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Proteomic analysis of the pH response in the fungal pathogen *Candida glabrata*

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Micro-organisms must adapt to environmental change to survive, and this is particularly true for fungal pathogens such as *Candida glabrata*. *C. glabrata* is found both in the environment and in diverse niches in its human host. The ambient pH of these niches varies considerably, and therefore we have examined the response of *C. glabrata* to changes in ambient pH using a proteomic approach. Proteins expressed in *C. glabrata* cells growing at pH 4.0, 7.4 or 8.0 were compared by 2-DE, and 174 spots displaying reproducible and statistically significant changes in expression level were identified by peptide mass fingerprinting, thereby extending our 2-DE map of the *C. glabrata* proteome to a total of 272 identified spots. Proteins involved in glucose metabolism, the TCA cycle, respiration and protein synthesis were expressed at lower levels during growth at pH 7.4 and/or 8.0, whereas proteins involved in stress responses and protein catabolism were expressed at higher levels under these alkaline conditions. Our data suggest that *C. glabrata* perceives low pH as less stressful than higher pH. This contrasts with another opportunistic fungal pathogen of humans, *Candida albicans*

Keywords:
Ambient pH / *Candida glabrata* / pH responses / Virulence

1 Introduction

The haploid, asexual ascomyceteous yeast, *Candida glabrata*, is a major opportunistic fungal pathogen of humans [1]. This fungus is found in the environment, for example on rotten fruit or in fruit juices, and is also part of the gastrointestinal microflora in many healthy human beings. Depending on various predisposing risk factors in the human host, this pathogenic yeast is capable of causing a broad range of diseases. These include relatively mild superficial skin and mucosal infections and agonising episodes of recurrent mycotic vaginitis in otherwise healthy women [2, 3]. In severely immunocompromised patients, *C. glabrata* is currently the second most common cause of systemic and disseminated candidiasis in humans [4, 5]. The risk of infection is increased in patients with indwelling devices such as plastic catheters as *C. glabrata* is able to form biofilms on artificial surfaces [6].

The occurrence of *C. glabrata* in such diverse habitats suggests that it has the ability to adapt efficiently to changes in the environment. The diverse niches that *C. glabrata* occupies vary greatly with respect to their ambient pH, for example in the gastrointestinal tract, vaginal mucosa, skin and blood. Therefore, effective pH adaptation is crucial for this fungus to survive at these host sites and hence to display its observed virulence patterns. This pH adaptation presumably addresses the impact of pH variation upon protein activities, nutrient availability and the proton gradient across

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Abbreviation: PFM, Pan Fungal Media; **TCA**, tricarboxylic acid

the plasma membrane, as well as cell wall remodelling. However, little is known about the pH-response in *C. glabrata*, which, in contrast to *Candida albicans*, does not stimulate hypha formation during the infection process [7].

Environmental pH strongly influences morphological differentiation in *C. albicans*. Growth in the yeast form is favoured under acidic conditions, while filamentous growth is induced at mild alkaline pH [8–11]. Hypha formation in this fungus has been suggested to promote the penetration of human tissue and contribute to invasive disseminated disease [12–14]. This response to alkaline conditions is mediated by the Rim101/PacC pH signalling pathway, which is conserved in *S. cerevisiae*, *A. nidulans* and *C. albicans* [15–18]. pH regulation has not been studied in *C. glabrata*.

Recently, proteomics was used effectively in *C. glabrata* to characterise the global effects of inactivating a central transcription factor [19]. Thus in this study we have performed a systematic proteomic analysis to characterise the response of *C. glabrata* to changes in ambient pH. We show that proteins involved in stress responses and carbon metabolism are regulated in a pH-dependent manner in this fungal pathogen.

2 Materials and methods

2.1 Strains and growth conditions

Single colonies of *C. glabrata* ATCC2001 were inoculated into YPD medium [20] and grown for 24 h at 37°C. Four single clonal cultures were pooled, resuspended in sterile water and used to inoculate 600 mL of Pan Fungal Minimal Media (PFM, pH 7.4) at a density of 8×10^5 cfu/mL. The minimal medium PFM contains essential minerals, trace elements, vitamins, amino acids and 2% glucose (Ken Haynes, personal communication):

(i) Trace elements – 40 μg/L $Na_2B_4O_7.10H_2O$, 400 μg/L $CuSO_4.5H_2O$, 1.32 mg/L $FeSO_4.7H_2O$, 800 μg/L $MnSO_4.4H_2O$, 696.4 μg/L Na_2MoO_4, 8 mg/L $ZnSO_4.7H_2O$.

(ii) Salts – 0.52 mg/L KCl, 0.52 mg/L $MgSO_4.7H_2O$, 1.52 mg/L KH_2PO_4.

(iii) Vitamins – 500 μg/L thiamine, 1 mg/L nicotinic acid, 2 mg/L DL-pantothenic acid, 2.5 mg/L pyridoxine, 10 μg/L biotin.

(iv) Essential amino acids – 30 mg/L L-leucine, 30 mg/L L-lysine, 20 mg/L L-methionine, 20 mg/L L-histidine, 20 mg/L L-uracil.

(v) Nitrogen source – 5mM ammonium tartrate.

(vi) Carbon source −2% w/v glucose.

PFM medium was buffered to pH 7.4 or 8.0 with 100 mM Tris-HCl, or to pH 4.0 with 100 mM glycolic acid. *C. glabrata* cultures were grown in PFM at pH 7.4 for 16–18 h until they reached an OD_{600} of 0.5. Cells were collected and shifted to PFM at pH 4.0, 7.4 or 8.0. Cultures were grown at 37°C for 2 h and harvested by centrifugation (5 min, 4000 rpm, 4°C). Cell pellets were washed twice with ice cold water and stored at −80°C.

2.2 Protein extraction

Total soluble proteins were extracted as described previously [21]. Cells were disrupted with glass beads in 160 μL lysis solution 1 (7.5 M urea, 2.5 M thiourea, 1.25 mM EDTA, 1.75 μg/mL pepstatin A, 62.5 mM DTT, 25 mM Tris-Cl, pH 10.8 and protease inhibitor cocktail at 3.7 times the recommended concentration (Roche, Lewes, UK)) using a bead beater. After cell disruption, proteins were further solubilised by adding 40 μL of lysis solution 2 (20% CHAPS, 50% v/v glycerol, 10% v/v carrier ampholyte (pH 4–7)), and incubated on ice for 1 h. After repeated bead beating, cell debris was removed by centrifugation (20 min, 13 000 rpm, 4°C) and protein extracts were stored at −80°C.

