eno1, Pgk1, Met6 are also recognized by human or animal sera [21,22],

It is important to note that three proteins with antioxidant properties were highly expressed in biofilms (Table 3). These proteins included Sod1 (Cu,Zn superoxide dismutase) and 3 protein species of Tsa1 and 4 protein species of Ahp1 also found in the cytoplasm. Functional studies with these proteins will clarify their role in biofilm protection against oxidative host defenses.

This study also suggested that the f biofilms and planktonic organisms have similar proteins in the cytosol and display similar proteins on their cell surfaces with some differences in abundance among proteins. These differences in abundance generated a unique profile among the growth forms. The maintenance of a similar surface may have advantages for both the microbe and the host. Bacteria in biofilms are less susceptible to host defenses than planktonic organisms [58]. Initial studies report that *C. albicans* is influenced to form biofilms by human peripheral blood mononuclear cells and that fungal cells in biofilms are not phagocytosed [59]. Organisms shed from a biofilm are more vulnerable. Thus the presence of major antigenic proteins on the surface of cells that may be shed or breakaway from biofilms would expose the surface to host antibodies as well as cell mediated immune response. On the other hand, the microbe retains a surface that can bind to host ligands. While the covalent structure of the cell wall has been substantially unraveled [18,60], the contribution of the bulk of cell wall proteins that are non-glucan attached is still relatively unexplored.

Finally the knowledge of differential protein expression of *Candida albicans* biofilms together with the functional analysis of some important proteins involved in glucose and folate metabolism, oxidative and stress response and host response will be very useful in preventing *Candida albicans* biofilm development. Studies to unravel the functional relevance of some of these proteins in biofilm formation are underway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

BVA	Biological Variance Analysis
Cy	cyanine dyes
DIA	differential in-gel analysis
EDA	Extended Data Analysis
HCA	Hierarchical Clustering Analysis
PCA	Principal Component Analysis
SEM	Scanning electron microscopy
YNB	Yeast Nitrogen Base with amino acids

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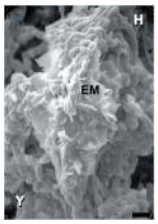

Fig. 1. SEM of biofilm

Biofilm formed on an acrylic strip with medium flow for 48 h was imaged. Yeast cells (Y), hyphae (H) and extracellular matrix (EM) were visible. The image was obtained at 1500×. The bar marker is 5 μm.

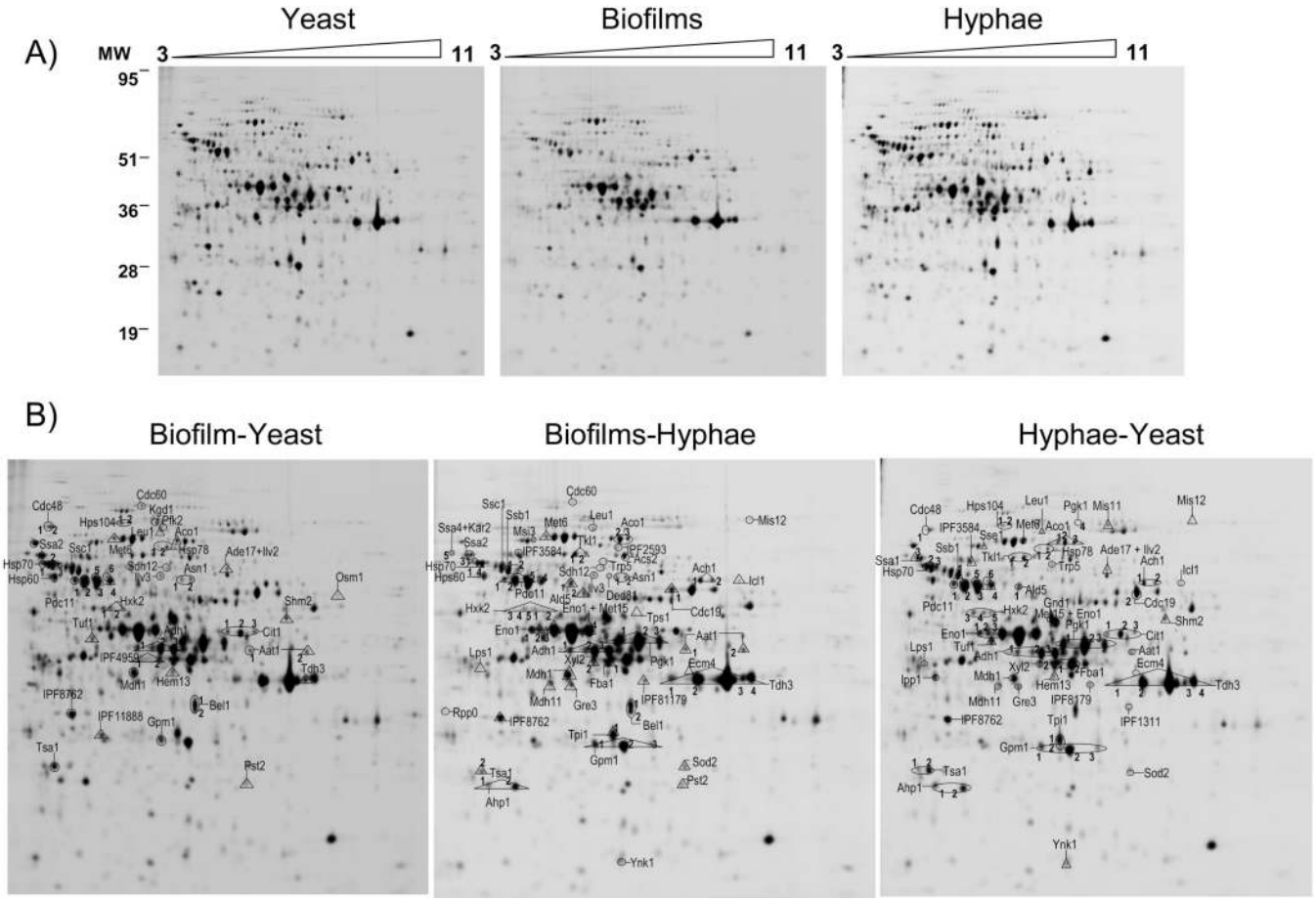

Fig 2. 2DE-DIGE analysis indicated that there are proteins differentially expressed in cytoplasmic extracts

A) 2-DE maps of *C. albicans* cytoplasmic proteins from yeast cells, hyphae and biofilms are shown. B) Each image shows the material pooled from each sample and used as the internal standard for all gels. Spots marked with triangles and circles correspond to over and under expressed proteins with respect to the reference condition.

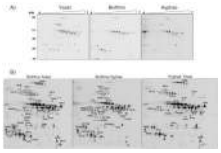

Fig 3. 2D-DIGE analysis indicates that there are proteins differentially expressed on biofilm and planktonic organism surfaces

A) 2-DE maps of *C. albicans* surface proteins from yeast cells, hyphae and biofilms are shown. B) Each image shows the material pooled from each sample and used as the internal standard for all gels. Spots **marked** with triangles or circles correspond to over or under expressed proteins respect to the reference condition.

