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Surfaceome of pathogenic yeasts, *Candida parapsilosis* and *Candida tropicalis*, revealed with the use of cell surface shaving method and shotgun proteomic approach*

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In the course of infections caused by pathogenic yeasts from the genus *Candida*, the fungal cell surface is the first line of contact with the human host. As the surface-exposed proteins are the key players in these interactions, their identification can significantly contribute to discovering the mechanisms of pathogenesis of two emerging pathogens from this genus, *C. parapsilosis* and *C. tropicalis*. Therefore, the aim of the present study was to identify the cell wall-attached proteins of these two species with the use of cell surface shaving and a shotgun proteomic approach. Different morphological forms of *C. parapsilosis* and *C. tropicalis* cells obtained after growth under various conditions were subjected to this treatment. This allowed to indicate the most abundant cell surface proteins on the basis of the normalized spectral abundance factors. In case of yeast-like forms these were, among others, proteins similar to a chitinase, glyceraldehyde-3-phosphate dehydrogenase and an inducible acid phosphatase for *C. parapsilosis*, and a constitutive acid phosphatase, pyruvate decarboxylase and glyceraldehyde-3-phosphate dehydrogenase for *C. tropicalis*. In case of pseudohyphal forms, proteins similar to a cell surface mannoprotein Mp65, chitinase and glycosylphosphatidylinositol-anchored transglycosylase Crh11 were identified at the cell surface of *C. parapsilosis*. The Rbt1 cell wall protein, a hyphally regulated cell wall protein and proteins from agglutinin-like sequence protein family were found as the most abundant on *C. tropicalis* pseudohyphae. Apart from the abovementioned proteins, several additional covalently bound and atypical cell wall proteins were also identified. These results extend the current knowledge regarding the molecular basis of virulence of these two non-albicans *Candida* species.

Key words: cell surface shaving, proteomics, fungal pathogens, cell wall, *Candida*

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INTRODUCTION

The most external part of microbial cells, such as the fungal cell wall or bacterial envelope, confers their shape, rigidity and mechanical strength, being also responsible for proper growth, morphogenesis and reproduction. Moreover, the cell surface also acts as a protective barrier, providing a dynamic response to stress and changes in environmental conditions. Because cell wall/envelope for a variety of prokaryotic and eukaryotic pathogens is also a key structure that participates in interactions with the human host during infection, thus playing an important role in pathogenesis, a progressively growing number of studies has been devoted to different types of molecules localized at the pathogens' cell surface (Hecker *et al*., 2010; Silhavy *et al*., 2010; Free, 2013; Demuyser *et al*., 2014).

At present, after the release of genome sequences for a number of pathogenic microorganisms and due to the expanding development of the PCR-based techniques, the detection of changes in the gene expression correlated with infection process is often used in the studies of bacterial or fungal pathogenesis (Sturtevant, 2000). However, one still should bear in mind a fact that mRNA is only the short-time messenger and that translated proteins might undergo different regulation and posttranslational modifications, resulting in changes of their function, structure and localization. In fact, proteins are the key players in the life of the cell under physiological and pathological conditions; thus, their actual exposure at the cell surface should be studied, not only the gene expression, especially in the case of atypical surface-associated proteins devoid of typical signal for extracellular localization, for these reasons sometimes called "moonlighting proteins" (Karkowska-Kuleta & Kozik, 2014). More suitable combined approaches, employing a wide range of proteomic techniques and genomic data, open new perspectives to understand the molecular mechanisms of host-pathogen interplay (Huang *et al*., 2002).

The complex characteristics of surfaceome — a set of surface-associated proteins being the most variable and complicated molecules that build the cells' envelopes — expand very rapidly, as a lot of data is now available about the proteins identified at the surface of Gram-positive and Gram-negative bacteria, non-pathogenic and pathogenic fungi and even a few multicellular pathogens (Desvaux *et al*., 2006; Solis & Cordwell, 2011; Olaya-Abril *et al*., 2014). The identification of this type of proteins with a classical proteomic approach involving fractionation and two-dimensional electrophoretic separation is not problem-free, because of their high glycosylation state, low abundance, insolubility and anchoring in the cell membrane or within the polysaccharide scaffold, as well as due to the possible contaminations of samples

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Abbreviations: Als, agglutinin-like sequence; ASL, alkali sensitive linkages; CGD, Candida Genome Database; DTT, dithiothreitol; GPI, glycosylphosphatidylinositol; MS/MS, tandem mass spectrometry; NSAF, normalized spectral abundance factor; YAPD, yeast extract-animal peptone-dextrose; YPD, yeast extract-peptone-dextrose

with proteins from other cell compartments (Pitarch *et al*., 2002). Thus, there has been an insistent necessity to develop new identification methods. A novel approach used for discovering the secrets of pathogens' cell surface is the shaving of the live cells with trypsin for a short period of time, with an assurance that the cells are still not damaged and the cell membrane remains intact, to avoid the cytoplasmic contamination (Olaya-Abril *et al*., 2014).

This new method designated as surfaceomics, combined with a shotgun proteomic approach, was successfully used for studying the cell surface proteinaceous components of different bacterial species, including *Streptococcus pyogenes* (Severin *et al*., 2007), *Staphylococcus aureus* (Solis *et al*., 2010) and *Escherichia coli* (Walters & Mobley, 2009). Also for the non-pathogenic yeast *Saccharomyces cerevisae* (Insenser *et al*., 2010), opportunistic pathogen *Candida albicans* (Hernáez *et al*., 2010; Vialás *et al*., 2012; Gil-Bona *et al*., 2015b) and pathogenic fungus *Cryptococcus neoformans* (Eigenheer *et al*., 2007), a number of surface-exposed proteins was identified with this approach.

Nowadays, in addition to the two most common fungal pathogens from the genus *Candida* — *C. albicans* and *C. glabrata* — a problem of increased incidence of severe infections caused by two other species with different virulence attributes — *C. parapsilosis* and *C. tropicalis* — has alarmingly emerged (Arendrup, 2013). The latter two species together account for over one fourth of all systemic candidiases in immunocompromised patients (Azie *et al*., 2012; Wang *et al*., 2014), which implies an urgent need to better describe the molecular mechanisms of their virulence. The structure and composition of the *C. albicans* and *C. glabrata* cell wall — being the first structure that contacts the host during invasion — have been already fairly well characterized (de Groot *et al*., 2008; Castillo *et al*., 2008; Klis *et al*., 2009), contrary to the two other non-albicans *Candida* species with great clinical importance.

Apart from polysaccharides, such as chitin, β-glucans and mannans, that serve as the mechanical scaffold of candidal cell wall, several groups of covalently bound or more loosely associated proteins are found at the *Candida* spp. cell surface. They are not only involved in a wide variety of physiological processes, but also act as important virulence factors that mediate the adhesion, biofilm formation, iron acquisition and proteolysis (Chaffin, 2008; Modrzewska & Kurnatowski, 2015). The aim of this work was to identify the cell surface-exposed proteins at the cell walls of different morphological forms of *C. parapsilosis* and *C. tropicalis* — unicellular, yeast-like cells or filamentous pseudohyphae — observed under different cultivation conditions that mimic cellular stress, nutrient starvation or host invasion. Such a comprehensive characterization could be used in the future to design new, broad-spectrum anti-candidal vaccines, as well as differentiating diagnostic methods, or to design new and effective antifungal therapy against candidiases caused by non-albicans *Candida* species.