2.3 2-DE

2-DE was performed as described previously [19]. After rehydration, IPGphor strips (pH 4–7) were used to separate proteins (500 μg) in the first dimension according to the following protocol: 200 V for 1 h, 500 V for 1 h, 1000 V for 1 h, followed by a gradient switch of 1000–8000 V for 0.5 h and 8000 V for 12 h, which adds to a total of 80 000 V·h. After isoelectric focussing, IPG strips were equilibrated in two steps: (a) 50 mM Tris-Cl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS, 0.025% bromophenol blue, 62.5 mM DTT for 25 min; and (b) 50 mM Tris-Cl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS, 0.025% bromphenol blue, 2.5% iodoacetamide for 25 min. Proteins were separated in the second dimension on precast 12.5% SDS polyacrylamide gels (20 cm × 26 cm, Amersham Biosciences, Little Chalfont, UK) at first with 2.5 W *per* gel for 1 h, followed by 19 W *per* gel until the run was completed. Gels were fixed (2% H_3PO_4, 50% EtOH) overnight, rinsed three times with water and equilibrated (1.3 M $(NH_4)_2SO_4$, 2% H_3PO_4, 34% MetOH) for 1 h, before they were stained with colloidal Coomassie (0.67 g/L). Gels were scanned, dried and stored until needed for mass spectrometric analysis.

2.4 2-D gel analysis and protein identification

2-D gels were prepared from four independent replicate cultures for each of the three different pH conditions (pH 4.0, 7.4 and 8.0). Spot detection and comparative analysis of the spot pattern were performed using *Phoretix*™ 2D Expression software (Nonlinear, Newcastle upon Tyne, UK) and GeneSpring software (Silicon Genetics, San Carlos, USA). To refine automatic spot matching, mismatched spots were corrected manually and spot volumes were normalised against total spot volume after background subtraction (mode of nonspot). Protein spots which showed statistically significant differences of greater or equal to two-fold in their mean spot volume on at least three of four gels (*T*-test: $p \leq 0.05$), or which were present/absent in one of the experimental conditions were excised from the gels. Proteins were digested with trypsin and peptide masses were determined

using a Voyager-DE STR mass spectrometer (MALDI-ToF) as described before [19]. After spectrum calibration and removal of trypsin autodigestion and keratin peaks, the monoisotopic masses were submitted to a MASCOT peptide mass fingerprint search (Matrix Science, London, UK). A local MASCOT server was used for searching a database built from the annotated *C. glabrata* ORF set in the Génolevures database (http://cbi.labri.fr/Genolevures/C_glabrata.php), while searches of fungal proteins in the NCBInr database were performed at www.matrixscience.com. Search parameters were: enzyme = trypsin; fixed modifications = carbamidomethyl (C); variable modifications = oxidation (M); mass tolerance ± 50 ppm; maximum missed cleavages = 1.

3 Results

3.1 Experimental approach

To characterise the proteomic response of *C. glabrata* to changes in ambient pH, cells were grown to mid-exponential phase in PFM at pH 7.4, a growth medium that has been developed by the Haynes laboratory for the standardisation of experiments involving the analysis of pH responses in yeasts and filamentous fungi (Ken Haynes, personal communication). These cells were harvested and transferred to fresh PFM at pH 4.0, 7.4 or 8.0. The growth of *C. glabrata* ATCC2001 was similar at pH 4.0 and 7.4, and slower at pH 8.0. Total soluble protein extracts were prepared and these were subjected to 2-DE to examine proteins with p*I* values of between 4.0 and 7.0, using procedures established previously to study the *C. glabrata* 2-D proteome [19]. Rather than examining new protein synthesis *per se*, our approach revealed significant changes in protein abundance under these growth conditions. Four independent experiments were performed.

To test the reproducibility of the replicate experiments the spot patterns on all of the gel images were subjected to principal components analysis using SIMCA-P software (Umetrics, Umea, Sweden) (Fig. 1). The four independent replicates for each experimental condition clustered closely together, with the pH 7.4 gels lying between the pH 4.0 and 8.0 replicates. Furthermore, the *C. glabrata* gels from this experiment clustered separately from *C. albicans* and *S. cerevisiae* 2-D gels [21]. This analysis indicates that the *C. glabrata* gels from each of the four independent pH experiments were highly reproducible.

3.2 pH-regulated clusters in *C. glabrata*

Quadruplicate gel images for each pH condition were analysed quantitatively using Phoretix 2D Expression software to select statistically significant ($p \leq 0.05$) changes of more than two-fold, relative to the expression levels at pH 4.0. Two examples of proteins that are pH-regulated in *C. glabrata* are illustrated in Fig. 2. Tmt1, which is a putative trans-aconitate

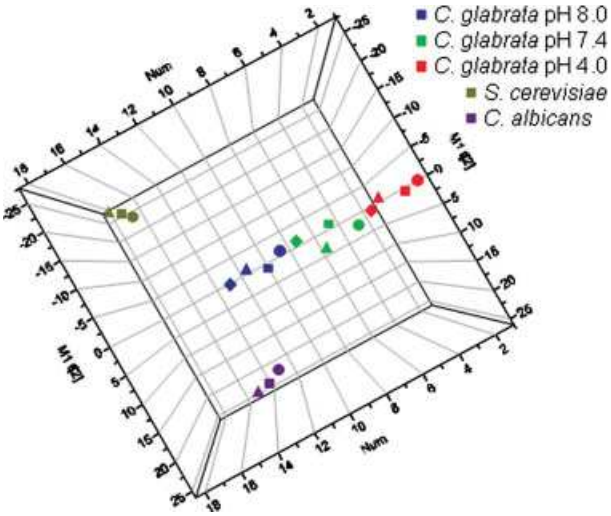

Figure 1. Principal components analysis of independent quadruplicate *C. glabrata* 2-D gels at pH 4.0 (red symbols), pH 7.4 (green symbols) and pH 8.0 (blue symbols) and replicate 2-D gels of *S. cerevisiae* (brown symbols) and *C. albicans* (purple symbols). The *S. cerevisiae* and *C. albicans* data are from Yin *et al.* [21].

