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Research Article

Revealing *Candida glabrata* biofilm matrix proteome: global characterization and pH response

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Candida glabrata is a clinically relevant human pathogen with the ability to form high recalcitrant biofilms that contribute to the establishment and persistence of infection. A defining trait of biofilms is the auto-produced matrix, which is suggested to have structural, virulent and protective roles. Thus, elucidation of matrix components, their function and modulation by the host environment is crucial to disclose their role in *C. glabrata* pathogenesis. As a major step toward this end, this study aimed to reveal, for the first time, the matrix proteome of *C. glabrata* biofilms, to characterize it with bioinformatic tools and to study its modulation by the environmental pH (acidic and neutral). The results showed the presence of several pH-specific matrix proteins (51 acidic- and 206 neutral-specific) and also proteins commonly found at both pH conditions (236). Of note, several proteins related to mannan and β-glucan metabolism, which have a potential role in the delivery/organization of carbohydrates in the matrix, were found in both pH conditions but in much higher quantity under the neutral environment. Additionally, several virulence-related proteins, including epithelial adhesins, yapsins and moonlighting enzymes, were found among matrix proteins. Importantly, several proteins seem to have a non-canonical secretion pathway and Pdr1 was found to be a potential regulator of matrix proteome. Overall, this study indicates a relevant impact of environmental cues in the matrix proteome and provides a unique resource for further functional investigation of matrix proteins, contributing to the identification of potential targets for the development of new therapies against *C. glabrata* biofilms.

Introduction

Candida species are opportunistic human pathogens capable of causing a broad range of infections [1–3]. Although *Candida albicans* is the main cause of candidiasis, recent epidemiologic surveys have pointed to an increase in infections caused by non-*Candida albicans Candida* species, mainly *Candida glabrata* [1,2]. Importantly, *C. glabrata* causes complicated infections that range from agonizing episodes of vulvovaginal candidiasis to life-threatening candidemia and that are associated with high morbidity and mortality (∼50%) rates [1,2,4]. The high clinical relevance of *C. glabrata* infections, is mainly attributed to the intrinsic and acquired antifungal resistance of this species [5,6], as well as to its ability to display several virulence factors, one of the most important is the ability to form biofilms on mucosae and medical devices [7,8]. Of note, the development of *C. glabrata* biofilms in venous central catheter is a predictor for mortality in patients with blood stream candidiasis [9,10]. Indeed, biofilm-related infections caused by *C. glabrata* are extremely difficult to treat due to the high resilience of its biofilms to antifungals and host defences [10,11].

One of the main challenges faced by *Candida* species during commensal and pathogenic lifestyles is the wide range of environmental pH in host niches, from acidic (vagina and stomach) to neutral and slightly alkaline (bloodstream and gut) [12,13]. Additionally, we have recently shown that the pH has

a significant impact on *C. glabrata* biofilm formation and modulates the amount of matrix produced [14]. The extracellular matrix is a defining trait of biofilms that derives from direct synthesis, secretion of components and lysis of a fraction of biofilm cells [15]. This structure has been shown to mediate adhesive and cohesive interactions, to provide a mechanically stable infrastructure for biofilm accumulation, to control biofilm dispersion and to protect biofilms from the host immunity and antifungals [16].

In the matrix of *C. glabrata* biofilms, carbohydrates and proteins have been mostly found, although lipids and nucleic acids were also described in lower quantities [17]. A relevant role of matrix carbohydrates, mainly β-glucans, in the antifungal resistance of *C. glabrata* biofilms has been demonstrated [17,18]. Proteins identified in the matrix of *C. albicans* biofilms were proposed to contribute to the delivery and organization of matrix carbohydrates, to act as a digestive system that provides a nutrient source for biofilm cells, to impair immunity host response and may be also related with the control of biofilm dispersion [19]. Importantly, some matrix proteins of *C. albicans* biofilms have been shown to be promising targets for anti-biofilm therapeutics [20,21]. However, proteins of *C. glabrata* biofilm matrix have been largely understudied and in contrast with *C. albicans*, the biofilm matrix proteome was not described yet. To cover this relevant scientific flaw, the aim of this study was to reveal the complex proteome of *C. glabrata* biofilm matrix, to characterize it with bioinformatic tools and to study its modulation by the environmental pH. This study is essential to increase the understanding of the role of matrix proteins in *C. glabrata* pathogenesis and their modulation by niche-specific environmental cues.

Methods

Initial culture conditions

In this study, the reference strain *C. glabrata* ATCC 2001 was used. *Candida glabrata* cells were grown on Sabouraud dextrose agar (SDA; Merck) for 48 h at 37°C, followed by 18 h in Sabouraud dextrose broth (SDB; Merck) under agitation (120 rev/min). The cells were recovered by centrifugation and washed twice with ultrapure sterile water. Then, the pellet was resuspended in Roswell Park Memorial Institute medium (RPMI; Sigma–Aldrich), buffered with 3-(N-Morpholino) propanesulfonic acid (MOPS; Sigma–Aldrich) and adjusted to pH 7 or pH 4 (with lactic acid).