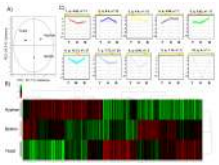

Fig 4. Unsupervised multivariate analysis of DIGE results from cytoplasmic extracts

A) PCA shows the clustering of the 12 individual Cy3- and Cy5-labeled DIGE spots maps (red is the biofilms group, green is the hyphal group and black is the yeast cell group) in three groups differentiated by two principle component. Each data point in the PCA plots describes the global expression values for the subset of proteins whose ratios varied 1.5 fold or more (ANOVA $p < 0.05$). B) HCA settings are Pearson distance measure and average linkage. Dendrogram of experimental groups clustering is showed in the right and of individual proteins is showed on the top with a relative expression values displayed as heat map. C) K-means clustering was made using Spotfire software. The total score was 53.28. For each cluster quality measure (q) and the number of spot proteins in the cluster are displayed

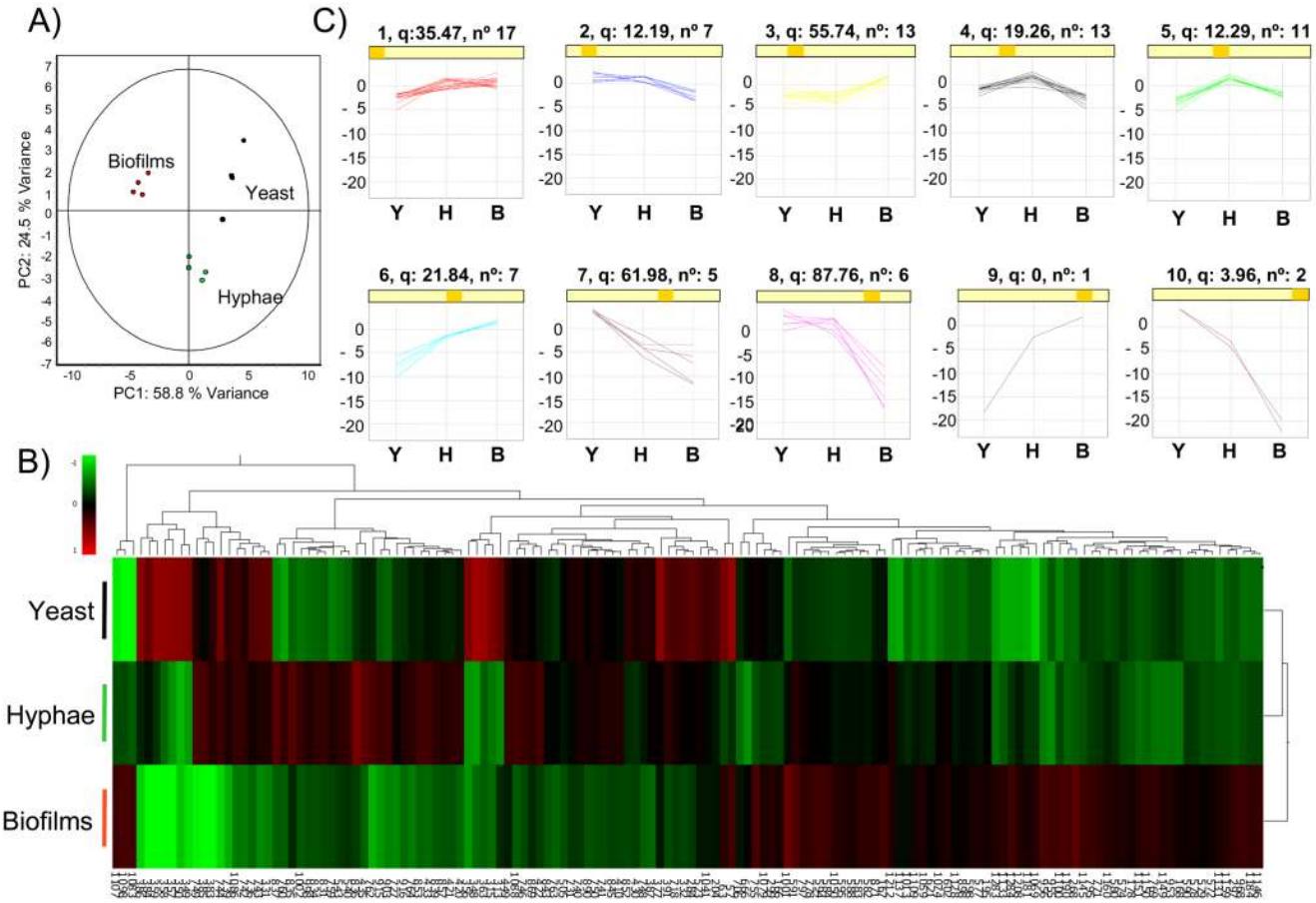

Fig 5. Unsupervised multivariate analysis of DIGE results from surface extracts

A) PCA shows the clustering of the 12 individual Cy3- and Cy5-labeled DIGE spots maps (red is the biofilms group, green is the hyphal group and black is the yeast cell group) in three groups differentiated by two principle component. Each data point in the PCA plots describes the global expression values for the subset of proteins whose ratios varied 1.5 fold or more (ANOVA $p < 0.05$). B) HCA settings are Pearson distance measure and average linkage. Dendrogram of experimental groups clustering is showed in the right and of individual proteins is showed on the top with a relative expression values displayed as heat map. C) K-means clustering was made using Spotfire software. The total score is 315. For each cluster quality measure (q) and the number of spot proteins in the cluster are displayed.