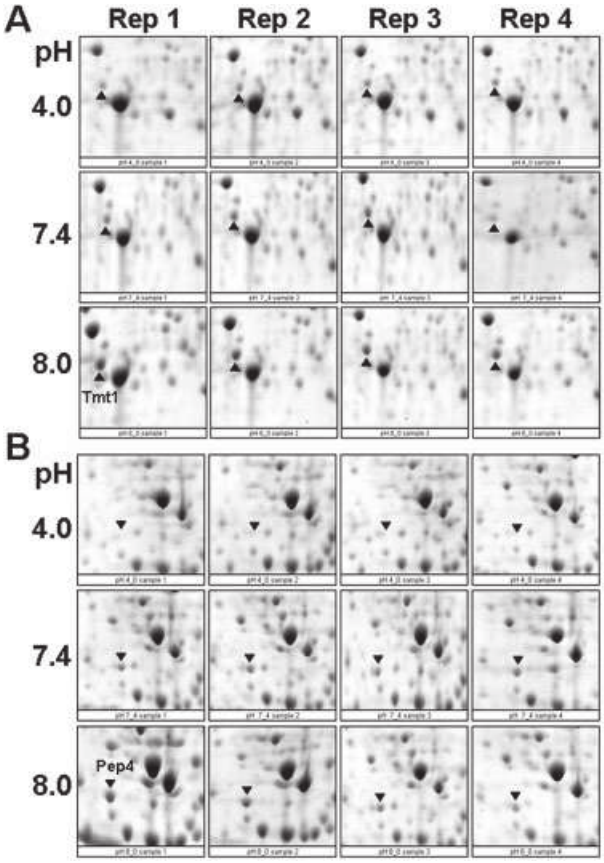

Figure 2. Reproducible changes in Tmt1 and Pep4 levels in replicate extracts from *C. glabrata* cells grown at an ambient pH of 4.0, 7.4 or 8.0. Arrows indicate the positions of the Tmt1 and Pep4 spots in (A) and (B), respectively.

3-methyltransferase, was reproducibly up-regulated at pH 8.0 relative to pH 4.0. In contrast, the vacuolar endopeptidase Pep4 was up-regulated at both pH 7.4 and 8.0.

A total of 174 spots on the 2-D gels were picked for analysis. The proteins were clustered on the basis of their expression patterns at pH 4.0, 7.4 and 8.0 making use of GeneSpring (Fig. 3A). Identifications were obtained for 154 (88.5%) of these 174 spots by PMF. Multiple hits were obtained for some proteins, yielding a total of 87 distinct full-length proteins that were identified (red lines in Fig. 3A).

In general *C. glabrata* cells growing at pH 7.4 or 8.0 showed more similar expression patterns than cells growing at pH 4.0. The pH-regulated proteins fell into four main clusters (Fig. 3). Cluster 1 contained thirteen proteins that are expressed at significantly lower levels at pH 7.4 and 8.0 compared with pH 4.0 (Table 1). Five of these proteins (Aco1, Tdh3, Krs1, Mdh1, Pdc1) are involved in organic acid metabolism and carbon metabolism (glycolysis/gluconeogenosis and the tricarboxylic acid (TCA) cycle). Also, Rip1, an ubiquinol-Cytochrome *c* reductase, which transfers electrons from ubiquinol to Cytochrome *c* during respiration was down-regulated

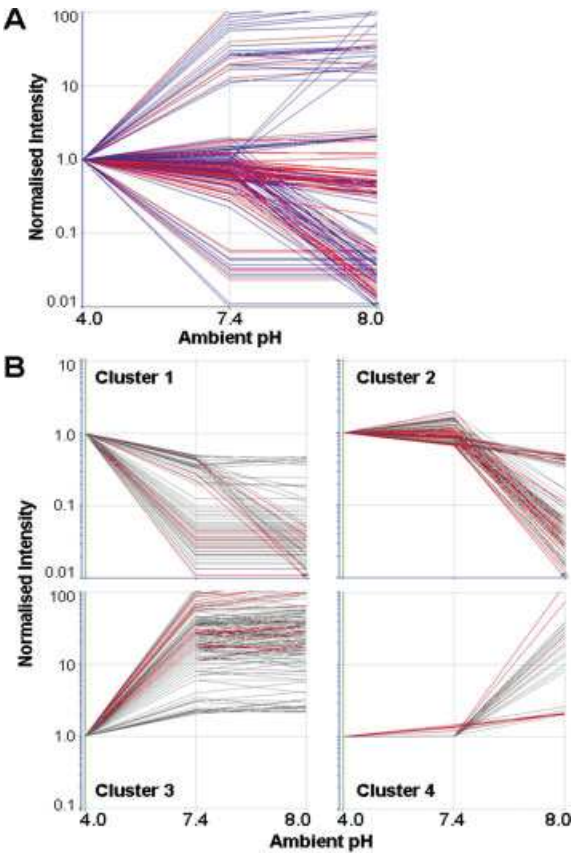

Figure 3. Clustering of pH-regulated proteins in *C. glabrata* based on their expression patterns. (A) The expression levels of the 174 analysed spots are presented relative to their expression at pH 4.0. Red lines represent the expression patterns of the 87 distinct proteins (see text). (B) Expression patterns for individual proteins in the four main pH-regulated clusters.

at pH 7.4 and 8.0. Additionally we identified proteins involved in protein folding (Sse1, Hsp82) and protein complex assembly (Hsp82, Cox12) in this cluster of proteins that are expressed at lower levels at pH 7.4 and 8.0.