MATERIALS AND METHODS

Yeast strains and culturing. The *C. parapsilosis* strain CDC 317 (ATCC MYA-466) and *C. tropicalis* strain T1 (ATCC MYA-344) were purchased from American Type Culture Collection (Manassas, VA, USA). Cells were grown as yeast-like forms in YPD medium, pH 6.0 (1% yeast extract, 2% soybean peptone and 2% glucose) (Sigma, St. Louis, MO, USA) at 30°C for 16 h. To induce formation of pseudohyphal forms, both strains were cultivated in a defined medium RPMI 1640, pH 7.4 (PAA Laboratories GmbH, Pasching, Austria) at 37°C for 48 h or in YPD buffered medium with lowered content of animal-derived peptone (YAPD), pH 7.0 (0.1% yeast extract, 0.2% peptone from animal proteins, 2% glucose and 10 mM NaH_2PO_4) (Sigma) at 37°C for 48 h. In certain cases, a sterile filtered human plasma was added to YPD or RPMI 1640 medium to a final concentration of 10% (v/v).

Cell surface shaving with trypsin. *C. parapsilosis* or *C. tropicalis* cells (5×10^8), grown in the appropriate culturing medium were separated by centrifugation (5 minutes, 3000 rpm), observed under microscope to confirm their existence as the yeast-like form after YPD-culturing or the filamentous form after YAPD- or RPMI 1640-culturing, and then washed three times with 1 ml of 25 mM ammonium bicarbonate buffer (NH_4HCO_3). The cell pellet was then resuspended in 100 µl of the same buffer with 5 mM dithiothreitol (DTT). Sequencing-grade trypsin (10 µg) (Promega, Madison, WI, USA) was added to the cell suspension for incubation at 37°C for 5 minutes. After that, cells were centrifuged (5 minutes, 6000 rpm) and the supernatant was filtered through a filter with 0.22 µm pores and subjected to further incubation with trypsin for 5 hours at the same temperature. The cell membrane integrity of the remaining cells was tested by staining with SYTOX® Green (Invitrogen Life Technologies, Carlsbad, CA, USA) and Trypan Blue (Sigma). After incubation, trifluoroacetic acid (TFA) (Sigma) was added to the supernatant to a final concentration of 0.1%, to stop the enzymatic reaction. The sample was then incubated on ice for 15 minutes and after that centrifuged (15 minutes, 12 000 rpm), dried in a Speed-Vac (Martin Christ, Osterode am Harz, Germany) and frozen until further use.

Protein identification with LC-MS/MS and further statistical analyses. The peptides were separated and analyzed with a HCTUltra ETDII ion-trap mass spectrometer equipped with an electrospray ionization ion source (Bruker, Bremen, Germany) and coupled to an ultra-high-performance liquid chromatography Dionex Ultimate 3000 system. All peptide samples were first dissolved in 100 µl of loading buffer (10% acetonitrile with 0.1% formic acid), centrifuged (10 minutes, 10000 rpm) and, after transferring to fresh vials, separated on a 100 mm × 2.1 mm Accucore C18 column (particle size of 2.6 µm) (Thermo Fisher Scientific, Waltham, MA, USA), with a gradient of 10–60% of 0.1% formic acid in 80% acetonitrile for 60 minutes with a flow rate of 0.2 ml/min. The mass spectrometer was operated in a standard MS/MS mode with simultaneous fragmentation of the most intensive precursor ions by collision-induced dissociation and electron-transfer dissociation. Mascot Generic format (.mgf) files were generated by pre-processing the raw data with Data Analysis 4.0 software (Bruker). The lists of obtained peaks were searched against the nonredundant protein database of the NCBI with taxonomy restriction to Fungi (26 490 256 sequences for all entries, 1 935 595 sequences for fungal proteins) or SwissProt protein database with taxonomy restriction to *Homo sapiens* (548 872 sequences for all entries, 20 282 sequences for human proteins) using an in-house Mascot server (v.2.3.0; Matrix Science, London, UK). The following search parameters were applied: enzyme specificity – trypsin; permitted number of missed cleavages – 2; fixed modification – carbamidomethylation (C); variable modifications – oxidation (M); protein mass

– unrestricted; peptide mass tolerance of ±0.3 Da and fragment mass tolerance of ±0.5 Da.

After peptide identification a final dataset was created on the basis of the classical mathematical normalization, named the normalized spectral abundance factor (NSAF) that allows to estimate the relative protein abundances. The NSAF was calculated using the following formula

$$NSAF = \frac{\frac{SpC}{L}}{\sum_{i=1}^{N}\left(\frac{SpC}{L}\right)}$$

where the total number of spectral counts (SpC), divided by the protein's length (L) was divided by the sum of the total number of MS/MS spectra for all proteins identified in the experiment in one sample.

RESULTS

Identification of *C. parapsilosis* and *C. tropicalis* cell surface-exposed proteins

A rapid and simple approach — cell surface shaving with trypsin, combined with shotgun proteomics — was applied in the present study to identify the cell surface-exposed proteins from different morphological forms of two opportunistic pathogens, *C. parapsilosis* and *C. tropicalis* (Fig. 1). Our observations indicated that in rich, complete YPD medium, the cells of both species existed as unicellular, yeast-like forms, while in the two other media, as filamentous pseudohyphal forms. The YAPD medium, characterized by the reduced amount of nitrogen due to the animal peptone, primarily stimulates the production of proteinases (Rapala-Kozik *et al.*, 2010), although at the temperature of 37°C and pH 7.0 it also causes the generation of filamentous forms. However, in YAPD medium the cells were elongated to a relatively lesser extent than in the case of the other growth conditions used — a defined, cell cultivating medium RPMI 1640 — that is typically used for inducing pseudohyphal growth of *Candida* spp. at 37°C (Hoyer *et al.*, 1995).

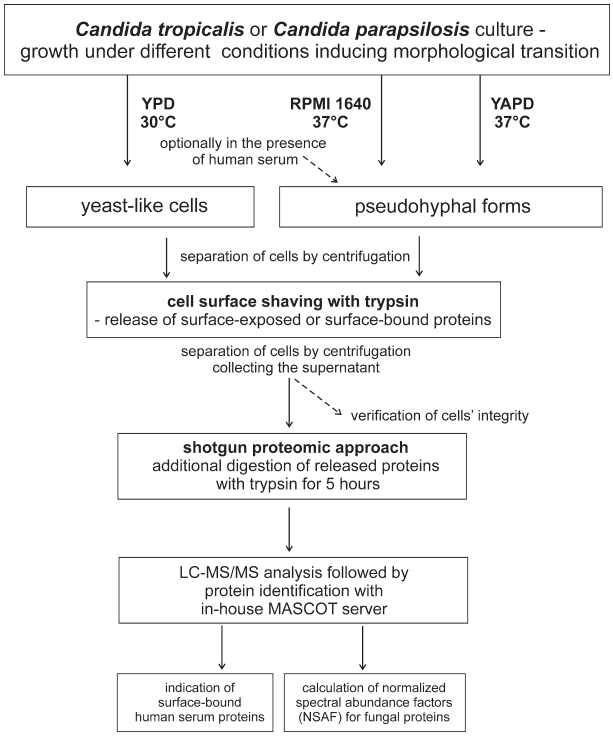

Figure 1. The workflow strategy for the identification of fungal surface-exposed proteins or human proteins bound to fungal surface, using the cell surface shaving method and shotgun proteomic approach.