Biofilm formation and matrix extraction

The development of biofilms and matrix extraction were performed as previously described [14]. Briefly, the cellular suspensions prepared at pH 7 and pH 4 were adjusted to 1×10^5 cells/ml and placed into wells of 24-wells polystyrene microtiter plates (Orange Scientific). The plates were incubated for 24 h at 37°C under 120 rev/min. Developed biofilms were washed with sterile water, scraped from the wells and sonicated (Ultrasonic Processor) for 30 s at 30 W in order to separate the matrix from biofilm's cells, through mechanical disruption [22]. Previously, a correlation curve of time and/or strength of sonication vs. quantity of protein detected in the matrix was performed to define the suitable sonication conditions (Supplementary Figure S1). To avoid the possibility of contamination of matrix proteins with proteins from intracellular and *C. glabrata* cell wall, the integrity of yeast cells was evaluated by microscopy observation after sonication (data not shown). The biofilm suspensions were then centrifuged at 5000g for 5 min and the matrix-containing supernatant was filtered through a 0.2 μm nitrocellulose filter. The protein quantity in the samples was measured with the BCA Kit (Bicinchoninic Acid, Sigma–Aldrich), as previously described [14], and the samples were processed and analyzed as described next.

Proteomic analysis

To identify matrix proteins, two independent matrix replicates obtained at each pH condition were analyzed by nano Liquid chromatography tandem mass spectrometry (LC–MS/MS). First, the protein-containing samples were digested following an already described procedure [23] with some modifications. Briefly, protein extracts were diluted in 8 M urea/100 mM Triethylamonium bicarbonate (TEAB), centrifuged and washed in the same solution. Next, protein samples were reduced and alkylated with 10 mM tris(2-carboxyethyl)phosphine (TCEP), 40 mM Carboxylic acid amide (CAA), in 8 M urea/100 mM TEAB for 30 min in the dark at 30°C, centrifuged, followed by a first wash in 8 M urea/100 mM TEAB and a second wash in 50 mM TEAB. Protein digestion with trypsin/Lys-C mix was performed overnight at 37°C in 50 mM TEAB. The reaction was stopped with 1% Trifluoroacetic acid (TFA). Peptides were then recovered by centrifugation followed by an additional

centrifugation step with 0.1% TFA. Next, peptide samples were cleaned-up and concentrated by chromatography. Protein identification was then performed by nano LC–MS/MS, in an equipment composed by an Ultimate 3000 liquid chromatography system coupled to a Q-Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Scientific) [24]. Data acquisition was controlled by Xcalibur 4.0 and Tune 2.8 software (Thermo Scientific).

Bioinformatic analyses

The raw data of LC–MS/MS analysis were processed using Proteome Discoverer 2.2.0.388 software (Thermo Scientific) and searched against the UniProt database [25] for the taxonomic selection *C. glabrata*. The Sequest HT search engine was used to identify tryptic peptides. The ion mass tolerance was 10 ppm for precursor ions and 0.02 Da for fragmented ions. Maximum allowed missing cleavage sites was set to 2 and peptide confidence was set to high. The processing node Percolator was enabled with the following settings: maximum delta Cn 0.05; decoy database search target FDR 1%, validation of based on *q*-value. The proteins of all matrix samples were identified and those commonly found in both replicates of each condition were selected for further analyses.

The Venny tool 2.1.0, provided by BioinfoGP (Bioinformatics for Genomics and Proteomics) [26], was used to compare the matrix proteins found at pH 7 with those found at pH 4. Venny was also used to compare the matrix proteome of *C. glabrata* biofilms with that of *C. albicans* [19,27,28] as well as with the secretome of *C. glabrata* [29], *C. albicans* [27] and *Saccharomyces cerevisiae* [30] species. For these comparisons the orthologous proteins of *C. albicans* and *S. cerevisiae* in *C. glabrata* were previously obtained in PathoYeastract database [31]. Moreover, the PathoYeastract was also used to search for transcriptional regulators of genes coding *C. glabrata* biofilm matrix proteins. Additionally, the predictive secretory nature of matrix proteins was analyzed using the Fungal Secretome Database (FSD) [32] and Fungal Secretome KnowledgeBase (FunSecKB) [33].

Functional, molecular and statistical analyses

The functional description of all identified proteins was obtained with FungiFun 2.2.8 tool [34] using the classification ontology provided by the Functional Catalogue (FunCat) database [35]. Additionally, a functional enrichment analysis was conducted on FungiFun, using the whole proteome of *C. glabrata* ATCC 2001 as background and the Fisher-exact test with Benjamini–Hochberg correction [34,36], as a statistical test. Furthermore, the set of proteins obtained at different environmental conditions and annotated with similar functional categories were statistically compared using the 'N − 1' Chi-squared test [37,38]. All tests were performed with a confidence level of 95%.

Table 1 Number of *Candida glabrata* biofilm matrix proteins identified at pH 4 and/or pH 7

	Candida glabrata biofilm matrix proteins		
	pH 4-specific	pH 7-specific	both conditions
Total	51	206	236
Reported in *C. glabrata* planktonic secretome [29]	6	3	61
Orthologs in *C. albicans* biofilm matrix [19,27]	17	141	181
Orthologs in *C. albicans* and *S. cerevisiae* planktonic secretome [27,30]	6	22	51
Predicted extracellular localization by orthology [27]	2	9	17
Orthologs in *C. albicans* biofilm extracellular vesicles [28]	15	91	150
Predicted secretory nature [32,33]	25	98	131
Predicted GPI-anchor [41]	7	2	23

The table presents the number of *C. glabrata* biofilm matrix proteins identified exclusively in biofilms developed at pH 4, pH 7, at both conditions and also the number of proteins previously reported in the biofilm matrix or extracellular region of *Candida* species and other fungi as well as with predicted secretory nature and GPI-anchor.