Cluster 2 contained 29 proteins that were expressed at significantly lower levels at pH 8.0 compared to pH 4.0 (Table 2). To identify proteins that were down-regulated at pH 8.0 but not at pH 7.4, we selected those proteins whose expression levels at pH 7.4 were within 0.67-fold of their levels at pH 4.0 (*i.e.*, apparently not regulated from pH 4.0 to 7.4), but were decreased more than two-fold at pH 8.0. A number of proteins identified in this cluster play important roles in energy generation. In particular enzymes involved in glucose catabolism (Tkl1, Fba1, Cdc19, Pgi1) are represented in this group as well as proteins that take part in the TCA-cycle (Lsc1) or fermentation (Adh3, Dld1, Pdc1). Moreover cell redox homeostasis seems to be modulated by the down-regulation of key enzymes and regulators (Trr1, Ahp1, Tsa1). Functions involved in the cytoskeleton, chromosomal segregation, cell polarisation and endocytosis (Act1, Nuf2) were also included in this cluster of proteins that were down-regulated specifically at alkaline pH. Some of these changes might relate to the slower growth of the *C. glabrata* cells at pH 8.0.

Cluster 3 contained 20 proteins that were expressed at significantly higher levels at pH 7.4 and 8.0 compared with pH 4.0 (Table 3). This cluster included proteins involved in cell signalling (Bcy1), endocytosis (Lsp1, Pil1) and protein folding and turnover (Pdi1, Pep4). We also observed the strong induction of both subunits of phosphofructokinase 1 (Pfk1, Pfk2), which is indispensable for glycolysis.

Cluster 4 contained ten proteins that were expressed at significantly higher levels at pH 8.0 compared to pH 4.0 (Table 4). This cluster contains proteins that were up-regulated significantly at pH 8.0, but not at pH 7.4 (*i.e.* whose levels were less than 1.5-fold up-regulated at pH 7.4 compared with pH 4.0). Some of these changes in this cluster might relate to the slower growth of the *C. glabrata* cells at pH 8.0. Strikingly, most proteins identified in this cluster are involved in the closely related processes of cytoskeletal organisation (Aip1, Pph21) and intracellular transport (Sec28, Vma2, Ssa3). Two others, which were expressed at higher levels are involved in protein catabolism (Lap4, Rpt3).

In addition we identified 15 *C. glabrata* proteins that did not belong to one of the above clusters, but nevertheless displayed significant pH regulation (Table 5). For example, the four clusters did not include proteins that were slightly up-regulated at pH 7.4, but significantly down-regulated at pH 8.0. These 15 unclustered proteins included proteins involved in a range of interesting functions. The oxidative stress protein, catalase A (Cta1) was down-regulated 38-fold at pH 8.0 compared to pH 4.0, but was not significantly regulated during the shift from pH 4.0 to 7.4 (although its fold-regulation lay outside the cut-off for cluster 2). A second stress protein, Rhr2, was also down-regulated at pH 8.0. The translation elongation factor EF-2 (Yef3) was up-regulated

Table 1. pH-regulated proteins in Cluster 1 (down at pH 7.4 and 8.0)

Protein name			Fold-regulation		Function	PMF quality metrics[a)]		
Biological	Formal	Ortholog[b)]	pH 7.4	pH 8.0		Hit ratio	Coverage (%)	ELDP
Aco1[c)]	CAGL-CDS0863.1	Ylr304c	−36	−36	Aconitate hydratase	8/17	15	4
Cox12	CAG57814	Ylr038c	−3.6	−72	Cytochrome *c* oxidase	5/20	68	3
Get3	CAGL-CDS3157.1	Ydl100c	−91	−91	Homologue of YDL100c (ATPase)	9/20	27	3
Hsc82[c)]	CAGL-CDS1077.1	Ymr186w	−27	−27	Heat shock protein	6/10	10	4
Krs1[c]	CAGL-CDS1555.1	Ydr037w	−23	−23	Lysyl-tRNA synthetase	11/27	19	7
Mdh1[c)]	CAGL-CDS3282.1	Ykl085w	−39	−39	Malate dehydrogenase	5/17	12	1
Nfu1	CAGL-CDS3894.1	Ykl040c	−22	−22	Iron homeostasis	4/16	18	2
Pdc1	CAGL-CDS1705.1	Ylr044c	−4.5	−170	Pyruvate decarboxylase	14/26	33	10
Rip1	CAGL-CDS4323.1	Yel024w	−2.2	−120	Ubiquinol-Cytochrome *c* reductase iron-sulfur protein	7/23	31	1
Sse1[c)]	CAGL-CDS1116.1	Ypl106c	−2.6	−26	Heat shock protein of HSP70 family	13/29	24	5
Tdh3	CAGL-CDS3304.1	Ygr192c	−100	−100	Glyceraldehyde-3-phosphate dehydrogenase	9/17	34	7
Tpd3	CAGL-CDS1444.1	Yal016w	−30	−30	Ser/thr protein phosphatase 2A, regulatory chain A	17/27	37	13
Ymr315w[c)]	CAGL-CDS3170.1	Ymr315w	−2.2	−20	Hypothetical protein	4/11	15	4

a) PMF quality metrics [39]: hit ratio, number of masses matched to the candidate protein/number of masses submitted to the search; coverage equals the total percent of candidate protein sequence covered by the matched peptides; excess of limit-digested peptides (ELDP) equals the number of matched peptides having no missed cleavages minus the number of matched peptides containing a missed cleavage site. An ELDP value >1 strongly indicates a correct match [39].
b) *S. cerevisiae* proteins having the highest homology by the WU-BLAST2 program (http://seq.yeastgenome.org/cgi-bin/blast-sgd.pl).
c) Possible fragment of full-length protein (according to 2-D gel position and PMF coverage).

1.6-fold at pH 7.4, but down-regulated 16-fold at pH 8.0. Also the translational regulator Gcn3 was down-regulated 16-fold at pH 8.0. Proteins that were down-regulated at pH 8.0 also included Cpr1 (a peptidyl-prolyl *cis–trans* isomerase involved in protein folding), Ilv5 (isoleucine/valine synthesis) and proteins involved in glucose metabolism (Eno1, Pgk1, Rhr2, Tpi1).

3.3 Specific functional categories are enriched in pH-regulated expression clusters

We examined whether specific functional categories were significantly enriched in the groups of pH-regulated proteins that we identified in *C. glabrata*. No such enrichment was observed for the group of 15 unclustered proteins. However, each of the four pH-regulated clusters displayed a statistically significant enrichment in specific functional categories defined by the GO terms (Table 6).