A slightly modified method for digestion of fungal surface-exposed proteins was used in this study in comparison to that described previously for *C. albicans* by Hernáez *et al.* (2010), Vialás *et al.* (2012) and Gil-Bona *et al.* (2015b). Namely, proteins released from the cell wall during the five-minute incubation with trypsin, after separation of the supernatant from fungal cells, were further incubated with trypsin at 37°C for additional five hours to improve tryptic-digestion efficiency and to increase the number of peptides produced. The crucial condition during the whole treatment was to maintain the intact cell membrane without cell lysis, in order to avoid cytoplasmic contamination; therefore the cell integrity was confirmed after each experiment with specific staining.

This applied approach allowed us to identify 16, 9 and 12 proteins for *C. parapsilosis* and 13, 13 and 10 proteins for *C. tropicalis*, assigned to cells grown in YPD, YAPD and RPMI 1640 medium, respectively. The calculation of NSAF for each protein identified in a particular sample enabled us to select and rank the relatively most abundant fungal proteins for each growth medium (Zybailov *et al.*, 2007). All abbreviated names and protein descriptions included in this study were taken from NCBI protein database and Candida Genome Database (CGD) (Inglis *et al.*, 2012). Although the genome sequences for these two particular *Candida* spp. strains were recently released (Butler *et al.*, 2009), their proteome is still poorly characterized and described, therefore the identified *C. parapsilosis* and *C. tropicalis* hypothetical proteins were compared and assigned to orthologous genes and/or proteins from *C. albicans*.

As shown in Tables 1 and 2, some of the identified proteins were common for all tested conditions, such as *C. parapsilosis* orthologs of Phr2, Mp65, Pir1 and Ecm33, and *C. tropicalis* ortholog of Tdh3. Some proteins were unique, as for example proteins similar to *C. albicans* Rbe1, Plb5, Eng1 and Tos1 identified in *C. parapsilosis* and proteins similar to *C. albicans* Rbt1, Hyr3 and Als1 identified in *C. tropicalis*, which were present at the cell surface only after growth in the RPMI 1640 medium.

Within the particular sets of proteins derived from the cells cultured under defined conditions, the percentage content of proteins equipped with glycosylphosphatidylinositol (GPI) anchor that links them covalently to the cell wall was determined on the basis of structural annotations located in CGD. For both investigated species, the highest content of GPI-linked proteins was detected for the cell walls of pseudohyphae grown in RPMI 1640 medium. It was 50% and 30%, respectively — which corresponds to 6 and 3 proteins — for *C. parapsilosis* and *C. tropicalis*. What is more, for the latter species, no additional GPI-anchored protein was identified for other tested conditions, whereas for *C. parapsilosis* 4 (25%) and 2 (22%) proteins found at the surface of cells grown in YPD or YAPD broth, respectively, were assigned as equipped with GPI remnant.

In order to determine the functional characteristics of identified proteins, for each of them the specific function was assigned on the basis of manually curated Gene Ontology (GO) annotations from CGD. The results of these

Table 1. Mass spectrometry analysis of *C. parapsilosis* proteins identified at the cell surface after growth under different conditions.
After cell surface shaving with trypsin and additional digestion of obtained proteins for 5 hours, peptides were analyzed using the Dionex Ultimate 3000 UHPLC system coupled to an HCTUltra ETDII mass spectrometer. The obtained lists of peaks were searched against the NCBI protein database using an in-house Mascot server (SC – sequence coverage).

YPD medium								
Accession	Protein	Description	Molecular mass [kDa]	pI	Score	Number of peptides	SC	NSAF
gi|354547255	hypothetical protein CPAR2_502140 [*Candida parapsilosis*]	Similar to *C. albicans* GPI-linked chitinase (Cht2)	61.3	4.23	312	6	10%	0.006089
gi|354545590	hypothetical protein CPAR2_808670 [*Candida parapsilosis*]	Similar to *C. albicans* glyceraldehyde-3-phosphate dehydrogenase (Tdh3)	36.3	6.19	383	9	33%	0.005255
gi|354546478	hypothetical protein CPAR2_208530 [*Candida parapsilosis*]	Similar to *C. albicans* putative inducible acid phosphatase (Pho100)	35.7	4.57	499	9	31%	0.005099
gi|354545372	hypothetical protein CPAR2_806490 [*Candida parapsilosis*]	Similar to *C. albicans* protein with internal repeats (Pir1)	41.5	4.60	338	7	13%	0.004866
gi|354544096	hypothetical protein CPAR2_108560 [*Candida parapsilosis*]	Similar to *C. albicans* GPI-anchored cell wall protein (Ecm33)	44.7	4.24	289	6	14%	0.004515
gi|354547623	hypothetical protein CPAR2_401600 [*Candida parapsilosis*]	Similar to *C. albicans* 1,3-beta-glucosyltransferase (Bgl2)	34.1	4.33	288	8	28%	0.004282
gi|354548203	hypothetical protein CPAR2_407410 [*Candida parapsilosis*]	Similar to *C. albicans* cell surface mannoprotein (Mp65)	39.5	4.75	283	6	19%	0.003879
gi|354543937	hypothetical protein CPAR2_106940 [*Candida parapsilosis*]	Similar to *C. albicans* HSP70 family chaperone (Ssa2)	69.9	5.04	156	3	6%	0.003368
gi|354544206	hypothetical protein CPAR2_109660 [*Candida parapsilosis*]	Similar to *C. albicans* glycosidase (Phr2)	60.8	4.33	199	3	7%	0.002951
gi|354547143	hypothetical protein CPAR2_501020 [*Candida parapsilosis*]	Similar to *C. albicans* putative pyruvate decarboxylase (Pdc11)	62.6	5.28	165	3	8%	0.002951
gi|354545680	hypothetical protein CPAR2_200500 [*Candida parapsilosis*]	Similar to *C. albicans* orf19.7596, protein with a phosphoglycerate mutase family domain	37.7	5.84	163	5	14%	0.002824
gi|354547299	hypothetical protein CPAR2_502580 [*Candida parapsilosis*]	Similar to *C. albicans* alcohol dehydrogenase (Adh1)	43.5	8.57	132	4	10%	0.002801
gi|354545390	hypothetical protein CPAR2_806670 [*Candida parapsilosis*]	Similar to *C. albicans* secreted yeast cell wall protein (Ywp1)	53.4	4.23	148	3	5%	0.002721
gi|354547257	hypothetical protein CPAR2_502160 [*Candida parapsilosis*]	Similar to *C. albicans* cell wall protein (Scw11)	49.9	4.19	86	3	11%	0.002414
gi|354546348	hypothetical protein CPAR2_207210 [*Candida parapsilosis*]	Similar to *C. albicans* enolase (Eno1)	47.0	5.59	135	3	10%	0.002289
gi|354544910	hypothetical protein CPAR2_801850 [*Candida parapsilosis*]	Similar to *C. albicans* Ala-, Leu- and Ser-rich protein (Op4)	42.0	4.84	158	3	17%	0.002164
YAPD medium								
gi|354543937	hypothetical protein CPAR2_106940 [*Candida parapsilosis*]	Similar to *C. albicans* HSP70 family chaperone (Ssa2)	70.0	5.04	269	7	15%	0.031902