The Gene Ontology (GO) Slim Mapper tool of *Candida* Genome Database (CGD) [27] was also used to categorize the proteins according to their molecular activity and cellular localization ontologies. Furthermore, the molecular interactions between proteins were analyzed using STRING 11.0 database [39] and then the proteins were clustered with the Cytoscape tool [40]. The minimum required interaction score of STRING analysis was set to 0.4 (medium confidence).

Results

Environmental pH modulates the matrix proteome

The identification of *C. glabrata* matrix proteins by LC–MS/MS revealed a total of 493 proteins, which include 236 proteins found in the matrix of biofilms developed at pH 4 and 7, 206 exclusively found in neutral conditions and 51 proteins exclusively found in acidic conditions (Table 1). The systematic names of all matrix proteins are listed in the Supplementary Table S1, along with the information on their pH-specificity.

Species- and biofilm-specific matrix proteins

The matrix proteins found in this study were compared with those recently identified in *C. glabrata* planktonic secretome [29]. This analysis revealed an overlap of 70 proteins (including six acidic-specific and three neutral-specific), corresponding to 58% of the reported planktonic secretome (Figure 1 and Supplementary Table S1). Additionally, matrix proteins were compared with those previously reported in the biofilm matrix or extracellular region of other *Candida* species and fungal genera. The comparison with *C. albicans* revealed that 339 proteins found in this study (including 17 acidic-specific and 141 neutral-specific) are orthologs of proteins previously found in the matrix of *C. albicans* biofilms (Table 1 and Supplementary Table S1) [19,27,28]. Additionally, the comparison with orthologous proteins secreted by *C. albicans* or *S. cerevisiae* planktonic cells showed an overlap of 114 proteins (Table 1 and Supplementary Table S1) [27,30]. Moreover, the comparison with orthologs secreted by other fungi (including *Candida parapsilosis*, *Candida dubliniensis*, *Candida auris*, *Aspergillus nidulans*, *Schizosaccharomyces pombe* and *Neurospora crassa*) revealed an overlap of 28 proteins (Table 1 and Supplementary Table S1) [27]. Of note, the majority of secreted proteins previously reported in *Candida* species or other fungi were identified in uncontrolled or unbuffered pH environments. The combination of the various comparative analyses revealed that 122 matrix proteins found in this study (including 27 acidic-specific and 55 neutral-specific) were not reported before in the extracellular region or biofilm matrix of other fungal species (highlighted in bold in the Supplementary Table S1). Additionally, of the 122 proteins found to be species-specific 112 are also potentially biofilm-specific (not reported in *C. glabrata* planktonic secretome) (Supplementary Table S1).

Environmental pH modulates the functional distribution of matrix proteins

The sets of proteins found in the matrix of *C. glabrata* biofilms developed at pH 4 (287 proteins) and pH 7 (442 proteins) were clustered according to the biological processes they are involved in, based on the classification of FunCat database (through FungiFun tool) [35]. This analysis revealed that matrix proteins, at both pH conditions, are involved in 15 different main functional processes with an enrichment of 'metabolism', 'protein with binding function' and 'cell rescue, defence and virulence' classes (Figure 2 and Supplementary Table S2). Furthermore, the percentage of proteins of each class found in the matrix of biofilms formed at pH 4 was similar to that found at pH 7 (Figure 2). However, the same functional analysis performed in the sets of pH-specific proteins (51 acidic-specific and 206 neutral-specific proteins) revealed some differences (Figure 2 and Supplementary Table S2), especially in the 'metabolism' class, whose percentage was statistically higher in neutral than acidic-specific proteins (50% vs. 33.3%) (Figure 2). Additionally, a significantly higher percentage of unclassified proteins was found among acidic-specific than neutral-specific sets (Figure 2).

To extend the functional analysis of matrix proteins, the FungiFun tool was also used to identify the statistically enriched sub-classes in relation to the functional distribution of the entire *C. glabrata* background. This analysis revealed statistical enrichment of 14, 11 and 25 functional sub-classes in the sets of proteins exclusively identified at pH 4, pH 7 and under both conditions, respectively (Supplementary Table S2). The most enriched sub-class in the three sets was 'carbohydrate metabolism', followed by 'biogenesis of the cell wall' in the set of proteins identified under both pH conditions and by 'protein processing' and 'stress response' functions in proteins found to be neutral- and acidic-specific, respectively (Supplementary Table S2). Among proteins involved in the carbohydrate metabolism, several proteins displaying functions involved in β-glucan and mannan

Biofilm matrix proteome vs. planktonic secretome
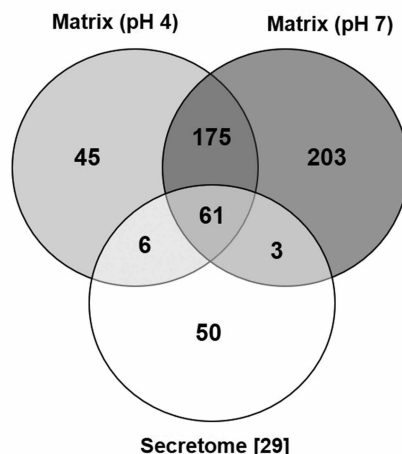
Figure 1. Overlap of *Candida glabrata* biofilm matrix proteins with the reported planktonic secretome.
The number proteins found in the matrix of *C. glabrata* ATCC 2001 biofilms developed at pH 4 and/or pH 7 are herein shown along with their overlap with the planktonic secretome previously reported [29].

metabolism were identified (Figure 3), which have a potential role in the delivery of these carbohydrates to the matrix [19].