Relative to the expression levels at pH 4.0, proteins involved in protein synthesis, folding and complex assembly were down-regulated at pH 8.0 (clusters 1 and 2), whereas protein catabolism was up-regulated at pH 7.4 and 8.0 (clusters 3 and 4) (Table 6). Microtubule cytoskeleton organisation and biogenesis was down-regulated at pH 8.0 (cluster 2), whereas actin cytoskeleton organisation and biogenesis was up-regulated at pH 8.0 (cluster 4). Also, the enzymes involved in amine metabolism (Aco1, Krs1, His4, Arg1, Sam2) were down-regulated at basic pH (clusters 1 and 2). However, one of the most striking effects was upon carbon metabolism. Enzymes involved in glucose catabolism (Glk1, Fba1, Pgi1, Cdc19/Pyk1, Pdc1, Tdh3 and Tkl1), the TCA cycle (Aco1, Lsc2 and Mdh1) and respiration (Aco1, Rip1, Mdh1, Dld1, Lsc2) were down-regulated at pH 8.0 and in some cases also at pH 7.4 (clusters 1 and 2). In contrast, the glycolytic enzyme phosphofructokinase 1 (Pfk1 and Pfk2) was expressed at lower levels at pH 4.0 relative to pH 7.4 and 8.0. Clearly the change in ambient pH from 7.4 to 4.0 has a significant impact upon the expression of carbon metabolic enzymes in *C. glabrata*.

3.4 Extended 2-DE map of the *C. glabrata* proteome

The new protein identifications that we obtained in this study were added to our 2-DE map of the *C. glabrata* proteome. The new 2-DE map, which contains 272 identified features representing 174 *C. glabrata* proteins (Fig. 4), extends our previous map with 112 new identified spots. The new map is available at www.abdn.ac.uk/ims/facilities/proteomics/ and the list of proteins on the *C. glabrata* 2-DE map is available in Supporting Information.

4 Discussion

In this study we examined the proteomic response of *C. glabrata* to changes in ambient pH. We examined the pH responses of this fungal pathogen because it must respond effectively to changes in ambient pH if it is to thrive in the

Table 2. pH-regulated proteins in cluster 2 (down at pH 8.0 but not at pH 7.4)

Protein name			Fold-regulation		Function	PMF quality metrics[a)]		
Biological	Formal	Ortholog[b)]	pH 7.4	pH 8.0		Hit Ratio	Coverage (%)	ELDP
Act1[c)]	CAGL-CDS2867.1	Yfl039c	−1.4	−85	Actin	6/15	22	4
Adh1[c)]	CAGL-CDS3139.1	Yol086c	−1.2	−2.0	Alcohol dehydrogenase I	8/12	26	6
Ahp1	CAGL-CDS4636.1	Ylr109w	−1.5	−2.3	AHP1 alkyl hydroperoxide reductase	3/7	26	1
Arg1	CAGL-CDS2634.1	Yol058w	−1.5	−2.3	Argininosuccinate synthetase	12/28	34	8
Atp2	CAGL-CDS2034.1	Yjr121w	−0.60	−34	F1F0-ATPase complex, F1 beta subunit	20/40	44	16
Cdc19[c)]	CAGL-CDS2071.1	Yal038w	−1.4	−2.0	Pyruvate kinase	11/24	24	5
Dld1	CAGL-CDS1640.1	Ydl174c	−1.5	−76	D-lactate ferricytochrome C oxidoreductase	9/28	19	5
Eft2	CAGL-CDS0703.1	Ydr385w	−1.3	−25	Elongation factor 2	8/22	11	4
Fba1	CAGL-CDS3065.1	Ykl060c	−1.3	−2.8	Fructose-bisphosphate aldolase	6/26	20	4
Glk1[c)]	CAGL-CDS2099.1	Ycl040w	−0.78	−18	Aldohexose specific glucokinase	11/16	26	7
His4[c)]	CAGL-CDS0806.1	Ycl030c	−1.1	−9.0	Phosphoribosyl-AMP cyclohydrolase/ phosphoribosyl-ATP pyrophosphatase/ histidinol dehydrogenase	13/23	21	3
Hsp60[c)]	CAGL-CDS1672.1	Ylr259c	−1.4	−18	Heat shock protein 60, mitochondrial precursor	18/31	32	6
Ipp1	CAGL-CDS3706.1	Ybr011c	−1.5	−56	Inorganic pyrophosphatase, cytoplasmic	5/20	38	5
Lsc2[c)]	CAGL-CDS2643.1	Ygr244c	−0.78	−28	Succinate-CoA ligase beta subunit	10/34	23	4
Muk1	CAGL-CDS1670.1	Ypl070w	−0.93	−27	Heat shock protein	5/12	12	−1
Nab1a	CAGL-CDS4484.1	Ygr214w	−0.90	−27	Ribosomal protein p40	3/18	31	5
Nuf2[c)]	CAGL-CDS2318.1	Yol069w	−1.0	−16	NUF2 spindle pole body protein	4/17	12	4
Pdc[c)]	CAGL-CDS1705.1	Ylr044c	−1.1	−2.1	Pyruvate decarboxylase	12/23	28	8
Pdr13[c)]	CAGL-CDS1860.1	Yhr064c	−1.1	−42	Regulator protein (Ssz1), pleiotropic drug resistance	13/19	24	3
Pgi1[c)]	CAGL-CDS1740.1	Ybr196c	−0.87	−28	Glucose-6-phosphate isomerase	8/18	14	6
Pst2[c)]	CAGL-CDS4456.1	Ydr032c	−1.1	−42	Unknown function	4/9	28	0
Sam2	CAGL-CDS2876.1	Ydr502c	−1.2	−48	S-adenosylmethionine synthetase	9/19	26	1
Scp160[c)]	CAGL-CDS3486.1	Yjl080c	−0.77	−10	Required for maintenance of exact ploidy	17/20	15	11
Ssb2[c)]	CAGL-CDS1430.1	Ynl209w	−1.4	−120	Heat shock protein of HSP70 family, cytosolic	5/11	12	5
Tef1[c)]	CAGL-CDS2328.1	Ypr080w	−1.0	−36	Translation elongation factor eEF1 alpha-A chain	8/18	26	6
Tif1[c)]	CAGL-CDS2789.1	Ykr059w	−1.2	−2.3	Translation initiation factor	10/20	29	8
Tkl1[c)]	CAGL-CDS1156.1	Ypr074c	−0.50	−23	Transketolase	10/20	17	4
Trr1[c)]	CAGL-CDS3413.1	Ydr353w	−0.97	−36	Thioredoxin reductase (NADPH)	5/12	15	5
Tsa1[c)]	CAGL-CDS4485.1	Yml028w	−1.0	−39	Thioredoxin peroxidase	6/12	36	6

a) PMF quality metrics [39]: hit ratio, number of masses matched to the candidate protein/number of masses submitted to the search; coverage equals the total percent of candidate protein sequence covered by the matched peptides; excess of limit-digested peptides (ELDP) equals the number of matched peptides having no missed cleavages minus the number of matched peptides containing a missed cleavage site. An ELDP value >1 strongly indicates a correct match [39].