gi|354544096	hypothetical protein CPAR2_108560 [*Candida parapsilosis*]	Similar to *C. albicans* GPI-anchored cell wall protein (Ecm33)	44.7	4.24	336	7	14%	0.021383
gi|354543776	hypothetical protein CPAR2_105340 [*Candida parapsilosis*]	Similar to *C. albicans* ATP synthase alpha subunit (Atp1)	59.0	8.53	170	5	10%	0.019295
gi|354546348	hypothetical protein CPAR2_207210 [*Candida parapsilosis*]	Similar to *C. albicans* enolase (Eno1)	47.0	5.59	270	5	15%	0.01739
gi|354544206	hypothetical protein CPAR2_109660 [*Candida parapsilosis*]	Similar to *C. albicans* glycosidase (Phr2)	60.8	4.33	211	4	7%	0.015972
gi|354547001	hypothetical protein CPAR2_213780 [*Candida parapsilosis*]	Similar to *C. albicans* chaperones of Hsp70 family (Kar2)	74.6	4.89	144	3	5%	0.014476
gi|354545372	hypothetical protein CPAR2_806490 [*Candida parapsilosis*]	Similar to *C. albicans* protein with internal repeats (Pir1)	41.5	4.60	292	5	12%	0.01411
gi|354548203	hypothetical protein CPAR2_407410 [*Candida parapsilosis*]	Similar to *C. albicans* cell surface mannoprotein (Mp65)	39.5	4.75	191	4	11%	0.010498
gi|354545590	hypothetical protein CPAR2_808670 [*Candida parapsilosis*]	Similar to *C. albicans* glyceraldehyde-3-phosphate dehydrogenase (Tdh3)	36.3	6.19	154	4	13%	0.009482
RPMI 1640								
gi|354548203	hypothetical protein CPAR2_407410 [*Candida parapsilosis*]	Similar to *C. albicans* cell surface mannoprotein (Mp65)	39.5	4.75	569	11	24%	0.013221
gi|354547255	hypothetical protein CPAR2_502140 [*Candida parapsilosis*]	Similar to *C. albicans* GPI-linked chitinase (Cht2)	61.2	4.23	387	7	10%	0.013208
gi|354547549	hypothetical protein CPAR2_400860 [*Candida parapsilosis*]	Similar to *C. albicans* GPI-anchored cell wall transglycosylase (Crh11)	52.0	4.09	298	6	8%	0.0095
gi|354544096	hypothetical protein CPAR2_108560 [*Candida parapsilosis*]	Similar to *C. albicans* GPI-anchored cell wall protein (Ecm33)	44.7	4.24	270	6	12%	0.00839
gi|354544910	hypothetical protein CPAR2_801850 [*Candida parapsilosis*]	Similar to *C. albicans* Ala-, Leu- and Ser-rich protein (Op4)	42.0	4.84	194	5	11%	0.00792
gi|354545390	hypothetical protein CPAR2_806670 [*Candida parapsilosis*]	Similar to *C. albicans* secreted yeast cell wall protein (Ywp1)	53.4	4.23	149	4	5%	0.00675
gi|354545372	hypothetical protein CPAR2_806490 [*Candida parapsilosis*]	Similar to *C. albicans* protein with internal repeats (Pir1)	41.5	4.60	306	4	12%	0.00517
gi|354544127	hypothetical protein CPAR2_108890 [*Candida parapsilosis*]	Similar to *C. albicans* endo-1,3-beta-glucanase (Eng1)	118.9	4.71	122	3	2%	0.00426
gi|354548325	hypothetical protein CPAR2_700650 [*Candida parapsilosis*]	Similar to *C. albicans* cell wall protein (Rbe1)	34.2	5.07	115	3	9%	0.00318
gi|354545615	hypothetical protein CPAR2_808920 [*Candida parapsilosis*]	Similar to *C. albicans* phospholipase 5 (Plb5)	94.7	4.24	63	1	1%	0.00286
gi|354544206	hypothetical protein CPAR2_109660 [*Candida parapsilosis*]	Similar to *C. albicans* glycosidase (Phr2)	60.8	4.72	95	1	6%	0.001829
gi|354547406	hypothetical protein CPAR2_503650 [*Candida parapsilosis*]	Similar to *C. albicans* protein similar to alpha agglutinin anchor subunit (Tos1)	47.3	3.82	95	1	1%	0.00143

Table 2. Mass spectrometry analysis of *C. tropicalis* proteins identified at the cell surface after growth under different conditions.
After cell surface shaving with trypsin and additional digestion of obtained proteins for 5 hours, peptides were analyzed using the Dionex Ultimate 3000 UHPLC system coupled to an HCTUltra ETDII mass spectrometer. The obtained lists of peaks were searched against the NCBI protein database using an in-house Mascot server (SC – sequence coverage).

YPD medium								
Accession	Protein	Description	Molecular mass [kDa]	pI	Score	Number of peptides	SC	NSAF
gi|255722347	hypothetical protein CTRG_00890 [*Candida tropicalis* MYA-3404]	Similar to *C. albicans* putative constitutive acid phosphatase (Pho113)	53.0	4.41	449	11	20%	0.014472
gi|255729208	pyruvate decarboxylase (Pdc11) [*Candida tropicalis* MYA-3404]	Involved in amino acid catabolism and the fermentation of glucose to ethanol	62.5	5.32	297	8	17%	0.012358
gi|255732890	glyceraldehyde-3-phosphate dehydrogenase (Tdh3) [*Candida tropicalis* MYA-3404]	Involved in glycolysis and gluconeogenesis, also in adhesion of symbiont to host	36.2	6.19	384	13	34%	0.004598
gi|255727881	enolase 1 (Eno1) [*Candida tropicalis* MYA-3404]	Involved in glycolysis and gluconeogenesis, major cell-surface antigen	47.0	5.54	401	9	35%	0.007928
gi|255722371	hypothetical protein CTRG_00902 [*Candida tropicalis* MYA-3404]	Similar to acid phosphatase	53.0	4.35	309	6	14%	0.012834
gi|255729820	conserved hypothetical protein CTRG_04132 [*Candida tropicalis* MYA-3404]	Similar to *C. albicans* putative inducible acid phosphatase (Pho100)	35.4	4.17	340	5	18%	0.003531
gi|255727428	phosphoglycerate kinase (Pgk1) [*Candida tropicalis* MYA-3404]	Involved in glycolysis and gluconeogenesis	44.7	5.98	258	3	18%	0.002105
gi|255725432	protein with internal repeats precursor (Pir1) [*Candida tropicalis* MYA-3404]	O-glycosylated protein required for cell wall stability	40.7	4.96	135	3	7%	0.000747
gi|255728227	hypothetical protein CTRG_03336 [*Candida tropicalis* MYA-3404]	Similar to *C. albicans* putative repressible vacuolar alkaline phosphatase (Pho8)	55.2	4.54	108	2	9%	0.002750
gi|255721433	hypothetical protein CTRG_00432 [*Candida tropicalis* MYA-3404]	Similar to *C. albicans* protein with similarity to RNase T2 enzymes (Rbt7)	37.3	4.63	124	3	16%	0.002846
gi|255732774	triosephosphate isomerase (Tpi1) [*Candida tropicalis* MYA-3404]	Abundant glycolytic enzyme	26.8	5.50	186	3	20%	0.011204
gi|255725714	hypothetical protein CTRG_02093 [*Candida tropicalis* MYA-3404]	Similar to *C. albicans* cell surface mannoprotein (Mp65)	39.1	4.77	116	2	11%	0.003242
gi|255724848	white colony protein (Wh11) [*Candida tropicalis* MYA-3404]	Involved in pathogenesis and phenotypic switching	7.0	4.85	152	4	78%	0.002094
YAPD medium								
gi|255727773	heat shock protein (Ssb1) [*Candida tropicalis* MYA-3404]	May be involved in folding of newly-made polypeptide chains	66.6	5.30	568	15	25%	0.011095
gi|255731011	heat shock protein 2 (Ssa2) [*Candida tropicalis* MYA-3404]	Involved in protein folding and vacuolar import of proteins	70.1	4.97	583	12	20%	0.013201
gi|255726910	elongation factor 3 (Cef3) [*Candida tropicalis* MYA-3404]	Involved in translational elongation and termination	117.1	5.49	301	7	9%	0.007746
gi|255725194	elongation factor 1-alpha (Tef1) [*Candida tropicalis* MYA-3404]	Involved in the interaction with host	50.3	9.14	520	15	25%	0.009863