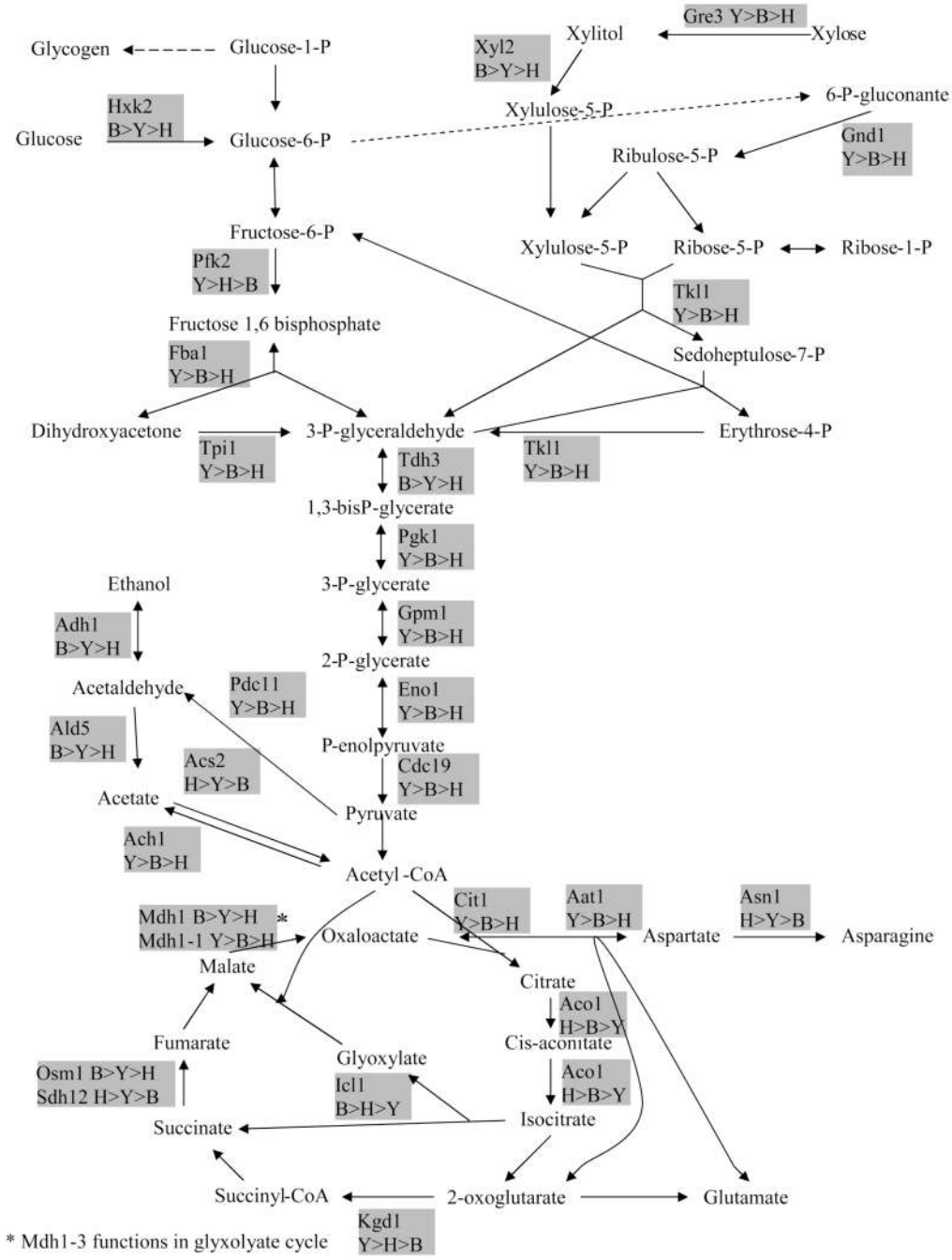

Fig 6. Hexose fermentative and aerobic metabolism

Pathways for glucose metabolism, glycolysis, gluconeogenesis, fermentation and aerobic metabolism are shown. Only the proteins that were differently abundant are shown although additional enzymes may also catalyzed the reaction or additional proteins may be required for the reaction. Below or beside each protein the relative abundance of the protein in the three growth conditions is indicated by symbols B (biofilms), H (hyphae), Y (yeast cells). The location of reactions, cytoplasm or mitochondrion is not indicated. The pathways were based on pathways available through CGD [39].

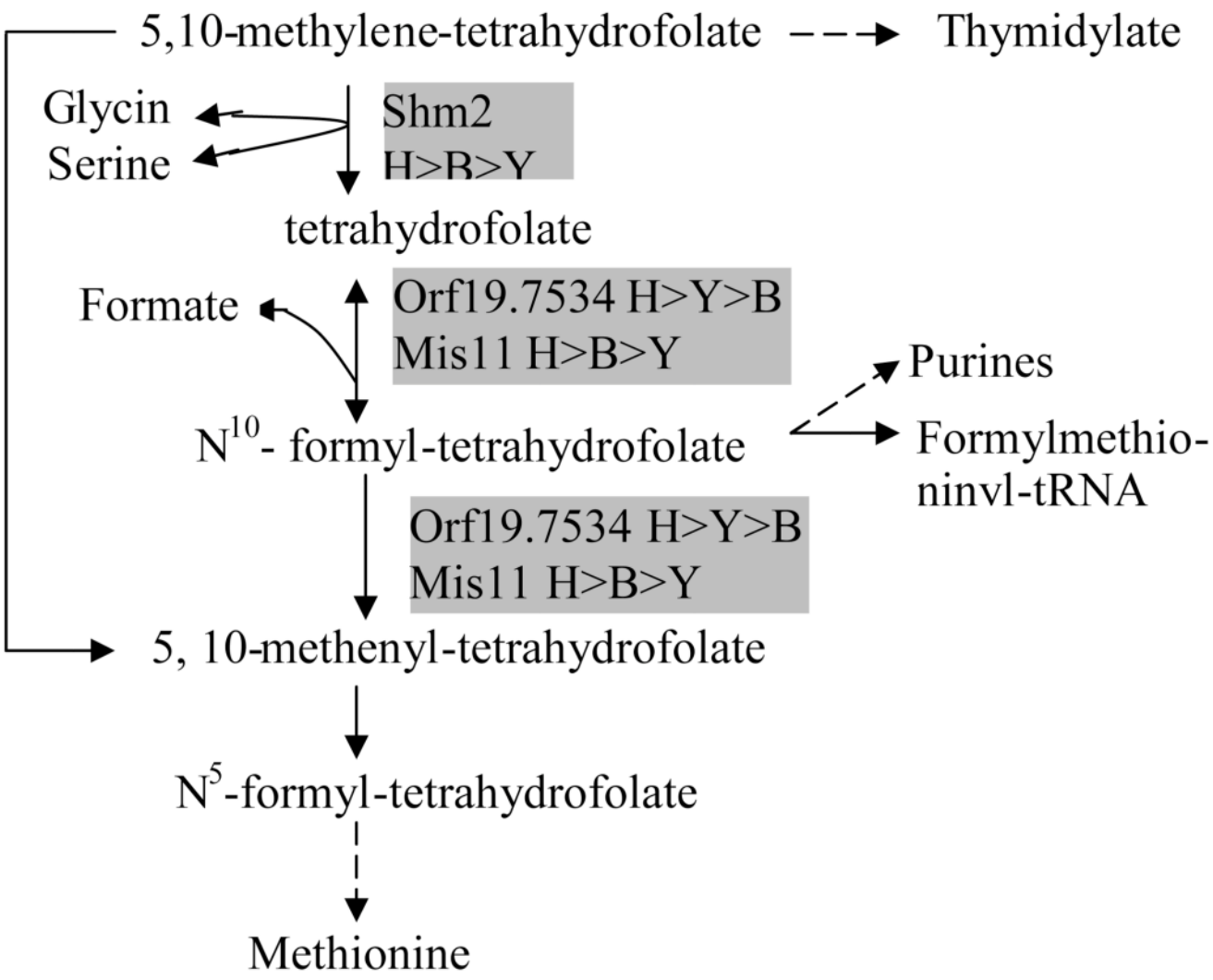

Figure 7. Folate and one carbon metabolism

Partial pathways for folate and one-carbon metabolism are shown. Only the proteins that were differentially abundant are shown. Below or beside each protein the relative abundance of the proteins in the three growth conditions is indicated by symbols B (biofilms), H (hyphae), Y (yeast cells). The location of reactions, cytoplasm or mitochondrion is not indicated. Tetrahydrofolate compounds may also be found and participate in reactions as polyglutamates and this is not indicated. The pathways were based on pathways available through CGD [39,61].

Table 1

Summary of proteins of interest in the comparison Biofilms vs Yeast cells , Biofilms vs Hyphae and Hyphae vs Yeast cells in cytoplasmic and cell surface extracts.

EXTRACT	COMPARISON	TOTAL NUMBER	OVER EXPRESSED	UNDER EXPRESSED	IDENTIFIED PROTEINS
Cytoplasmic	Biofilms-Yeast cells	175	93	82	54
	Biofilms-Hyphae	218	125	93	83
	Hyphae-Yeast cells	276	120	156	83
Cell surface	Biofilms-Yeast cells	70	38	32	53
	Biofilms-Hyphae	51	14	37	43
	Hyphae-Yeast cells	40	27	13	30

Table 2

Functional classifications of proteins of interest in cytoplasmic extract comparison between Biofilms (B) vs Yeast cells (Y), B vs Hyphae (H) and H vs Y. Average ratios, Student T-test p-values and analysis of variants (One way ANOVA) p-values are calculated using Decyder™ software v6.5.