b) *S. cerevisiae* proteins having the highest homology by the WU-BLAST2 program (http://seq.yeastgenome.org/cgi-bin/blast-sgd.pl).

c) Possible fragment of full-length protein (according to 2-D gel position and PMF coverage).

diverse niches it occupies. In its human host *C. glabrata* survives in the relatively acidic environments of the oral cavity, stomach, duodenum and vaginal tracts, as well as in more basic niches such as in the intestine, bloodstream and kidneys, for example. In this respect, *C. glabrata* is similar to another major fungal pathogen of humans, *C. albicans*. In *C. albicans* pH adaptation has been shown to occur following exposure to host cells [22, 23] and pH signalling is essential for the virulence of this pathogen [24]. The same is true for the filamentous fungal pathogen *Aspergillus fumigatus* [25]. Differential pH regulation of essential cell wall functions affects the ability of *C. albicans* to infect vaginal tissue and internal organs [26]. Furthermore, pH sensing appears to be critical for tissue invasion during systemic *Candida* infections [27]. *C. glabrata* and *C. albicans* are evolutionarily divergent, these species being separated by the ancient whole genome duplication event that occurred during the evolution of yeast species [28]. Nevertheless, given that both are prevalent opportunistic fungal pathogens and that both can occupy similar niches in their human host, we reasoned that they might display some similarities in their global responses to ambient pH.

The global pH responses of *C. albicans* have been examined by transcript profiling [29]. Bensen *et al.* observed that *C. albicans* genes involved in a number of functional categories were regulated in response to ambient pH. These

Table 3. pH-regulated proteins in cluster 3 (up at pH 7.4 and 8.0).

Protein name			Fold-regulation		Function	PMF quality metrics[a)]		
Biological	Formal	Ortholog[b)]	pH 7.4	pH 8.0		Hit ratio	Coverage (%)	ELDP
Anc1	CAGL-CDS4480.1	Ypl129w	19	15	Transcription initiation factor	6/17	23	4
Bcy1	CAGL-CDS2738.1	Yil033c	57	65	cAMP dependent protein kinase, regulatory subunit	5/16	20	3
Cdc48 [c)]	CAGL-CDS0734.1	Ydl126c	17	18	Microsomal protein of CDC48/PAS1/SEC18 family of ATPases	12/18	19	10
Dos1	CAGL-CDS3022.1	Ydr068w	27	19	Involved in genome stability	5/23	18	1
Fmp29	CAGL-CDS1287.1	Yer080w	27	32	Homologue of YER080w	6/21	11	2
Lsp1	CAGL-CDS3497.1	Ypl004c	86	120	Long chain base-responsive inhibitor of protein kinases Phk1p and Phk2p, acts along with the Pil1p	12/26	49	8
Met6	CAGL-CDS0893.1	Yer091c	12	12	5-Methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	12/24	22	12
Nas6	CAGL-CDS4155.1	Ygr232w	11	19	Regulatory, nonATPase subunit of the 26S proteasome	5/13	31	1
Pdi1	CAGL-CDS1942.1	Ycl043c	62	94	Protein disulfide-isomerase	9/22	17	5
Pep4	CAGL-CDS2671.1	Ypl154c	68	100	Aspartyl protease Saccharopepsin precursor (EC 3.4.23.25)	14/22	28	4
Pfk1	CAGL-CDS0454.1	Ygr240c	26	32	6-Phosphofructokinase, alpha subunit	10/18	13	4
Pfk2	CAGL-CDS0484.1	Ymr205c	19	26	6-Phosphofructokinase, beta subunit	10/15	11	8
Pil1	CAGL-CDS3503.1	Ygr086c	96	130	Long chain base-responsive inhibitor of protein kinases Phk1p and Phk2p, acts along with Lsp1p	9/20	43	9
Prt1	CAGL-CDS1029.1	Yor361c	26	29	Translation initiation factor eIF3 subunit	13/19	17	9
Rfa1	CAGL-CDS1363.1	Yar007c	27	32	DNA replication factor A, 69 KD subunit	8/21	16	6
Sun1	CAGL-CDS3844.1	Yhr200w	27	34	26S proteasome regulatory subunit	8/24	39	4
Tal1	CAGL-CDS3297.1	Ylr354c	30	34	Transaldolase	11/17	31	7
Tif5	CAGL-CDS2734.1	Ypr041w	75	140	Translation initiation factor eIF5	7/21	19	7
Yll029w	CAGL-CDS2738.1	Yll029w	12	12	Homologue of YLL029w	9/24	14	5
Ynl134c	CAGL-CDS2951.1	Ynl134c	35	42	Possible alcohol dehydrogenase	10/18	42	8

a) PMF quality metrics [39]: hit ratio, number of masses matched to the candidate protein/number of masses submitted to the search; coverage equals the total percent of candidate protein sequence covered by the matched peptides; excess of limit-digested peptides (ELDP) equals the number of matched peptides having no missed cleavages minus the number of matched peptides containing a missed cleavage site. An ELDP value >1 strongly indicates a correct match [39].

b) *S. cerevisiae* proteins having the highest homology by the WU-BLAST2 program (http://seq.yeastgenome.org/cgi-bin/blast-sgd.pl).

c) Possible fragment of full-length protein (according to 2-D gel position and PMF coverage).

include carbohydrate, amino acid and lipid metabolism, electron transport, signal transduction, ion transport, cell wall structure, hyphal development and protein synthesis, folding and degradation. For several reasons a number of these functional categories are likely to be under-represented in our proteomic analysis of pH responses in *C. glabrata*. Firstly, cell wall proteins and integral membrane proteins such as ion transporters would not have been extracted under the lysis conditions we used, and would not have been resolved well on our 2-D gels. Secondly, although *C. glabrata* can undergo morphogenesis [30], it does not form hyphae in response to ambient pH. Therefore we would not have expected (pseudo)hyphal proteins to be highlighted by our analyses. Despite the significant differences between transcript profiling and proteomics, and despite the evolutionary divergence of *C. albicans* and *C. glabrata*, some common themes have emerged from these global analyses.