Consistent with their potential role, the carbohydrate-related proteins included an abundance of proteins with predicted hydrolase or transferase activities, which were the most common molecular activities among all matrix proteins (Supplementary Table S3). Furthermore, several virulence-related proteins, including adhesins and aspartyl proteases (yapsins) were also found in the matrices (Figure 3). Additionally, various moonlighting proteins with original intracellular functions such as glycolysis, essential metabolism, translation and stress response, and potential extracellular functions such as adhesion and host immunity evasion, were also found in
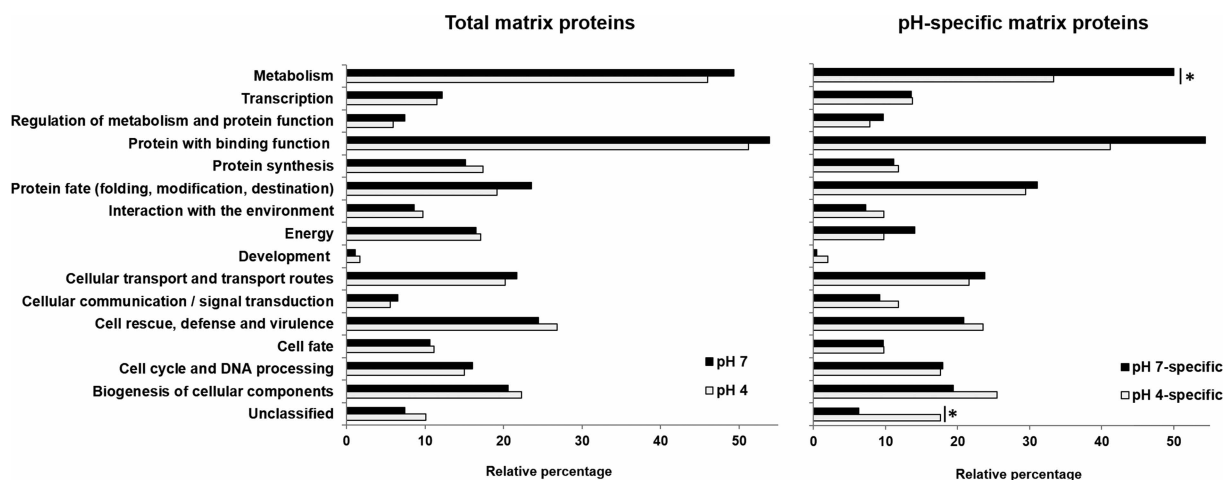

Figure 2. Functional distribution of matrix proteins identified in *Candida glabrata* biofilms developed at pH 4 and pH 7.
The total proteins found in the matrix of *C. glabrata* ATCC 2001 biofilms developed at pH 4 and pH 7 (left graph) and the proteins specifically found at each pH (right graph) were clustered according to their biological function annotated in CGD. The percentages shown correspond to the number of proteins included in each functional class compared with the total or specific number of proteins found in the biofilm matrix at each pH. The results that gave rise to this figure are fully detailed in Supplementary Table S2. Asterisks represent statistical difference between the results obtained at pH 7 and pH 4 (* *P*-value ≤0.05).
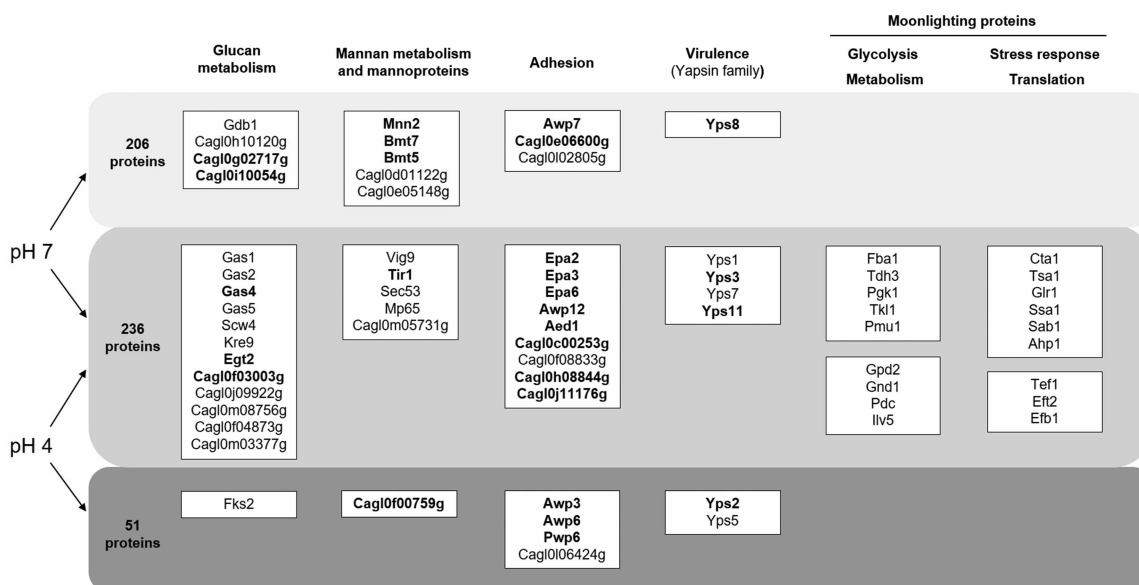
Figure 3. Matrix proteins of *Candida glabrata* biofilms developed at pH 4 and pH 7.
Schematic representation of *C. glabrata* proteins found in the matrix of biofilms developed at pH 4 and pH 7 (exclusively at each pH and common in both conditions). The scheme presents matrix proteins annotated to functions with high relevance for matrix synthesis and role. Proteins first time associated with the matrix in this study (no orthologs identified in the matrix of other species) are highlighted in bold. The data that support this figure are detailed in Supplementary Tables S1 and S2.

this study (Figure 3). Of note, some matrix proteins, especially those related to adhesion, were found for the first time associated with the matrix of *Candida* biofilms in this study (highlighted in bold in Figure 3), i.e. their orthologs (if any) were not previously reported in the biofilm matrix of other *Candida* species, and thus are potential new matrix factors specifically displayed by *C. glabrata* biofilms.