gi|255720819	ATP synthase beta chain, mitochondrial precursor (Atp2) [*Candida tropicalis* MYA-3404]	Involved in transport of protons against an electrochemical gradient, using energy from ATP hydrolysis	44.5	4.89	527	13	35%	0.006271
gi|255732890	glyceraldehyde-3-phosphate dehydrogenase (Tdh3) [*Candida tropicalis* MYA-3404]	Involved in glycolysis and gluconeogenesis, also in adhesion of symbiont to host	36.2	6.19	518	13	45%	0.00429
gi|255727881	enolase 1 (Eno1) [*Candida tropicalis* MYA-3404]	Involved in glycolysis and gluconeogenesis, major cell-surface antigen	47.0	5.54	376	9	25%	0.005685
gi|255721021	malate dehydrogenase, mitochondrial precursor (Mdh1) [*Candida tropicalis* MYA-3404]	Participates in the citric acid cycle	34.8	6.39	458	9	37%	0.010552
gi|255721795	heat shock protein 60, mitochondrial precursor (Hsp60) [*Candida tropicalis* MYA-3404]	Prevents aggregation and mediates protein refolding after heat shock	60.5	5.21	206	5	13%	0.004085
gi|255727899	transketolase 1 (Tkl1) [*Candida tropicalis* MYA-3404]	Involved in pentose-phosphate shunt	73.9	5.65	132	3	6%	0.002389
gi|255727428	phosphoglycerate kinase (Pgk1) [*Candida tropicalis* MYA-3404]	Involved in glycolytic process	44.7	5.98	169	4	11%	0.002916
gi|255723558	conserved hypothetical protein CTRG_06190 [*Candida tropicalis* MYA-3404]	Similar to *C. albicans* putative F0-ATP synthase FO subunit B (Atp5)	22.0	9.52	100	3	10%	0.000511
gi|255721461	hypothetical protein CTRG_00446 [*Candida tropicalis* MYA-3404]	Similar to *C. albicans* nascent polypeptide associated complex protein alpha subunit (Egd2)	19.4	4.63	126	2	21%	0.000879
RPMI 1640								
gi|255721523	predicted protein [*Candida tropicalis* MYA-3404]	Similar to *C. albicans* cell wall protein with similarity to Hwp1 (Rbt1)	99.0	4.00	329	8	6%	0.008288
gi|255722852	predicted protein CTRG_05838 [*Candida tropicalis* MYA-3404]	Similar to *C. albicans* hyphally regulated cell wall protein (Hyr3)	194.8	3.65	142	3	1%	0.006937
gi|255722623	conserved hypothetical protein CTRG_01028 [*Candida tropicalis* MYA-3404]	Similar to *C. albicans* cell-wall agglutinin-like protein (Als1)	79.5	4.46	231	6	6%	0.022078
gi|255729942	hypothetical protein CTRG_04193 [*Candida tropicalis* MYA-3404]	Similar to *C. albicans* cell wall acid trehalase (Atc1)	120.9	4.57	210	4	4%	0.006595
gi|255722347	hypothetical protein CTRG_00890 [*Candida tropicalis* MYA-3404]	Similar to *C. albicans* putative constitutive acid phosphatase (Pho113)	53.0	4.41	418	6	15%	0.003862
gi|255722371	hypothetical protein CTRG_00902 [*Candida tropicalis* MYA-3404]	Similar to acid phosphatase	53.0	4.35	381	5	15%	0.013154
gi|255725714	hypothetical protein CTRG_02093 [*Candida tropicalis* MYA-3404]	Similar to *C. albicans* cell surface mannoprotein (Mp65)	39.1	4.77	300	6	28%	0.012869
gi|255729820	conserved hypothetical protein CTRG_04132 [*Candida tropicalis* MYA-3404]	Similar to *C. albicans* putative inducible acid phosphatase (Pho100)	35.4	4.17	287	4	13%	0.003101
gi|255732521	alcohol dehydrogenase 2 (Adh2) [*Candida tropicalis* MYA-3404]	Required for the reduction of acetaldehyde to ethanol during fermentation	37.2	6.54	165	3	9%	0.017396
gi|255732890	glyceraldehyde-3-phosphate dehydrogenase (Tdh3) [*Candida tropicalis* MYA-3404]	Involved in glycolysis and gluconeogenesis, also in adhesion of symbiont to host	36.2	6.19	136	3	12%	0.002994