					B vs Y		B vs H		H vs Y		
Master No.	**Protein ID**	**Protein encoded by**	**Gel Isoform**	**Name**	**Av. Ratio**	**T-test**	**Av. Ratio**	**T-test**	**Av. Ratio**	**T-test**	**1- ANOVA**
Metabolic Process											
Amino acid metabolism											
292	CA0653	CaMET6	1	5-methyl tetrahydropteroyltriglutamate-homocysteine methyltra	1,97	0,0074	1,68	0,047	1,17	0,48	0,0009
295	CA0653	CaMET6	2	5-methyl tetrahydropteroyltriglutamate-homocysteine methyltra	−1,34	0,015	1,33	0,011	−1,78	0,00059	0,000012
260	CA5842	CaLEU1		3-isopropylmalate dehydratase	4,91	0,00026	−2	0,0002	9,83	4,10E-05	2,50E-08
486	Mix CA4513	CaADE17		5-aminoimidazole-4-carboxamide ribotide transformylase	1,79	0,017	−1,27	0,0065	2,28	0,0027	0,000034
541	CA3616	CaASN1	1	Asparagine synthetase	−1,62	0,00095	−1,74	0,00011	1,08	0,14	5,4E-07
542	CA3616	CaASN1	2	Asparagine synthetase	−1,55	0,00041	−1,54	0,00012	−1,01	0,74	4E-07
436	CA1580	CaTRP5		Tryptophan synthase	−1,17	0,025	−2,14	5,80E- 05	1,82	6,90E-05	6,30E-08
523	CA4802	CaILV3		Dihydroxyacid dehydratase	−1,86	0,00033	−2,3	5,60E- 05	1,24	0,0014	4,50E-08
897	Mix CA2565	CaMET15		O-acetylhomoserine O-acetylserine sulphydrylase	−1,19	0,073	2,36	0,00012	−2,81	4,80E-05	1,1E-07
1021	CA2661	CaAAT1	1	Aspartate aminotransferase	−2,8	0,00025	1,76	0,00073	−4,92	1,10E-05	1,60E-08
1027	CA2661	CaAAT1	2	Aspartate aminotransferase	2,41	0,00079	1,83	0,0017	1,31	0,00088	1,4E-06
486	Mix CA0428	CaILV2		acetolactate synthase	1,79	0,017	−1,27	0,0065	2,28	0,0027	0,000034
215	CA2250	CaMIS11		Mitochondrial C1-tetrahydrofolate synthase precursor	1,25	0,069	−1,38	0,012	1,72	0,00093	0,00004
201	CA5869	CaMIS12		Mitochondrial C1-tetrahydrofolate synthase precursor	−1,43	0,017	−2,92	0,00025	2,05	0,0001	4,7E-07
201	CA5869	CaMIS12		Mitochondrial C1-tetrahydrofolate synthase precursor	−1,43	0,017	−2,92	0,00025	2,05	0,0001	4,7E-07
Phosphate metabolism											
1156	CA0870	CaIPP1		Inorganic pyrophosphatase	−1,4	0,002	1,35	0,0074	−1,89	0,00021	0,000002
1804	CA2645	CaYNK1		Nucleoside diphosphate kinase	−1,36	0,022	−2,05	0,00036	1,51	0,00096	2,6E-06
C compound and carbohydrate metabolism											
176	CA1691	CaPGK1	4	Phosphoglycerate kinase	−1,25	0,056	1,26	0,13	−1,57	0,02	0,0011
950	CA1691	CaPGK1	1	Phosphoglycerate kinase	1,1	0,26	1,94	0,0016	−1,76	0,0013	0,000008
958	CA1691	CaPGK1	3	Phosphoglycerate kinase	−1,33	0,053	1,59	0,0031	−2,11	0,0002	6,6E-06

					B vs Y		B vs H		H vs Y		
Master No.	**Protein ID**	**Protein encoded by**	**Gel Isoform**	**Name**	**Av. Ratio**	**T-test**	**Av. Ratio**	**T-test**	**Av. Ratio**	**T-test**	**1- ANOVA**
971	Mix CA1691	CaPGK1	2	Phosphoglycerate kinase + ubiquinol-cytochrome-c reductase	−1,46	0,0045	1,67	0,0012	−2,44	5,50E-05	4E-07
230	CA3112	CaPFK2		6-phosphofructokinase, beta subunit	−1,81	0,0019	−1,29	0,038	−1,4	0,023	0,000058
811	CA0895	CaSHM2		Serine hydroxymethyltransferase precursor; mitochondrial	6,92	4,90E- 05	−1,29	0,0046	8,94	5,60E-06	5,40E-09
868	CA3874	CaENO1	1	Enolase I	−1,12	0,25	2,39	0,00035	−2,68	0,00015	5,6E-07
869	CA3874	CaENO1	2	Enolase I	1,24	0,17	2,25	0,0046	−1,81	0,0054	0,000064
880	CA3874	CaENO1	3	Enolase I	1,07	0,53	2,62	0,00066	−2,44	0,00052	2,2E-06
897	Mix CA3874	CaENO1	4	Enolase I	−1,19	0,073	2,36	0,00012	−2,81	4,80E-05	1,1E-07
988	CA4765	CaADH1[b]	1	Alcohol dehydrogenase	2,33	0,00017	4,91	5,10E- 05	−2,1	0,0002	1,60E-08
997	CA4765	CaADH1	2	Alcohol dehydrogenase	1,77	0,00049	4,67	5,10E- 05	−2,64	1,10E-05	5,80E-09
1004	CA4765	CaADH1	3	Alcohol dehydrogenase	1,77	0,00095	4,93	5,10E- 05	−2,79	1,30E-05	8,40E-09
532	CA2474	CaPDC11 [a,b]	6	Pyruvate decarboxylase	−2,74	0,0056	−1,01	0,7	−2,71	0,0049	0,000042
540	CA2474	CaPDC11[b]	5	Pyruvate decarboxylase	−2,87	0,00031	1,65	0,0052	−4,74	0,00014	2,8E-07
545	CA2474	CaPDC11	1	Pyruvate decarboxylase	−2,83	0,00037	3,08	0,0013	−8,72	6,50E-05	1,6E-07
553	CA2474	CaPDC11	4	Pyruvate decarboxylase	2,97	0,00025	−1,61	0,0024	4,77	1,10E-05	2,60E-08
563	CA2474	CaPDC11	2	Pyruvate decarboxylase	−3,55	6,60E- 05	2,75	0,0018	−9,76	6,50E-05	1,1E-07
576	CA2474	CaPDC11	3	Pyruvate decarboxylase	1,98	0,0024	−1,42	0,016	2,81	0,00032	2,6E-06
627	CA3483	CaCDC19	1	Pyruvate kinase	1,19	0,068	1,68	0,0072	−1,41	0,026	0,00018
634	CA3483	CaCDC19	2	Pyruvate kinase	−1,19	0,1	−3,27	0,0001	2,74	2,10E-05	7,10E-08
654	CA3483	CaCDC19	3	Pyruvate kinase	−1,27	0,0048	1,22	0,0094	−1,55	0,0001	2,2E-06
1074	CA5180	CaFBA1	1	Fructose-bisphosphate aldolase	1,21	0,14	2,02	0,001	−1,67	0,00057	8,2E-06
1079	CA5180	CaFBA1	2	Fructose-bisphosphate aldolase	−1,29	0,005	1,34	0,0031	−1,72	0,00011	1,2E-06
1181	CA5892	CaTDH3	2	Glyceraldehyde-3-phosphate dehydrogenase	1,17	0,2	2,69	0,00054	−2,31	0,00095	2,9E-06
1188	CA5892	CaTDH3	4	Glyceraldehyde-3-phosphate dehydrogenase	1,03	0,85	1,84	0,0086	−1,78	0,0039	0,00011
1198	CA5892	CaTDH3	1	Glyceraldehyde-3-phosphate dehydrogenase	1,4	0,12	2,77	0,0022	−1,98	0,0042	0,000036
1193	CA5892	CaTDH3	3	Glyceraldehyde-3-phosphate dehydrogenase	1,7	0,015	2,79	0,001	−1,64	0,0081	0,000011
1453	CA5950	CaTPI1	1	Triose phosphate isomerase	−1,33	0,04	1,56	0,0024	−2,08	0,00031	5,7E-06
1502	CA5950	CaTPI1	2	Triose phosphate isomerase	−1,36	0,003	1,5	0,0012	−2,04	0,0002	7,6E-07
1486	CA4671	CaGPM1	1	Phosphoglycerate mutase	−1,91	0,0035	2,03	0,00035	−3,87	6,90E-05	2,3E-07
1496	CA4671	CaGPM1	2	Phosphoglycerate mutase	−1,46	0,0048	1,58	0,0011	−2,31	6,90E-05	4,6E-07
1498	CA4671	CaGPM1	3	Phosphoglycerate mutase	−1,12	0,41	1,83	0,0059	−2,05	0,0022	0,000054