Biofilm matrix proteins pose high molecular interaction

The molecular interactions between matrix proteins, including activation, binding, phenotype, reaction, inhibition, catalysis, posttranslational modification and transcriptional regulation, were predicted using STRING database [39]. Table 2 presents the results of STRING analysis, including the number of proteins with interaction, number of expected and obtained interactions and statistical results. The list of all interactions obtained in STRING analysis are presented in Supplementary Table S4, along with the types of interaction evidence. The results revealed statistical enrichment of protein–protein interactions among acidic (2129 interactions) and neutral (3774 interactions) matrix proteins, mainly of binding and catalysis type. The abundance of binding type interactions between the proteins is in accordance with the enrichment of proteins with binding function found in the functional analyses (Figure 2). Furthermore, the statistical enrichment of interactions between matrix proteins indicates that they have more interactions among themselves than what would be expected for a random set of proteins of similar size, drawn from the genome. As such, although the protein composition of *C. glabrata* biofilm matrix is multifaceted in many aspects, most of the proteins identified in the matrix are putatively functionally connected with one or more molecules within the matrix proteome.

Matrix proteins have conventional and unconventional secretion pathways

The predictive secretory nature of matrix proteins, including the presence of a hydrophobic signal sequence at the N-terminus, was analyzed using FSD [32] and FunSecKB [33] databases. These analyses showed that 254 proteins found in this study are annotated has to have a predictive secretory nature in at least one of these databases (Table 1 and Supplementary Table S1). Furthermore, 32 proteins were previously reported to have a predicted C-terminal sequence for Glycosylphosphatidylinisotol (GPI) modification [41] (Table 1 and

**Table 2 Molecular interactions of *Candida glabrata* biofilm matrix proteins identified at pH 4 and pH 7**

STRING network results	pH 4	pH 7
Total proteins	287	442
Proteins with interaction	245	396
Number of obtained interactions	2129	3774
Number of expected interactions	1120	2241
Average protein degree	14.8	17.1
Avg. local clustering coefficient	0.46	0.42
PPI enrichment P-value	$<1.0 \times 10^{-16}$	$<1.0 \times 10^{-16}$

The table presents the results of the STRING analysis of the molecular interactions among proteins found in the matrix of *C. glabrata* biofilms developed at pH 4 and 7. The minimum confidence interaction score was set to 0.4 (medium). PPI means protein–protein interaction. Detailed information on all interactions is provided in Supplementary Table S4.

Supplementary Table S1). Additionally, 256 matrix proteins are orthologs of proteins previously found in extracellular vesicles of *C. albicans* biofilms (Table 1 and Supplementary Table S1) [28]. Of note, 53% of the 237 proteins that do not have a predictive secretory nature or GPI-anchor are orthologs of proteins found in *C. albicans* biofilm extracellular vesicles (Supplementary Table S1).

Putative genetic regulators of the matrix proteome

The PathoYeastract database [42] was used to search for transcriptional activators of genes that encode matrix proteins found in this study. In this analysis 24 transcription factors were found and Pdr1 was the one that presented the highest number of potential targets among matrix proteins (102 proteins), followed by Haa1 (Figure 4). The targets of all transcription factors are listed in Supplementary Table S5. Additionally, the
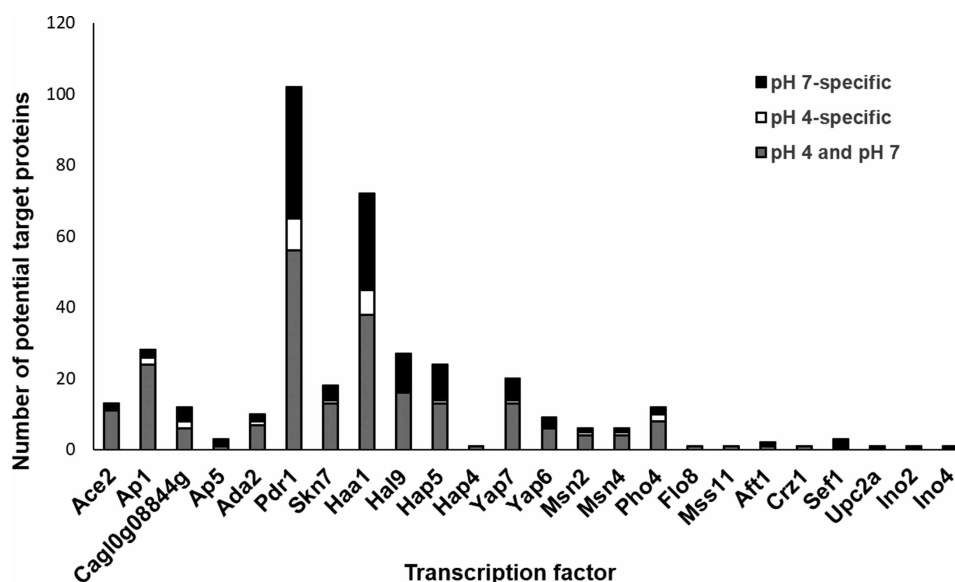
Figure 4. Potential regulators of *Candida glabrata* biofilm matrix proteins.
Transcription factors and respective number of targets among proteins identified in the matrix of *C. glabrata* biofilms developed at pH 4 and 7 (specific at each pH and common), according to PathoYeastract. The results that gave rise to this figure are fully detailed in Supplementary Table S5.

molecular interactions of Pdr1 targets found in both pH environments (56 proteins) were analyzed in STRING database [39] and then clustered with the Cytoscape tool [40], in combination with the biological function of each protein (Figure 5). This analysis revealed 117 molecular interactions between the 56 proteins (*P*-value = 1.11×10^{-16}), including an intertwined network of interactions between proteins with different biological functions (Figure 5). The list of interactions is presented in Supplementary Table S4.