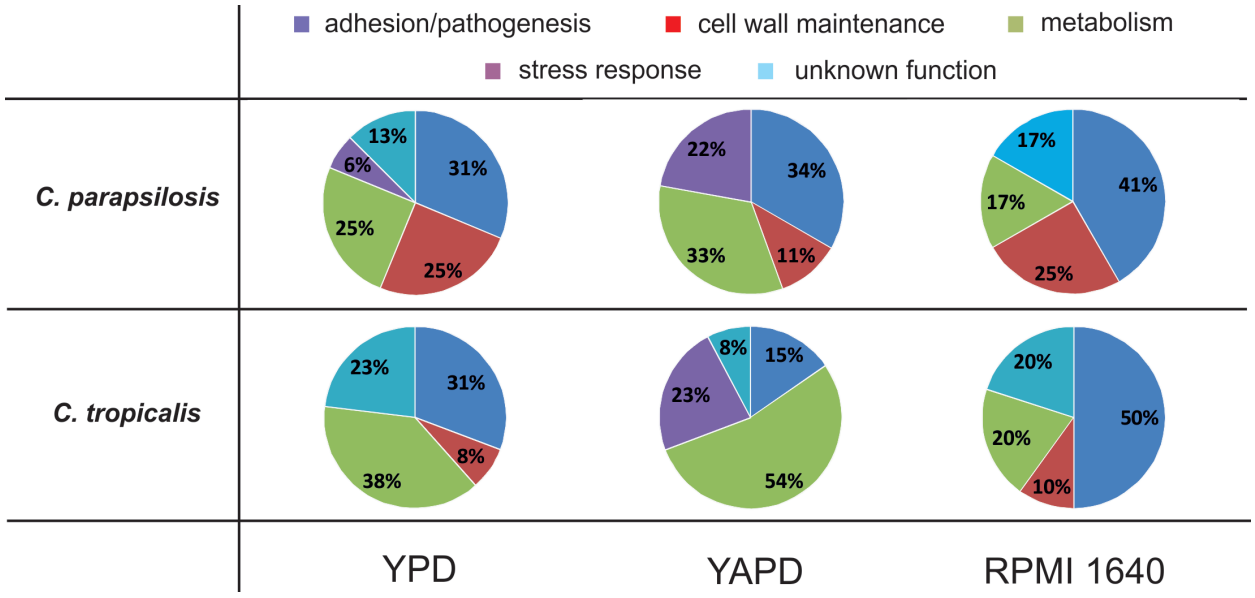

Figure 2. Functional classification of *C. parapsilosis* or *C. tropicalis* surface-exposed proteins identified with the cell surface shaving method and mass spectrometry.
Protein functions have been assigned on the basis of descriptions from NCBI protein database and orthologs' description in the Candida Genome Database.

analyses are summarized in Fig. 2, demonstrating some dependence between the functional profile of fungal surfaceome and the type of culture conditions and medium used. In case of *C. parapsilosis* cells, grown in RPMI 1640 medium, the most abundant group of proteins was that containing the adhesins and other virulence factors (41%). For *C. parapsilosis* cells cultured in YPD or YAPD, the pathogenesis-related proteins were also the most frequent (31% and 34%, respectively). However, for unicellular, yeast-like forms two groups of proteins — those involved in the cell wall maintenance or in the basic metabolism — were also strongly represented (both at 25%). What drew our particular attention in case of *C. parapsilosis* cells cultured in YAPD — a nitrogen-deficient medium with lysate of animal proteins — was that the largest number of proteins involved in the stress response (22%) was present at the fungal cell surface in comparison to other conditions applied. Similarly, for *C. tropicalis* cells grown in YAPD medium, 23% of proteins were assigned as molecules involved in cell response to stress conditions and, moreover, it was the only sample wherein proteins from this group were found. Apart from that, about half of all identified proteins (54%) were enzymes from basic metabolic pathways.

A comparable number of *C. tropicalis* proteins was identified as responsible for both physiological metabolism and pathogenesis (38% and 31%, respectively) in case of yeast-like forms grown in YPD broth. Importantly, 50% of proteins present at the cell surface of *C. tropicalis* pseudohyphae generated in RPMI 1640, were assigned as playing a role in the pathogenesis and adherence. It should be noted, however, that almost in each tested sample from both species there were some proteins with unknown/unassigned function and for this reason the described proportions could possibly slightly change after further characterization of these proteins.

Human proteins bound at the cell surface of *C. parapsilosis* and *C. tropicalis*

An approach similar to that described above, based on the shaving of the cell surface with trypsin followed by additional, prolonged digestion, was also applied to *C. parapsilosis* and *C. tropicalis* cells grown in YPD or RPMI 1640 medium in the presence of human plasma at 37°C for 48 hours. The final identification of peptides obtained was classified as derived from human plasma proteins that were apparently adsorbed to the fungal cell surface and, as previously, from fungal proteins exposed at the cell surface of pseudohyphae (Table 3). A significantly lesser amount of candidal proteins found in these peptide samples as compared to the above-described determinations — six for *C. parapsilosis* and three for *C. tropicalis* — was probably a result of quite a large number of bound human proteins, apparently covering the surface of fungal cell and reducing its availability for trypsin. However, in spite of such difficulties, we were capable to indicate three proteins from each investigated *Candida* species, whose exposure at the cell surface was at least three-fold higher in the medium with human plasma than at the same conditions but without plasma. The differences have been calculated on the basis of NSAF obtained for the tested peptide samples. In case of *C. parapsilosis*, a protein from CFEM family and proteins similar to Ecm33 and Pir1 — two typical cell wall proteins — were more abundant at the cell surface after fungal contact with human plasma. In the case of *C. tropicalis*, apart from atypical protein Tdh3, two cell-surface proteins — a protein similar to Rbt1 and an adhesin from agglutinin-like sequence protein family — were indicated as exposed at the cell surface in greater quantities, if pseudohyphae were formed in plasma-containing medium.

In addition, interesting results were obtained after searching the SwissProt protein database with restriction to human proteins in order to find molecules attached to the surface of fungal cells after growth under these "bloodstream infection-mimicking" conditions. 20 different proteins for *C. parapsilosis* and 19 for *C. tropicalis* were identified as adsorbed to the surface of fungal cells. The referred sets of identified human proteins were rather comparable in case of both investigated species, indicating a significantly shared preference for apolipoproteins and complement components in bind-

Table 3. Identification of human plasma proteins attached to the cell surface of *C. parapsilosis* and *C. tropicalis* cells grown in YPD broth or RPMI 1640 medium supplemented with 10% of human plasma and indication of fungal proteins, whose exposure at the cell surface under these conditions was at least three-fold higher than in a medium without plasma (marked with ↑).
After cell surface shaving, peptides were analyzed using the Dionex Ultimate 3000 UHPLC system coupled to an HCTUltra ETDII mass spectrometer. The obtained lists of peaks were searched against the NCBI protein database and SwissProt protein database using an in-house Mascot server.

	Surface-bound human proteins	Surface-exposed fungal proteins
C. parapsilosis	alpha-1-antitrypsin alpha-2-HS-glycoprotein apolipoprotein A-1 apolipoprotein A-II apolipoprotein A-IV apolipoprotein B-100 apolipoprotein E clusterin complement C3 complement C4-A complement C5 complement component C9 complement factor B fibrinogen chain alpha gelsolin fibrinogen chain beta heparin cofactor 2 fibrinogen chain gamma paraoxonase/arylesterase 1 vitronectin serum albumin serum amyloid A-4 protein	hypothetical protein CPAR2_407410, similar to *C. albicans* cell surface mannoprotein (**Mp65**) hypothetical protein CPAR2_502140, similar to *C. albicans* GPI-linked chitinase (**Cht2**) hypothetical protein CPAR2_806490, similar to *C. albicans* protein with internal repeats (**Pir1**) ↑ hypothetical protein CPAR2_400860, similar to *C. albicans* GPI-anchored cell wall transglycosylase (**Crh11**) hypothetical protein CPAR2_108560, similar to *C. albicans* GPI-anchored cell wall protein (**Ecm33**) ↑ **CFEM5**, putative membrane protein with unknown function ↑
C. tropicalis	alpha-1-antitrypsin angiotensinogen apolipoprotein A-1 apolipoprotein A-IV apolipoprotein B-100 apolipoprotein C-III apolipoprotein E clusterin complement C3 complement C4-A complement C5 complement component C9 fibrinogen chain alpha gelsolin fibrinogen chain beta hemoglobin subunit beta fibrinogen chain gamma heparin cofactor 2 paraoxonase/arylesterase 1 vitronectin serum albumin	predicted protein similar to *C. albicans* cell wall protein with similarity to Hwp1 (**Rbt1**) ↑ conserved hypothetical protein similar to *C. albicans* cell-wall agglutinin-like sequence protein (**Als1**) ↑ glyceraldehyde-3-phosphate dehydrogenase (**Tdh3**) ↑