					B vs Y		B vs H		H vs Y		
Master No.	**Protein ID**	**Protein encoded by**	**Gel Isoform**	**Name**	**Av. Ratio**	**T-test**	**Av. Ratio**	**T-test**	**Av. Ratio**	**T-test**	**1- ANOVA**
737	CA0127	CaHXK2	3	Hexokinase II	1,25	0,03	2,65	0,0001	−2,11	0,00033	2,8E-07
742	CA0127	CaHXK2	1	Hexokinase II	1,77	0,0014	2,45	0,00023	−1,39	0,0014	4,4E-07
744	CA0127	CaHXK2	4	Hexokinase II	1,04	0,67	2,72	0,00012	−2,62	0,00026	4E-07
745	CA0127	CaHXK2	2	Hexokinase II	1,6	0,0033	2,39	0,00019	−1,5	0,0024	8,8E-07
746	CA0127	CaHXK2	5	Hexokinase II	1,06	0,54	2,49	0,0001	−2,35	0,00014	2,4E-07
1311	CA5940	CaGCY1		aldo/keto reductase	−1,24	0,069	1,45	0,0052	−1,79	0,00055	0,000016
803	CA5239	CaGND1		6-phosphogluconate dehydrogenase	−1,46	0,012	1,23	0,075	−1,79	0,0005	0,000028
804	CA4084	CaTPS1		Trehalose-6-phosphate synthase	1,41	0,0012	1,59	0,00044	−1,13	0,057	2,7E-06
1208	CA1592	CaGRE3		D-xylose reductase	−1,27	0,026	2,31	0,00041	−2,94	5,80E-05	2,3E-07
1055	CA6057	CaXYL2	1	D-xylulose reductase	1,75	0,00055	2,45	0,0001	−1,4	0,0019	1,5E-07
1063	CA6057	CaXYL2	2	D-xylulose reductase	1,75	0,00038	2,95	5,60E- 05	−1,68	0,00045	4,80E-08
384	Mix CA3924	CaTKL1	2	transketolase 1	−1,48	0,0061	1,73	0,00049	−2,57	4,50E-05	2,3E-07
400	CA3924	CaTKL1	1	Transketolase 1	−1,09	0,24	1,54	0,0005	−1,67	0,00021	1,9E-06
412	CA2858	CaACS2		Acetyl-coenzyme-A synthetase	−1,48	0,0017	−1,69	0,00054	1,14	0,011	1,7E-06
575	CA0345	CaACH1	2	Acetyl-coenzyme-A hydrolase	−1,31	0,15	1,72	0,01	−2,25	0,0014	0,000064
577	CA4159	CaALD5	1	Aldehyde dehydrogenase (NAD+)	1,11	0,2	1,8	0,00019	−1,62	0,00052	1,7E-06
578	CA0345	CaACH1	1	Acetyl-coenzyme-A hydrolase	−1,11	0,054	2,68	0,00019	−2,97	0,0001	1,1E-07
633	CA4159	CaALD5	2	Aldehyde dehydrogenase (NAD+)	1,39	0,0024	1,82	0,00012	−1,31	0,0026	6E-07
Lipid, fatty acid-b oxidation, and isoprenoid metabolism											
384	Mix CA1572	CaPOX4		Peroxisomal fatty acyl-CoA oxidase	−1,48	0,0061	1,73	0,00049	−2,57	4,50E-05	2,3E-07
Miscellaneous											
1143	CA0517	CaHEM13		Coproporphyrinogen III oxidase	2,06	0,0011	1,2	0,024	1,71	0,002	0,000004
1655	CA1673	CaPST2		1,4-benzoquinone reductase	1,71	0,0022	1,55	0,00055	1,1	0,23	0,000011
687	CA4570	CaOSM 1		Osm1p osmotic growth protein	1,77	0,012	2,07	0,0028	−1,17	0,037	0,000029
Glyoxylate and tricarboxilic acid cycles											
471	CA2470	CaSDH12		Succinate dehydrogenase	−1,55	0,0012	−1,96	0,00034	1,26	0,0021	6,3E-07
894	CA3909	CaCIT1	1	Citrate synthase; exon 2	−2,14	0,0011	1,35	0,029	−2,88	0,00038	2,2E-06

					B vs Y		B vs H		H vs Y		
Master No.	**Protein ID**	**Protein encoded by**	**Gel Isoform**	**Name**	**Av. Ratio**	**T-test**	**Av. Ratio**	**T-test**	**Av. Ratio**	**T-test**	**1- ANOVA**
895	CA3909	CaCIT1	3	Citrate synthase; exon 2	−3,58	0,00025	1,34	0,01	−4,82	4,10E-05	4,80E-08
905	CA3909	CaCIT1	2	Citrate synthase; exon 2	−4,26	7,70E- 05	1,32	0,0025	−5,61	2,10E-05	7,60E-09
1154	CA5164	CaMDH1		Mitochondrial malate dehydrogenase precursor	−2,16	0,0012	1,65	0,00041	−3,57	5,80E-05	1,5E-07
1191	CA5826	CaMDH11		Malate dehydrogenase	1,22	0,012	1,92	0,00024	−1,57	0,0002	4E-07
580	CA4446	CaICL1		Isocitrate lyase	−1,14	0,43	1,52	0,0021	−1,73	0,0041	0,00013
200	CA3149	CaKGD1		2-oxoglutarate dehydrogenase	−1,71	0,0005	−1,3	0,0072	−1,31	0,0032	0,000003
310	CA3546	CaACO1	1	Aconitate hydratase	1,52	0,00075	−1,41	0,011	2,15	0,00032	2,2E-06
313	CA3546	CaACO1	3	Aconitate hydratase	1,23	0,039	−1,63	0,0015	2,01	0,00084	5,3E-06
320	CA1911	CaMSI3		Heat shock protein of HSP70 family	−1,01	0,91	−1,63	0,0012	1,6	0,0014	0,000018
324	CA3546	CaACO1	2	Aconitate hydratase	1,13	0,14	−1,87	0,00029	2,11	2,10E-05	2E-07
Protein fate											
Protein folding and stabilization											
196	CA5135	CaHSP104	2	Heat shock protein	−2,2	0,00049	1,39	0,011	−3,05	3,20E-05	1,6E-07
207	CA5135	CaHSP104	1	Heat shock protein	−2,03	0,0034	1,34	0,064	−2,72	0,00012	4,3E-06
320	CA1911	CaMSI3		Heat shock protein of HSP70 family	−1,01	0,91	−1,63	0,0012	1,6	0,0014	0,000018
342	CA4684	CaHSP78	1	Heat shock protein of clpb family of ATP-dependent proteases; mitochondrial	−2,33	0,00087	1,47	0,0091	−3,42	2,70E-05	2,3E-07
346	CA4683	CaHSP78	1	Heat shock protein of clpb family of ATP-dependent proteases; mitochondrial	−3,5	0,0001	1,1	0,25	−3,87	4,50E-05	6,30E-08
368	Mix CA1230	CaHPS70	5	cahsp70 mRNA for heat shock	−1,38	0,0005	−1,74	0,0013	1,27	0,028	8,9E-06
387	CA2857	CaSSA2	3	Heat shock protein of HSP70 family	−1,37	0,0015	−2,64	0,00012	1,92	0,00036	1,5E-07
393	CA2857	CaSSA2		Heat shock protein of HSP70 family	−4,64	4,90E- 05	−3,11	5,20E- 05	−1,49	0,00072	5,80E-09
481	CA4474	CaSSC1		Mitochondrial heat shock protein 70-related protein	−2,02	0,00053	−1,59	0,0028	−1,26	0,024	3,9E-06
368	Mix CA0915	CaKAR2		DnaK-type molecular chaperone	−1,38	0,0005	−1,74	0,0013	1,27	0,028	8,9E-06
490	CA3534	CaSSB1	2	Heat shock protein 70	−1,13	0,27	−1,54	0,005	1,36	0,032	0,00032
495	CA3534	CaSSB1	1	Heat shock protein 71	1,02	0,82	−1,77	0,00038	1,8	0,00033	2,2E-06
528	CA1239	CaHSP60		Heat Shock Protein 60	−1,51	0,0032	−1,62	0,0049	1,07	0,43	0,000063
440	CA1230	CaSSA4	1	Cahsp70 mRNA for heat shock	2,09	0,0017	2,6	0,0015	−1,25	0,13	0,000012
441	CA1230	CaSSA4	3	Cahsp70 mRNA for heat shock	−1,61	0,0017	1,29	0,0049	−2,08	0,00011	6,3E-07
444	CA1230	CaSSA4	4	Cahsp70 mRNA for heat shock	1,39	0,0041	2,02	0,0015	−1,45	0,018	0,000017