Discussion

This study identified and characterized the matrix proteome of *C. glabrata* biofilms, something that was not examined before, and that is essential for a better understanding of the pathogenesis of this species. Additionally, the modulation of the proteome by host niche pH was also revealed, namely pH 7 and pH 4, the latter adjusted with lactic acid as an approximation to the vaginal acidity.

In this study, a total of 493 proteins were found in the matrix of *C. glabrata* biofilms developed under different conditions (Supplementary Table S1) which, to the authors' knowledge, are the first proteins being identified in the biofilm matrix of this species. Importantly, many of these proteins (339) possess orthologs previously reported in biofilm matrix of *C. albicans* biofilms (Table 1) [19,27], which may represent the core matrix proteome of these two species (Supplementary Table S1). As such, these proteins are an interesting cohort to search for relevant virulence determinants displayed by both species and thus to disclose potential targets for the development of new therapies with large spectre of action. Of note, 154 proteins were first time identified in the matrix of *Candida* biofilms in this study, and among them 122 proteins appear to be specifically secreted by *C. glabrata* (highlighted in bold in Supplementary Table S1), as they were not reported to be secreted by planktonic cells of various fungal species (Table 1). Additionally, 112 of these species-specific proteins were not previously reported in *C. glabrata* planktonic secretome [29] (Figure 1 and Supplementary Table S1) and thus may hold promise as potential diagnostic biomarkers for *C. glabrata* biofilms.

This study revealed that the environmental pH is a relevant modulator of *C. glabrata* matrix proteome. In fact, of the 493 proteins identified in this study, 236 were found in biofilms developed in neutral or acidic conditions, but 206 were exclusively found at pH 7 and 51 exclusively at pH 4 (Table 1). These results are in accordance with the higher amount of protein present in the matrix of *C. glabrata* biofilms developed under neutral ($\sim$73.9 mg/g$_{biofilm}$) than acidic conditions ($\sim$37.7 mg/g$_{biofilm}$), as previously reported by our team [14]. Interestingly, studies with *C. glabrata* planktonic cells found altered expression of genes encoding secreted proteins, in response to the acidic pH [29,43]. These results suggest that the modulation of the transcriptome by the environmental pH may have direct implications in the secretome. A microarray-based study with *C. glabrata* biofilms is currently being designed by our research team to infer about this possibility in relation to the matrix proteome.

The matrix proteins found in this study are annotated to several biological functions, including among others metabolism, virulence, transcription, transport and interaction with the environment (Figure 2). Despite the different predicted functions, a close molecular interaction, either direct or indirect, was found among the matrix proteomes analyzed (Table 2), indicating that these proteins are at least partially biologically connected as a group [39]. A relevant finding in the functional analysis was the enrichment of the carbohydrate metabolism function, regardless of pH condition (Supplementary Table S2). Accordingly, the carbohydrate metabolism was previously reported as the most enriched function in *C. glabrata* planktonic secretome [29] and *C. albicans* biofilm matrix proteome [19]. Importantly, a higher number of carbohydrate-related proteins was found in the matrix of biofilms developed at pH 7 than at pH 4 (Figure 3 and Supplementary Table S2). Consistently, a greater amount of carbohydrates was previously found by our research team, in the matrix of neutral than acidic *C. glabrata* biofilms [14]. These results suggest that matrix proteins involved in carbohydrate metabolism may have a role in the delivery of matrix carbohydrates. In fact, secreted glucanases and glucan transferases have been shown to be involved in the delivery and organization of β-glucans in *C. albicans* biofilm matrix [19,21]. Accordingly, among *C. glabrata* matrix proteins an abundance of hydrolases and transferases was found, including several proteins with predicted involvement in β-glucan modification, such as Gas1, Gas2, Gas4, Gas5, Scw4, Egt2, Kre9 and Fks2 [27] (Figure 3). Importantly, Gas2 was previously found to be required for normal *C. glabrata* biofilm formation [44] and a similar role was found for its potential ortholog in *C. albicans* (Phr1), which is crucial for delivery of (1,3)-β-glucans to *C. albicans* biofilm matrix [21]. Due to their high relevance, some glucan-modification enzymes identified in the matrix of *C. albicans* biofilms have been purposed as interesting targets to discover inhibitors for anti-biofilm therapeutics [19,21]. Additionally, several enzymes involved in mannan metabolism, including Mnn2, Bmt7, Bmt5, Vig9 and Sec53 were found in this