ing to the cell surface of *C. parapsilosis* and *C. tropicalis* as compared to other plasma proteins. In addition, the alpha-1-antitrypsin (a protease inhibitor from serpin family), three chains of fibrinogen, and vitronectin — a glycoprotein found not only in plasma, but also in extracellular matrix — as well as the most abundant plasma protein, namely albumin, were also found at the cell surface of both investigated species. A noticeable difference between these two species concerned alpha-2-HS-glycoprotein (cystatin type 3) and serum amyloid protein identification in case of *C. parapsilosis* cells and hemoglobin beta subunit- and angiotensinogen binding noticed in the case of *C. tropicalis* cells.

DISCUSSION

The candidal cell wall is a dynamic and complicated structure, in the large part built of polysaccharides but the proteins are thought to be the main players in the initiation of the contact with host proteins and cells, and in further development of infection (Chaffin, 2008). Therefore, the identification of surface-exposed proteins of two emerging fungal pathogens — *C. parapsilosis* and *C. tropicalis* — might provide new, important insights into molecular determinants of their pathogenesis. Recently, the cell wall proteomes of several pathogenic fungi have been extensively studied using a broad range of different fractionating methods combined with mass spectrometry (Karkowska-Kuleta & Kozik, 2015). Nevertheless, the complete proteomes of *C. parapsilosis* and *C. tropicalis* are not yet fully recognized; however, some first reports of the presence of enolase, elongation factor 1-beta, heat shock protein Ssb1, fructose-bisphosphate aldolase and glyceraldehyde-3-phosphate dehydrogenase at the cell surface of *C. parapsilosis* yeast-like cells under conditions of oxidative stress have recently been published (Ramírez-Quijas *et al.*, 2015).

In this work, an uncomplicated and fast method based on tryptic digestion of proteins exposed at the cell surface of live cells was used, enabling us to characterize the surfaceome of two investigated *Candida* species. In comparison to the most recent studies concerning the cell surface shaving of *C. albicans* yeast and hyphal cells (Gil-Bona *et al.*, 2015b), our analyses showed both similarities and differences between this most widespread opportunistic pathogen and the two investigated non-albicans *Candida* species. Among the twenty most abun-

dant surface-exposed proteins indicated by Gil-Bona *et al.* (2015b) for *C. albicans* yeast forms, we found orthologs of Tdh3 and Eno1 in both non-albicans *Candida* species and, additionally, of Mp65 and Adh1 in *C. parapsilosis*, and of Pgk1 and Wh11 in *C. tropicalis*. However, in comparison to the most abundant proteins identified at the cell surface of *C. albicans* filamentous forms, in the present study only Tdh3 ortholog was found in the highest abundance among *C. tropicalis* proteins. Nevertheless, many other proteins identified by us at the cell surface of *C. parapsilosis* and *C. tropicalis* have their orthologs at the surface of *C. albicans* yeast or hyphae listed by Gil-Bona *et al.* (2015b) as proteins present at the cell wall with a lower abundance.

Because Tdh3, Eno1, Pgk1 and Adh1 are enzymes derived from cytoplasm and involved in glycolysis, gluconeogenesis and fermentation, their presence at the cell surface, although usually considered as atypical, is often described in literature on *C. albicans* (Gil-Navarro *et al.*, 1997; Urban *et al.*, 2003; López-Villar *et al.*, 2006; Karkowska-Kuleta & Kozik, 2014). These proteins are also reported as immunogenic during infections in humans (Pitarch *et al.*, 2001; Fernández-Arenas *et al.*, 2004; Pitarch *et al.*, 2004) and as molecules involved in binding of human host proteins, i.e., plasminogen, kininogen, integrins and extracellular matrix proteins (Gozalbo *et al.*, 1998; Klotz *et al.*, 2001; Jong *et al.*, 2003; Crowe *et al.*, 2003; Karkowska-Kuleta *et al.*, 2011). Admittedly, a question how such proteins are exported from cytoplasm is still relevant. Different mechanisms have been proposed to clarify this issue, including secretion of extracellular vesicles, membrane flipping, ineligible entry to secretory vesicles or exosome release (Nombela *et al.*, 2006; Nickel & Rabouille, 2009; Gil-Bona *et al.*, 2015a). On the other hand, there is still a concern, that such cytoplasmic proteins might originate from damaged cells; however, a strict control of cell viability during experiments performed in this study allowed to largely avoid such artifacts.

Apart from those four abovementioned proteins, some other cytoplasmic proteins have been identified in this study at *C. parapsilosis* or *C. tropicalis* cell surface, including heat shock proteins, elongation factors and subunits of ATP synthase, especially in nitrogen-limited medium YAPD. The increased presence of this group of proteins at the cell surface might lead to a conclusion, that these particular growth conditions not only induce filamentation, but might also stimulate the fungal cell's response to environmental stress and trigger the adaptive changes within the cell wall (Lee *et al.*, 2014; Rane *et al.*, 2014; Ramírez-Quijas *et al.*, 2015).

Furthermore, a well represented group of proteins involved in the cell wall building and maintenance was observed in this study both for *C. tropicalis* and *C. parapsilosis* cells. These proteins are mainly covalently bound to the cell wall, in contrast to those discussed above. Therefore, they are often called typical or classical cell wall proteins. There are at least three main types of linkages between proteins and a polysaccharide scaffold, including GPI-anchoring, alkali-sensitive linkages (ASL) and disulfide bridges; however, there is a large body of evidence that different mixed types of bonds occur within such the complicated structure of the cell wall (de Groot *et al.*, 2004; Pitarch *et al.*, 2008; Klis *et al.*, 2009; Boisramé *et al.*, 2011). Several of these typical proteins can be involved in adhesion to host proteins, thus contributing to fungal virulence and invasion of a human organism. One example is glucanase Mp65, a cell surface mannoprotein bound to *C. albicans* cell wall via ASL and disulfide bridges (Caminero *et al.*, 2014) that also acts as an antigen and an adhesin strongly related to *C. albicans* virulence (Sandini *et al.*, 2011). We found an orthologous protein similar to Mp65 both at the surface of *C. parapsilosis* yeast-like cells and pseudohyphae — where it was the most abundant protein — as well as at the surface of both morphological forms of *C. tropicalis*. As Mp65 is involved in the adhesion to plastics (Sandini *et al.*, 2007), this observation might partially elucidate the high ability of *C. parapsilosis* to adhere to and form biofilms at the artificial surfaces, including a variety of medical devices (Trofa *et al.*, 2008). Other important proteins involved in the cell wall maintenance were identified at the cell surface of non-albicans *Candida* species with the use of trypsin shaving. For *C. parapsilosis*, at least the following orthologous proteins should be mentioned: Cht2, Ecm33, Phr2 and Crh11 anchored to cell wall via GPI remnant (de Groot *et al.*, 2004; Castillo *et al.*, 2008), Eng1 and Bgl2 attached to the cell wall *via* ASL and/or disulfide bonds (Caminero *et al.*, 2014) and Pir1, a structural protein attached to cell wall β-1,3-glucan through ASL (de Groot *et al.*, 2004). An ortholog of the latter was also identified in *C. tropicalis* cell wall.