					B vs Y		B vs H		H vs Y		
Master No.	**Protein ID**	**Protein encoded by**	**Gel Isoform**	**Name**	**Av. Ratio**	**T-test**	**Av. Ratio**	**T-test**	**Av. Ratio**	**T-test**	**1- ANOVA**
451	CA1230	CaSSA4	2	Cahsp70 mRNA for heat shock	−1,98	0,00064	1,32	0,035	−2,61	0,00016	1,3E-06
Protein synthesis											
1315	CA4588	CaASC1	1	Protein of the 40S ribosomal subunit	−1,65	0,0016	−1,62	0,00077	−1,02	0,66	3,1E-06
1339	CA4588	CaASC1	2	protein of the 40S ribosomal subunit	−1,71	0,008	−1,73	0,005	1,01	0,79	0,000091
1221	CA5200	CaRPP0		Ribosomal protein L10; cytosolic	−1,46	0,0017	−1,29	0,018	−1,13	0,12	0,000086
530	CA4302	CaDED81		Asparaginyl-tRNA synthetase	−1,28	0,016	−1,57	0,0043	1,23	0,052	0,000095
411	CA5065	IPF3584		Pab1p mRNA polyadenylate-binding protein	1,03	0,66	−1,57	0,00077	1,61	0,0013	7,8E-06
116	CA2061	CaCDC60		Cytosolic leucyl-tRNA synthetase	−1,59	0,0023	−1,69	0,0023	1,07	0,34	0,000014
359	CA4301	IPF2593		amino acid-tRNA ligase homolog	−1,36	0,0041	−1,61	0,00078	1,19	0,016	5,4E-06
936	CA4909	CaTUF1		Translation elongation factor TU	2,01	0,0019	−1,3	0,02	2,61	0,00015	1,3E-06
Cell rescue, defense and virulence											
1583	CA2719	CaSOD2		Manganese-superoxide dismutase	−1,38	0,011	1,73	0,00029	−2,38	0,00011	4E-07
1584	CA5714	CaTSA1	1	Tsa1p thiol-specific antioxidant-like protein	−1,14	0,46	1,48	0,065	−1,69	0,013	0,0016
1590	CA5714	CaTSA1	2	Tsa1p thiol-specific antioxidant-like protein	−1,59	0,01	1,58	0,005	−2,51	0,00014	2,7E-06
Biogenesis of cellular components											
1151	CA2386	CaECM4		Involved in cell wall biogenesis and architecture	1,24	0,24	2,48	0,0027	−2	0,0023	0,000032
Protein metabolism											
233	CA3333	CaCDC48	1	Microsomal ATPase (by homology)	−1,74	0,00085	−1,1	0,19	−1,59	0,00068	4,6E-06
235	CA3333	CaCDC48	2	Microsomal ATPase (by homology)	−1,54	0,00068	−1,5	0,0015	−1,03	0,46	3,2E-06
971	Mix CA4783	IPF3358		Ubiquinol-cytochrome-c reductase	−1,46	0,0045	1,67	0,0012	−2,44	5,50E-05	4E-07
Experimentally uncharacterized proteins											
1644	CA4127	CaAHP1	1	Unknown function	−1,22	0,039	1,64	0,00087	−2	0,00021	1,6E-06
1649	CA4127	CaAHP1	2	Unknown function	−1,12	0,24	1,76	0,0004	−1,97	0,00033	2,1E-06
1461	CA4416	IPF11888		Unknown function	2,22	0,0044	1,48	0,0077	1,5	0,019	0,000037
1356	CA4220	IPF8762		Unknown function	−2,16	0,0017	10,35	5,10E- 05	−22,39	1,10E-05	5,40E-09

					B vs Y		B vs H		H vs Y		
Master No.	**Protein ID**	**Protein encoded by**	**Gel Isoform**	**Name**	**Av. Ratio**	**T-test**	**Av. Ratio**	**T-test**	**Av. Ratio**	**T-test**	**1- ANOVA**
1103	CA0622	CaLSP1		Unknown function	1,02	0,8	2,66	0,0001	−2, 6	9,40E-05	1,5E-07
1196	CA3836	IPF8179		putative esterase	−1, 42	0,014	1,67	0,002	−2, 36	0,0002	0,000002

[a] absent or not detected in biofilms

[b] absent or not detected in hyphae

[c] absent or not detected in yeast cells

Table 3

Proteins of interest in surface extract comparison between Biofilms (B) vs Yeast cells (Y), B vs Hyphae (H), and H vs Y

Average ratios, Student T-test p-values and analysis of variants (One way ANOVA) p-values are calculated using Decyder™ software v6.5.

Master	Protein	Protein	Gel		B vs Y		B vs H		H vs Y		
No.	ID	encoded by	Isofom	Name	Av. Ratio	T-test	Av. Ratio	T-test	Av. Ratio	T-test	1-ANOVA
192	CA0653	CaMET6	1	5-mthy dropteroyltriglutamate-homocysteine methyltransferase	2.39	0.036	1.5	0.31	1.6	0.16	0.017
195	CA0653	CaMET6	2	5-mthy dropteroyltriglutamate-homocysteine methyltransferase	2.54	0.027	1.07	0.7	2.38	0.09	0.013
196	CA0653	CaMET6	3	5-mthy dropteroyltriglutamate-homocysteine methyltransferase	2.6	0.036	−1.35	0.53	3.52	0.06	0.0079
197	CA0653	CaMET6	4	5-mthy dropteroyltriglutamate-homocysteine methyltransferase	2.49	0.031	−1.8	0.33	4.48	0.04	0.005
198	CA0653	CaMET6	5	5-mthy dropteroyltriglutamate-homocysteine methyltransferase	1.75	0.071	−1.3	0.24	2.27	0.05	0.0078
289	CA1230	CaSSA4		cahsp70 mRNA for heat shock	−3.65	0.049	−1.56	0.46	−2.34	0.06	0.011
313	CA3534	CaSSB1	1	heat shock protein 70	−3.63	0.042	2.38	0.17	−8.64	0.04	0.0048
315	CA3534	CaSSB1	2	heat shock protein 70	−6.66	0.038	1.29	0.83	−8.6	0.04	0.0052
331	CA3534	CaSSB1	3	heat shock protein 70	−7.86	0.018	−4.31	0.04	−1.82	0.13	0.0024
349	CA2474	CaPDC11	2	Pyruvate decarboxylase	−21.84	0.033	−1.26	0.46	−17.34	0.04	0.005
350	CA2474	CaPDC11	1	Pyruvate decarboxylase	−16.25	0.033	1.35	0.82	−21.87	0.04	0.0039
356	CA2474	CaPDC11	3	Pyruvate decarboxylase	−8.64	0.027	1.43	0.88	−12.39	0.03	0.0024
357	CA2474	CaPDC11[a]	4	Pyruvate decarboxylase	−26.14	0.013	−2.15	0.24	−12.14	0.03	0.0014
358	CA2474	CaPDC11[a]	5	Pyruvate decarboxylase	−77.82	0.003	−9.17	0.01	−8.48	0.04	0.00027
359	CA2474	CaPDC11	6	Pyruvate decarboxylase	−44.43	0.004	−7.85	0.02	−5.66	0.04	0.00027
383	CA2474	CaPDC11	9	Pyruvate decarboxylase	−25.03	0.006	−23.37	0.00027	−1.07	0.29	0.00027
384	CA2474	CaPDC11	8	Pyruvate decarboxylase	−31.64	0.005	−10.45	0.00027	−3.03	0.06	0.00027
385	CA2474	CaPDC11[a]	10	Pyruvate decarboxylase	−17.8	0.013	−29.74	0.00044	1.67	0.13	0.00055
386	CA2474	CaPDC11	7	Pyruvate decarboxylase	−22.89	0.01	−4.7	0.03	−4.87	0.04	0.00081
387	CA2474	CaPDC11	11	Pyruvate decarboxylase	−3.95	0.042	−2.79	0.02	−1.41	0.23	0.0079
412	CA3852	CaTUP1		general transcription repressor	2.29	0.013	−1.04	0.85	2.38	0.05	0.005
433	CA0692	CaPGM2		Phosphoglucomutase	−2.16	0.061	−4.57	0.02	2.12	0.06	0.0035
435	CA5986	CaINO1		myo-inositol-1-phosphate synthase	−1.75	0.11	−5.73	0.03	3.28	0.05	0.004
524	CA5239	CaGND1		6-phosphogluconate dehydrogenase	−2.48	0.042	−3.16	0.13	1.28	0.22	0.01
542	CA5738	CaLYS9		Lysine biosynthesis	1.64	0.11	−2.63	0.17	4.3	0.05	0.0079
590	CA3874	CaENO1	1	Enolase I (2-phosphoglycerate dehydratase)	2.27	0.033	1.85	0.04	1.23	0.17	0.0061