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Molecular interactions between Pdr1 targets
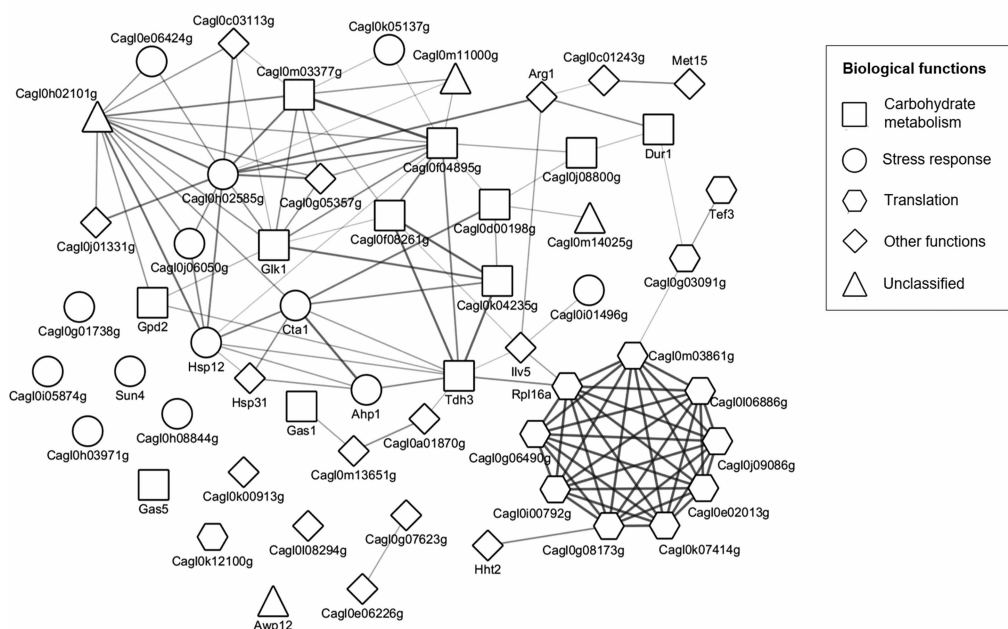
Figure 5. Molecular interactions between Pdr1 targets encoding biofilm matrix proteins.

The matrix proteins found *C. glabrata* ATCC 2001 biofilms developed at pH 4 and 7 (simultaneously) and that are encoded by target genes of Pdr1, are herein distributed according to their molecular interactions annotated in STRING. Geometrical forms represent proteins and the respective biological function annotated in FunCat: stress response (circle), carbohydrate metabolism (square), translation (hexagon), unknown (triangle) and other functions (diamond). Lines represent interaction between proteins and their thickness the level of confidence (higher thickness represent higher interaction score). The minimum confidence interaction score was set to 0.4 (medium). The results that gave rise to this figure are detailed in Supplementary Table S4 (part III).

study (Figure 3), suggesting a potential role in the delivery of mannans to the matrix, which were recently found to be a significant matrix carbohydrate component [17]. Interestingly, some proteins involved in glucan and mannan metabolism, including Gas4, Egt2, Mnn2, Bmt5, Bmt7 and Tir1, were found for the first time associated with the matrix in this study (no orthologs reported in the biofilm matrix of other *Candida* species) (all highlighted in bold in Figure 3). Similarly, several glucan and mannan-related proteins previously identified in the matrix of *C. albicans* biofilms [19] were not found in this study. As such, these results may explain the differences in the amount and organization of glucans and mannans reported in the matrices of *C. albicans* and *C. glabrata* biofilms [14,17]. Future functional investigations of glucan and mannan-related matrix proteins identified in this study will clear their potential as targets to control *C. glabrata* biofilms.

A relevant finding of this study was the identification of several matrix proteins with predicted virulence-related roles. These proteins include aspartyl proteinases, referred as yapsins (Yps1, Yps2, Yps3, Yps5, Yps7, Yps8 and Yps11), adhesion-related (Epa2, Epa3, Epa6, Awp3, Awp6, Awp7, Awp12, Aed1, Pwp6 and others) and proteins with predicted role in the protection against host immunity (Icl1 and Cagl0f05137g) [27] (Figure 3). The high number of adhesion-related proteins suggests a role of matrix proteins in the adhesion to the host cells, as previously reported for proteins secreted by *C. albicans* and bacteria [45–47]. Nevertheless, few adhesion-related proteins have been found in the matrix of *C. albicans* biofilms [19]. Indeed, almost all adhesin-like proteins identified in this study are potential new matrix factors specifically displayed by *C. glabrata* biofilms (highlighted in bold in Figure 3). It may be speculated that this species requires higher contribution of adhesion factors due to its lack of filamentation ability, which is crucial for adhesion and tissue invasion in *C. albicans*. Additionally, seven of the 11 yapsins known in *C. glabrata* were found in the biofilm matrix (Figure 3), including some also found in the planktonic secretome recently revealed [29]. Yapsins are essential for virulence and have a role in pH and vacuole homoeostasis, survival in macrophages, cell wall

integrity and energy production [43,48–50]. Importantly, yapsins are required for survival in the presence of weak acids (including lactic acid) and their encoding genes were found to be up-regulated in response to low pH, along with other genes involved in stress response and protein modification [43]. Accordingly, stress response and protein modification functions were found to be enriched among pH 4-specific matrix proteins in this study, and two yapsins (Yps2 and Yps5) were exclusively found in this set (Figure 3). The identification of yapsins in the biofilm matrix reinforces their relevance in the virulence of *C. glabrata* species and future functional studies will be essential to decipher their specific role in the matrix.