It is clear that carbon metabolism is regulated in response to changing ambient pH in both *C. glabrata* (Table 6) and in *C. albicans* [29]. We found that glucose catabolic enzymes (Glk1, Fba1, Pgi1, Cdc19/Pyk1, Pdc1, Tdh3 and Tkl1) and enzymes of the TCA cycle (Aco1, Lsc2 and Mdh1) were expressed at higher levels in *C. glabrata* cells growing at pH 4.0 (Tables 1 and 2). In *S. cerevisiae* gluconeogenic, glyoxylate cycle and TCA cycle genes are repressed by glucose, even at low glucose concentrations [21, 31, 32]. Given that *C. glabrata* displays the Crabtree Effect like other species in the evolutionary *Saccharomyces* clade [33], one might have expected TCA cycle enzymes to have been repressed in our glucose-containing medium. However, increased glucose catabolism might be required to generate energy for the maintenance of intracellular pH homeostasis when the environment is acidic. Certainly central carbon metabolism is regulated in a niche-specific fashion during *C. albicans* infections [34].

Table 4. pH-regulated proteins in cluster 4 (up at pH 8.0, but not at pH 7.4)

Protein name			Fold-regulation		Function	PMF quality metrics[a)]		
Biological	Formal	Ortholog[b)]	pH 7.4	pH 8.0		Hit ratio	Coverage (%)	ELDP
Aip1	CAGL-CDS1453.1	Ymr092c	1.0	73	Actin cytoskeleton component	11/30	21	7
Ape1	CAGL-CDS2014.1	Ykl103c	1.0	73	Vacuolar aminopeptidase I	11/30	25	9
Pph21	CAGL-CDS3003.1	Ydl134c	1.4	2.1	Protein ser/thr phosphatase	6/19	23	4
Sec28	CAGL-CDS3645.1	Yil076w	1.0	25	Epsilon-COP coatomer subunit	10/21	41	10
Sol3	CAGL-CDS4048.1	Yhr163w	1.0	130	Probable 6-phosphogluconolactonase	15/23	69	11
Ssa3	CAGL-CDS1280.1	Ybl075c	1.5	2.1	Heat shock protein of HSP70 family	17/25	27	5
Vma2	CAGL-CDS2020.1	Ybr127c	1.4	2.1	H+-ATPase V1 domain 60 KD subunit	13/27	35	9
Yer175c	CAGL-CDS3673.1	Yer175c	1.2	2.1	Hypothetical protein	10/27	35	10
Yjl123c	CAGL-CDS2461.1	Yjl123c	1.4	2.1	Hypothetical protein	7/27	18	5
Yta2	CAGL-CDS2557.1	Ydr394w	1.4	2.1	26S proteasome regulatory subunit	5/20	15	1

a) PMF quality metrics [39]: hit ratio, number of masses matched to the candidate protein/number of masses submitted to the search; coverage equals the total percent of candidate protein sequence covered by the matched peptides; excess of limit-digested peptides (ELDP) equals the number of matched peptides having no missed cleavages minus the number of matched peptides containing a missed cleavage site. An ELDP value >1 strongly indicates a correct match [39].
b) *S. cerevisiae* proteins having the highest homology by the WU-BLAST2 program (http://seq.yeastgenome.org/cgi-bin/blast-sgd.pl).

Table 5. Other pH-regulated *C. glabrata* proteins

Protein name			Fold-regulation		Function	PMF quality metrics[a)]		
Biological	Formal	Ortholog[b)]	pH 7.4	pH 8.0		Hit ratio	Coverage (%)	ELDP
Ald5	CAGL-CDS2056.1	Ymr170c	1.5	2.0	Aldehyde dehydrogenase	9/25	24	5
Atp1	CAGL-CDS1821.1	Ybl099w	1.8	2.3	F1F0-ATPase complex, F1 alpha subunit	13/36	28	5
Cox6[c)]	CAGL-CDS4818.1	Yhr051w	−1.9	−2.9	Cytochrome *c* oxidase subunit VI	5/17	21	1
Cpr1	CAGL-CDS4714.1	Ydr155c	−1.9	−71	Cyclophilin (peptidylprolyl isomerase)	6/13	45	2
Cta1[c)]	CAGL-CDS2032.1	Ydr256c	−1.5	−38	Catalase A	9/16	23	5
Eno1[c)]	CAGL-CDS2501.1	Ygr254w	1.3	−66	Enolase	13/28	32	11
Gcn3	CAGL-CDS3563.1	Ykr026c	−1.5	−2.2	Translation initiation factor eIF2B	5/22	27	5
Gin3	CAGL-CDS1983.1	Yml128c	1.8	2.3	Hypothetical protein	13/36	28	5
Ilv5[c)]	CAGL-CDS2775.1	Ylr355c	1.3	−66	Ketol-acid reducto-isomerase	7/28	23	5
Ino1[c)]	CAGL-CDS1849.1	Yjl153c	−1.6	−43	Myo-inositol-1-phosphate synthase	8/18	14	4
Pgk1[c)]	CAGL-CDS2665.1	Ycr012w	−1.6	−3.3	Phosphoglycerate kinase	5/16	20	3
Rhr2	CAGL-CDS4010.1	Yil053w	−1.5	−2.2	DL-glycerol phosphatase	6/22	38	4
Ssa2[c)]	CAGL-CDS1311.1	Yll024c	−1.8	−22	Heat shock protein of HSP70 family	5/11	9	−1
Tpi1	CAGL-CDS4014.1	Ydr050c	−1.7	−3.9	Triose-phosphate isomerase	7/18	33	1
Yef3[c)]	CAGL-CDS0384.1	Ylr249w	1.6	−16	Translation elongation factor eEF3	10/22	9	6

a) PMF quality metrics [39]: hit ratio, number of masses matched to the candidate protein/number of masses submitted to the search; coverage equals the total percent of candidate protein sequence covered by the matched peptides; excess of limit-digested peptides (ELDP) equals the number of matched peptides having no missed cleavages minus the number of matched peptides containing a missed cleavage site. An ELDP value >1 strongly indicates a correct match [39].
b) *S. cerevisiae* proteins having the highest homology by the WU-BLAST2 program (http://seq.yeastgenome.org/cgi-bin/blast-sgd.pl).
c) Possible fragment of full-length protein (according to 2-D gel position and PMF coverage).