Master No.	Protein ID	Protein encoded by	Gel Isofom	Name	B vs Y Av. Ratio	B vs Y T-test	B vs H Av. Ratio	B vs H T-test	H vs Y Av. Ratio	H vs Y T-test	1-ANOVA
598	CA3874	CaENO1	2	Enolase I (2-phosphoglycerate dehydratase)	2.43	0.031	1.31	0.33	1.85	0.07	0.0072
600	CA5618	CaLEU2		isopropyl malate dehydrogenase	2.07	0.082	−1.38	0.38	2.86	0.05	0.0086
658	CA1691	CaPGK1		Phosphoglycerate kinase	2.06	0.048	−1.24	0.55	2.54	0.04	0.0076
666	CA4765	CaADH1	1	alcohol dehydrogenase	1.04	0.37	3.84	0.01	−3.69	0.04	0.0017
677	CA4765	CaADH1	2	alcohol dehydrogenase	1.05	0.33	2.51	0.01	−2.39	0.05	0.0051
727	CA5040	CaBAT22	1	branched chain amino acid aminotransferase	−2.08	0.04	−3.87	0.01	1.86	0.05	0.0014
742	CA5180	CaFBA1	2	fructose-bisphosphate aldolase	−3.86	0.026	−4.57	0.05	1.18	0.24	0.0036
743	CA5180	CaFBA1	3	fructose-bisphosphate aldolase	−7.73	0.01	−4.74	0.01	−1.63	0.12	0.00087
744	CA5180	CaFBA1	5	fructose-bisphosphate aldolase	−18.1	0.006	−12.47	0.01	−1.59	0.16	0.00044
749	CA5180	CaFBA1[a]	4	fructose-bisphosphate aldolase	−10.54	0.013	−19.65	0.01	1.63	0.18	0.00044
759	Mix CA2582	CaTAL1		transaldolase (by homology)	1.42	0.15	−2.47	0.006	3.52	0.05	0.006
	Mix CA0685	CaADO1	1	adenosine kinase (by homology)	1.42	0.15	−2.47	0.006	3.52	0.05	0.006
760	CA0685	CaADO1	2	adenosine kinase	1.47	0.14	−3.7	0.0046	5.45	0.05	0.0046
762	CA4181	CaHOM6		homoserine dehydrogenase	−3.86	0.016	−10.33	0.00037	2.68	0.04	0.00037
764	CA0870	CaIPP1		inorganic pyrophosphatase	−2.53	0.04	−4.91	0.03	1.94	0.09	0.0039
771	CA5897	IPF2384	1	unknown function (ortholog ScPMU1)	2.68	0.032	2.53	0.03	1.06	0.25	0.005
778	CA5180	CaFBA1	1	fructose-bisphosphate aldolase	2.72	0.015	1.65	0.11	1.65	0.06	0.0024
796	CA5897	IPF2384	2	unknown function (ortholog ScPMU1)	2.25	0.07	−1.23	0.44	2.76	0.04	0.0072
834	CA2644	CaGRP2	1	Reductase	−1.15	0.33	−3.03	0.01	2.64	0.06	0.0098
835	CA5040	CaBAT22	2	branched chain amino acid aminotransferase	1.66	0.099	−3.37	0.06	5.6	0.04	0.0028
837	CA2644	CaGRP2	2	Reductase	1.02	0.27	−4.91	0.01	5.03	0.04	0.005
843	CA3588	CaSPE3	1	putrescine aminopropyltransferase	−1.64	0.084	−2.05	0.02	1.25	0.13	0.0065
853	CA2756	IPF18418	2	unknown function (ortholog ScYHI9)	−2.29	0.16	−4.39	0.0042	1.91	0.09	0.0063
857	CA2756	IPF18418	1	unknown function (ortholog ScYHI9)	−1.85	0.18	−2.89	0.0034	1.57	0.1	0.0079
892	CA4184	CaSNZ1		stationary phase protein by homology	1.76	0.034	−1.21	0.54	2.14	0.04	0.0068
898	CA1592	CaGRE3		D-xylose reductase	2.82	0.039	1.22	0.63	2.32	0.04	0.0061
915	CA3427	IPF11865		unknown function	−2.1	0.19	−6.44	0.01	3.07	0.05	0.0046
916	CA3588	CaSPE3	2	putrescine aminopropyltransferase	−1.94	0.17	−3.57	0.03	1.84	0.1	0.013
935	CA5788	CARHR2		DL-glycerol phosphatase	−1.48	0.23	−2.08	0.04	1.4	0.16	0.022
955	CA4220	IPF8762	1	unknown function	3.93	0.012	6.33	0.0038	−1.61	0.08	0.00029