Among some interesting putative GPI-anchored proteins, in this study we identified a protein similar to Plb5 at the surface of *C. parapsilosis* pseudohyphae. At the cell surface of *C. tropicalis* pseudohyphae, apart from an ortholog of Rbt1 — a GPI-linked protein with a similarity to Hwp1, required for fungal virulence (Braun *et al.*, 2000) — we also found two proteins from distinct large families gathering important adhesins, namely agglutinin-like sequence (Als) protein family and Iff/Hyr protein family. This observation suggests that like in the case of *C. albicans*, *C. tropicalis* filamentous forms possess strongly adhesive properties that help this pathogen to invade the host organism. It is well known that formation of filamentous forms is the essential step for full fungal pathogenicity (Diez-Orejas *et al.*, 1999; Thompson *et al.*, 2011).

The important role in *C. tropicalis* of a protein similar to Rbt1, a protein from Als protein family, and additionally Tdh3 during the invasion might be confirmed by the observed increase of the quantity of these proteins at the cell surface after growth in the medium containing human plasma, which mimics — to some extent — the contact with host proteins during bloodstream infection. In case of *C. parapsilosis*, an analogous effect was found for proteins similar to Pir1, Ecm33 and CFEM5. On the other hand, the investigation of surfaceome under these conditions preliminarily revealed a set of human proteins that can adhere to the fungal cell surface. Among them, as expected, the most abundant human plasma protein — albumin — was found, but several apolipoproteins, complement components and other proteins were also identified, not only ones most abundantly present, but also those less frequently represented in the plasma (Anderson & Anderson, 2002). Because a key role of the complement cascade in innate immunity is to recognize and eliminate pathogenic microorganisms from bloodstream after their opsonization or perforation of their cellular membranes via membrane-attacking complex engaging complement component C9 (Peitsch & Tschopp, 1991), its function for host defense during disseminated candidiasis is essential (Boxx *et al.*, 2010). Hence, the identification at the *C. parapsilosis* and *C. tropicalis* cell surface of several complement components — C3, C4-A, C5, C9 — after the contact of fungal cells with human plasma might be related to triggering the complement pathways activation, following the binding of its components to the fungal surface. However, it is well known,

at least in the case of *C. albicans*, that some surface-exposed proteins, such as Pra1, Gpm1 and Gpd2, might be involved in the binding of a few complement factors and regulators — C4BP, factor H and FHL1 — thereby participating in gaining control over this system by the fungus in order to inhibit complement activation and to facilitate immune evasion (Poltermann *et al*., 2007; Luo *et al*., 2011; Luo *et al*., 2013). There is also some evidence, that representatives of the second, most abundant group of proteins shown herein as bound to *C. parapsilosis* and *C. tropicalis* cell surface — namely apolipoproteins — might play an important role in the host resistance against fungal infections (Vonk *et al*., 2004). What is more, the plasma amyloid protein, found here as adhered to the cell surface of *C. parapsilosis*, is also a chemoattractant for polymorphonuclear cells, increasing their antifungal action (Badolato *et al*., 2000).

Apart from these strongly represented groups of human proteins — the complement components and apolipoproteins — fibrinogen and vitronectin were also identified as attached to the *C. parapsilosis* and *C. tropicalis* cell surface. As the latter is the important, adhesive protein present not only in plasma but also in the extracellular matrix, its binding may promote fungal adhesion to host tissues during the first step of infection and further dissemination of fungal cells within the organism via a circulatory system (Lopez *et al*., 2014). It should be noted that hijacking the important host system such as the coagulation cascade by pathogens greatly facilitates their pathogenicity and it is a well known process occurring during bacterial infections (Rivera *et al*., 2007).

One of the surface-bound human proteins identified in the case of *C. tropicalis* was hemoglobin, being not only the necessary and important source of iron for microorganism, but also the stimulator of increased adhesin exposure (i.e., of Hwp1) at the fungal cell surface and development of the hyphal forms as it has been previously described for *C. albicans* (Yan *et al*., 1998; Pendrak & Roberts, 2007). Quite similar results, corroborating with data obtained for *C. parapsilosis* and *C. tropicalis* and concerning the host plasma proteins' binding to fungal cell surface, were described for another important fungal pathogen of humans, *Paracoccidioides brasiliensis*. In the case of this species, complement factor C3, albumin, vitronectin, fibrinogen, alpha-1-antitrypsin, angiotensinogen, apolipoproteins B-100 and A-1, paraoxonase/arylesterase 1 and alpha-2-HS-glycoprotein were identified (Longo *et al*., 2013). Due to such reports it now becomes possible to recognize more thoroughly the various aspects of interactions between fungal pathogen and a human host.

To our knowledge, this work is the first study describing the surfaceome — the proteome of cell surface — of two non-albicans *Candida* species, i.e., *C. parapsilosis* and *C. tropicalis*, albeit for the former species there is now some information available about a few proteins exposed at the cell surface of the yeast-like cells during the oxidative stress (Ramírez-Quijas *et al*., 2015). Revealing the *Candida* spp. cell wall protein composition with the use of proteomic methods and mass spectrometry is a very important contribution to the exploration of potentially new virulence attributes of these fungi. However, addressing the physiological and functional questions related to the known proteome is also of a great importance. Recently, important attempts to indicate the immunogenic proteins of *C. parapsilosis* and *C. tropicalis* were made, with a finding of twelve proteins for each species as inducing the antibody response (Lee *et al*., 2014a; Lee *et al*., 2014b). In the current study we confirmed the presence of some of them at the cell surface of *C. parapsilosis* cells, i.e., proteins similar to Adh1, Atp1, Eno1, Tdh3 and Pdc11, and *C. tropicalis* cells, i.e., proteins similar to Atp2, Eno1, Tdh3 and Tpi1.

As the particular species of *Candida* genus are considered to differ from each another in molecular mechanisms of pathogenicity, drug resistance, epidemiology, risk factors among various groups of infected patients and the level of mortality (Krcmery & Barnes, 2002), these differences might be reflected in the set of proteins exposed at their cell surface. In the case of *C. tropicalis*, the frequent presence of alkaline and acid phosphatases was particularly interesting and correlated with previous findings (Sangar *et al*., 1975), and this feature distinguishes *C. tropicalis* from other *Candida* species diagnostically.

To summarize, the obtained results might markedly contribute to a better understanding of the complicated phenomenon — the host-pathogen interaction during candidal infections — and significantly expand the scope of designing new antifungal drugs, vaccines and diagnostic methods for other species of the *Candida* genus.

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