In this study 256 matrix proteins with predicted secretory nature and/or GPI-anchor were found (Table 1 and Supplementary Table S1) [32,33,41]. The mechanisms underlying the release of GPI-anchored proteins include proteolytic cleavage, release of precursors of cell wall-anchored proteins and routine shedding [51,52]. However, a high quantity of matrix proteins (237) does not contain secretion sequences or GPI-anchor, what suggests unconventional secretion pathways or accumulation of proteins after cell death [19]. Extracellular vesicles are unconventional secretion pathways already identified in various fungi including *C. albicans* [53–55]. Proteomic analyses of fungal EV have revealed the presence of cytoplasmic, plasma membrane, mitochondrial, vacuolar and even nuclear proteins [28,53,54]. Importantly, EV released by *C. albicans* biofilms were found to be a relevant source of matrix proteins [28]. In accordance, the matrix proteins found in this study are annotated to 23 predicted cellular localizations and the most enriched was the cytoplasm, followed by extracellular region, plasma membrane, ribosome, nucleus and cell wall [27] (Supplementary Table S3). Furthermore, 256 orthologous proteins of those previously identified in *C. albicans* biofilm EV were found in the matrices (Table 1), including 126 without a predictive secretory nature or GPI-anchor. These results point to a potential delivery of matrix proteins in *C. glabrata* biofilms through EV, and thus further studies to confirm the presence of these structures and their role in matrix protein cargo will be necessary to clear the secretion pathways of *C. glabrata* biofilm matrix proteins.

Among proteins with unconventional secretion pathways, those called moonlighting proteins have high relevance due to their ability to display very different functions in intracellular and extracellular/surface locations, that is not caused by gene fusions or splicing variations [56,57]. In this study, several proteins with potential moonlighting functions were identified in the matrix, under both pH conditions, including enzymes primarily involved in glycolysis (Fba1, Tdh3, Pgk1, Tkl1 and Pmu1) or other central metabolic pathways (Gpd2, Gnd1, Pdc, Ilv5) and proteins with a role in translation (Tef1, Eft2 Efb1) and intracellular stress response (Cta1, Tsa1 Glr1, Ahp1, Ssa1 and Ssb1) (Figure 3). Importantly, these proteins have been shown to play important extracellular functions, including adhesion to host cells, tissues and medical devices [58,59], binding to numerous proteinaceous targets within the host organism [60–62], evasion of the immune system [63,64] and oxidative stress response [65,66]. Furthermore, moonlighting proteins found in the matrix of bacterial biofilms were reported to interact with eDNA and stabilize the biofilms [67,68]. Indeed, moonlighting proteins are increasingly considered important virulence factors in fungi, bacteria and parasites, and thus being pointed as interesting new targets for the development of novel therapeutics [56,69,70]. However, most of these proteins, especially those playing roles in central metabolic pathways, such as glycolysis, are not suitable targets because their catalytic mechanisms are conserved in human hosts. Thus, the development of small molecules that block their secretion has been suggested as a promising strategy to control these virulence factors [70]. As such, elucidating how moonlighting proteins are secreted to the matrix of *C. glabrata* biofilms, might identify processes and proteins that are involved in this process and that could serve as novel targets for the development of new therapies for biofilm-related *C. glabrata* infections.

Although the majority of transcriptional regulators in *C. glabrata* does not have yet all their targets identified, several transcription factors are annotated as activators of genes that encode proteins found in this study (Figure 4) [42]. Importantly, Ace2 was previously found to regulate the secretion of various *C. glabrata* proteins, including some encoded by its transcriptional targets [42,71]. Of note, almost half of known target genes of Ace2 [42] encode proteins found in the biofilm matrix in this study (Figure 4). Moreover, Pdr1 was found to be the transcription factor with the highest number of target genes encoding matrix proteins, suggesting a potential role in the regulation of matrix proteome (Figure 4). Pdr1 is zinc finger transcription factor known to regulate multidrug resistance in *C. glabrata* [72]. Interestingly, a close molecular interaction was found among the potential target matrix proteins of Pdr1, and those involved in carbohydrate metabolism and stress response were found to be key proteins linking the interaction network (Figure 5). Further transcriptomic and proteomic analyses with mutant strains are being designed by our research team to identify regulators of *C. glabrata* matrix proteome.

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Conclusions

To the authors' knowledge, this is the first study to decipher the proteome of *C. glabrata* biofilm matrix and to show that it is highly modulated by the environmental pH. This study suggests important roles for matrix proteins, including in the delivery of matrix carbohydrates, adhesion and virulence, indicating a relevant involvement in the pathogenesis of *C. glabrata* species. Additionally, this study reinforces the high relevance of niche-specific environmental cues in the modulation of virulence features displayed by *C. glabrata*. Although many questions remain, the high-throughput and bioinformatic analyses described in this article represent a major step towards a better understanding of *C. glabrata* biofilm matrix and without doubt will serve as an excellent framework for future studies. Identification of matrix proteins and understanding their function represents a promissory path for the development of effective and much needed therapies against *C. glabrata* biofilms.

Data Availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [73] partner repository with the dataset identifier PXD022597 and 10.6019/PXD022597.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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CRediT Contribution

Sónia Silva: Conceptualization, Resources, Supervision, Validation, Project administration, Writing — review and editing. **Bruna Gonçalves:** Data curation, Software, Formal analysis, Investigation, Methodology, Writing — original draft, Writing — review and editing. **Nuno Miguel Azevedo:** Investigation, Methodology. **Hugo Osório:** Data curation, Validation. **Mariana Henriques:** Conceptualization, Supervision, Writing — review and editing.

Abbreviations

CGD, *Candida* Genome Database; FSD, Fungal Secretome Database; FunSecKB, Fungal Secretome KnowledgeBase; GPI, glycosylphosphatidylinisotol; TEAB, triethylamonium bicarbonate; TFA, trifluoroacetic acid.

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