Master No.	Protein ID	Protein encoded by	Gel Isofom	Name	B vs Y Av. Ratio	B vs Y T-test	B vs H Av. Ratio	B vs H T-test	H vs Y Av. Ratio	H vs Y T-test	1-ANOVA
956	CA4220	IPF8762	2	unknown function	6.01	0.013	6.23	0.0034	−1.04	0.25	0.00068
998	CA5950	CaTPI1	1	Triose phosphate isomerase	−1.01	0.28	−2.75	0.02	2.72	0.06	0.0094
1001	CA5526	CaASR2		unknown function	5.14	0.042	2.3	0.21	2.24	0.17	0.014
1002	CA5950	CaTPI1	2	Triose phosphate isomerase	1.02	0.27	−3.64	0.0048	3.72	0.04	0.0048
1005	CA1704	CaSOL3	1	weak multicopy suppressor of los1-1	2.41	0.039	1.09	0.57	2.21	0.06	0.0068
1007	CA1704	CaSOL3	2	weak multicopy suppressor of los1-1	2.6	0.041	−1.24	0.2	3.22	0.04	0.0039
1021	CA1503	CaRKI1		D-ribose-5-phosphate ketol-isomerase	7.49	0.04	2.02	0.19	3.71	0.1	0.0084
1031	CA4416	IPF11888		unknown function (ortholog ScPRX1)	−3.43	0.13	−3.13	0.01	−1.09	0.24	0.019
1036	CA0828	IPF17186	2	unknown function (ortholog ScHSP31)	1.28	0.2	−2.08	0.03	2.67	0.07	0.016
1038	CA0828	IPF17186	1	unknown function (ortholog ScHSP31)	1.06	0.25	−2.03	0.01	2.15	0.1	0.025
1069	CA0748	CaTFS1		cdc25-dependent nutrient- and ammonia- response cell-cycle regulator	7.94	0.031	2.22	0.23	3.58	0.06	0.0054
1083	CA5116	CaTPM2		Tropomyosin3-prime end	21.77	0.012	4.15	0.01	5.24	0.05	0.001
1098	CA3874	CaENO1	3	Enolase I (2-phosphoglycerate dehydratase)	18.99	0.003	3.75	0.01	5.06	0.04	0.00027
1131	CA5714	CaTSA1	3	Tsa1p thiol-specific antioxidant-like protein	3.66	0.013	2.55	0.04	1.44	0.15	0.0027
1150	CA4127	CaAHP1	1	Putative alkyl hydroperoxide reductase	3.05	0.02	3.81	0.01	−1.25	0.18	0.0019
1151	CA4127	CaAHP1	2	Putative alkyl hydroperoxide reductase	2.77	0.029	3.8	0.01	−1.37	0.19	0.0024
1160	CA4127	CaAHP1	3	Putative alkyl hydroperoxide reductase	2.38	0.042	1.92	0.02	1.24	0.16	0.0079
1163	CA4127	CaAHP1	4	Putative alkyl hydroperoxide reductase	1.65	0.13	1.3	0.46	1.21	0.6	0.024
1181	CA5409	CaCOF1	1	cofilin	5.26	0.031	1.82	0.29	2.89	0.09	0.0079
1190	CA3978	CaCPR3		cyclophilin	4.73	0.039	4.98	0.08	−1.05	0.29	0.0057
1199	CA5409	CaCOF1	2	cofilin	8.08	0.033	2.12	0.11	3.81	0.09	0.0068
1212	CA4120	CaSOD1		Cu,Zn-superoxide dismutase,	5.09	0.032	1.11	0.71	4.58	0.04	0.0037
1236	CA3676	CaRBP1		rapamycin-binding protein	8.11	0.042	2.85	0.07	2.85	0.1	0.009
1283	Mix CA5078	IPF15297		unknown function	4.91	0.014	3.72	0.0012	1.32	0.14	0.0013
	Mix CA5714	CaTSA1	1	Tsa1p thiol-specific antioxidant-like protein	4.91	0.014	3.72	0.0012	1.32	0.14	0.0013
1284	CA5714	CaTSA1	2	Tsa1p thiol-specific antioxidant-like protein	7.37	0.012	2.81	0.00068	2.63	0.04	0.00081

[a] absent or not detected in biofilms

[b] absent or not detected in hyphae

[c] absent or not detected in yeast cells

Table 4

Selected cellular processes, functions, and cellular locations that are associated with differential cytoplasmic protein abundance

This table is based on the GO Term Finder analysis (Supporting Information Table S7) with a selection or processes, functions and components that are significantly affected by the differentially abundant proteins.

PROCESS(ES)	B/H[a]	B/Y[a]	H/Y[a]	SOME ASSOCIATED DIFFERENTIALLY ABUNDANT PROTEINS
Acetate metabolism	UP[b]	DOWN[b]	DOWN	Acs2, Ach1, Ald5
Amino acid metabolism, amino acid biosynthesis, cellular amine biosynthesis	DOWN	DOWN NC[b]	UP	Asn1, Cdc60, Pdc11, Ilv3, Cit1, Trp5, Ded81, Orf19.7534
Carbohydrate catabolism, hexose metabolism, glycolysis, gluconeogenesis, pyruvate metabolism	UP	DOWN UP	DOWN	Adh1, Cdc19, Eno1, Fba1, Gnd1, Gpm1, Gre3, Hxk2, Pdc11,Pgk1, Tdh3, Tpi1, orf129.6757
Generation precursor metabolites and energy	UP	DOWN UP	DOWN	Sdh12, Pdc11, Pgk1, Cit1, Fba1, Kgd1, Pfk2, Tpil, Gpm1
Induction by symbiont of host defense response, positive regulation of defense response, modulation by organism of defense response of other organism during symbiotic interaction	UP	DOWN UP	DOWN	Met6, Cdcv19, Pgk1, Eno1, Adh1, Fba1, Hsp70, Tpi1, Tdh3, Adh1, Aco1, Pgk1
Multi-organism process	UP	DOWN	DOWN	Met6, Cdc19, Pgk1, Eno1, Adh1, Fba1, Hsp70, Tps1, Tpi1, Tdh3, Icl2, Tsa1, Gpm1, Ssa2,
Organic acid metabolism	DOWN	DOWN UP	DOWN	Acs2, Mis11, Cdc10, Sdh12, ilv3, Mdh1-1, Trp5, Shm2, Aco1, Ded81, Leu1, Oef19.7534
Pentose metabolism, arabinose and xylose metabolism	UP	DOWN	DOWN	Gre3, Gnd1, orf19.6757
Protein folding, refolding	DOWN	DOWN	DOWN UP	Ssa2, Ssc1, Msi3, Hsp60, Ssb1, Hsp104, Hsp78
Response to stimulus, response to stress, response to temperature stimulus or heat	UP	NC	DOWN	Met6, Ahp1, Lsp1, Sod2, Gre3, Hsp70, Gnd1, Hsp104, Tps1, Tsa1, Hsp78, Hxk2, orf19.6757
TCA cycle	DOWN	NC[b]	DOWN	Mdh1-1, Mdh1, Kgd1, Aco1, Cit1
FUNCTION(S)				
Aldo-keto reductase activity	UP	DOWN	DOWN	Gre3, orf19.6757
Adenyl nucleotide binding, adenyl ribonucleotide binding ATP binding,	DOWN	DOWN	UP	Ssa2, Msi3, Ded81
Aminoacyl-tRNA ligase activity	DOWN	DOWN	UP	Cdc60, Ded81, orf19.6701
Carbon-oxygen lyase activity	DOWN	DOWN UP	DOWN UP	Ilv3,Trp5, Aco1, Leu1, Pdc11, Fba1, Icl1
Hydro-lyase activity	DOWN	UP	UP	Ilv3, Trp5, Aco1, Leu1
Ligase activity	DOWN	UP	UP	Acs2, Asn1/asn1, Mis11, Cdc60, orf19.6701, Ded81, orf19.7534
Methenyltetrahydrofolate cyclohydro-lase activity, methylenetetrahydrofolate dehydrogenase activity, formate-tetrahydrofolate ligase activity	DOWN	NC	UP	Mis11, orf19.7534
Oxidoreductase activity	UP	DOWN UP	DOWN UP	Pxp2, Ahp1, Hem13, Sod2, Pst2, Adh1, Gre3, Gnd1, orf19.5180, Ald5, orf19.6757, Tdh3, Osm1, Tsa1, Mdh1, Mis11, Pst2, Mdh1-1, Osm1, Sdh12, Kgd1, orf19.7534
Protein binding	UP	DOWN	DOWN	Pgk1, Eno1, Adh1, Fba1, Hsp70, Hsp104, Tdh3:Tsa1, Gpm1, Ssa2, Ssc1, Msi3, Asc1
Unfolded protein binding	DOWN	DOWN	DOWN	Ssa2, Ssc1, Ssb1, Hsp70, Tsa1, Hsp78

[a] Ratio protein abundance Biofilm (B) to Hyphae (H) or Yeast cells (Y) or H to Y.

[b] Greater abundance is indicated by UP and less abundance by DOWN and no change by NC.

The presence of two terms indicates that either significant increase or decrease or no change in abundance was observed for proteins associated with the process(es).