There were two notable exceptions to the observation that glucose catabolic enzymes were induced in *C. glabrata* cells growing at pH 4.0 compared with cells growing at pH 7.4 or 8.0: Pfk1 and Pfk2 (Table 3). Significantly these are the *alpha* and *beta* subunits of phosphofructokinase1, which is specific to and essential for glycolysis. How might glycolytic flux be maintained in *C. glabrata* cells during growth at low pH in the face of decreased phosphofructokinase 1 levels? In *S. cerevisiae*, phosphofructokinase 1 activities are maintained by increased levels of the positive allosteric effector fructose-2,6-*bis*phosphate [35–37]. Therefore, analogous mechanisms might exist in *C. glabrata* to maintain phosphofructokinase 1 activity, and hence glycolytic flux, during growth in acidic conditions.

An interesting difference between the pH responses of *C. glabrata* and *C. albicans* lies in the behaviour of their protein

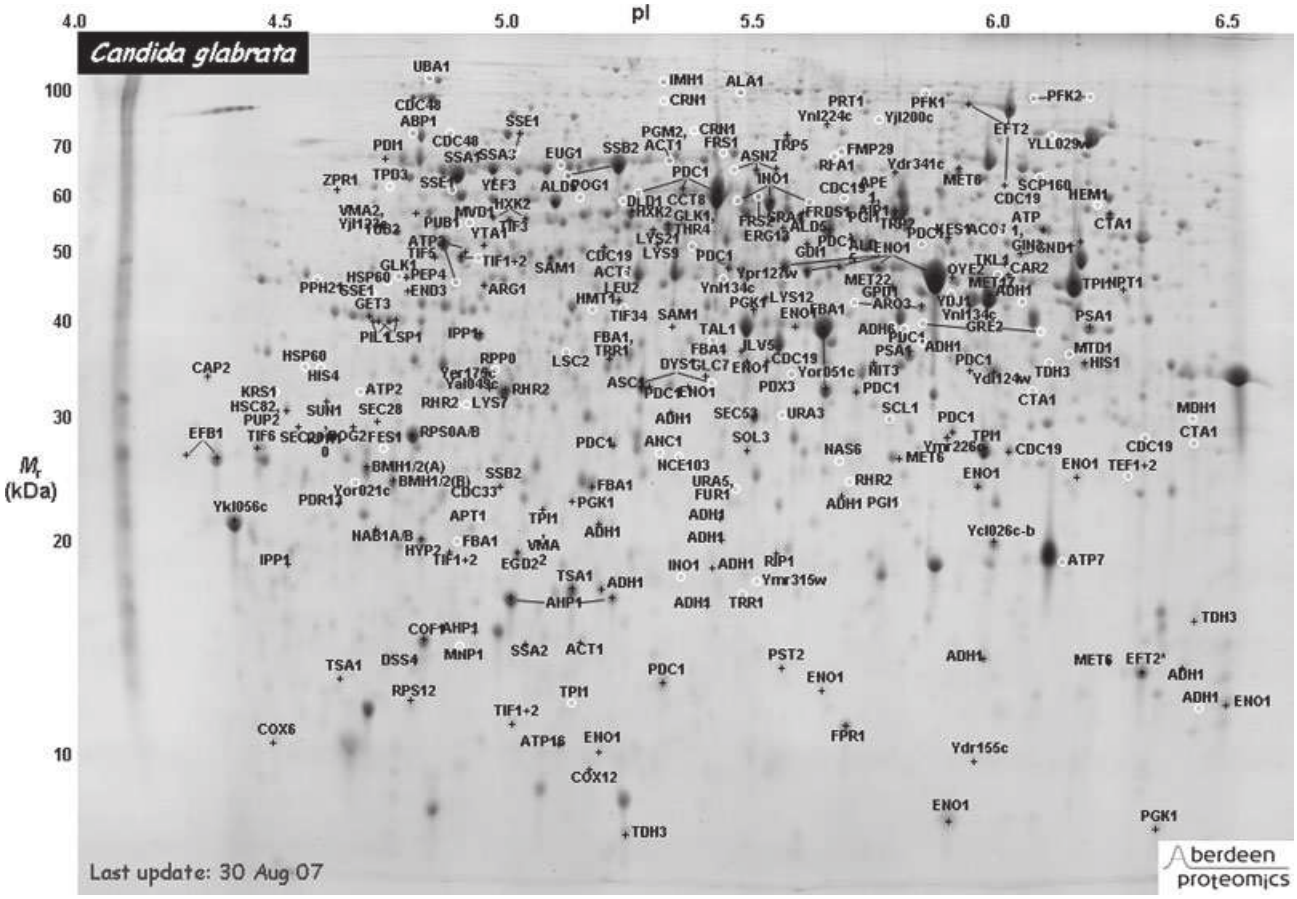

Figure 4. Updated 2-DE map of the *C. glabrata* proteome. Gene annotations are by homology to *S. cerevisiae*.

Table 6. Enrichment of specific functional categories in pH-regulated protein clusters

GO ID	GO term	Cluster 1	Cluster 2	Cluster 3	Cluster 4
		(▼) 7.4 and 8.0	(▼) 8.0	(▲) 7.4 and 8.0	(▲) 8.0
6007	Glucose catabolism		5.5E-06	6.8E-05	
6098	Pentose-phosphate shunt		6.0E-04		
6082	Organic acid metabolism	1.1E-04	1.4E-04		
15980	Energy derivation by oxidation of organic compounds		7.6E-07		
45333	Cellular respiration	5.0E-04	5.1E-02		
9308	Amine metabolism	5.7E-02	5.5E-02		
6796	Phosphate metabolism	4.5E-02			
6412	Protein biosynthesis		8.8E-02		
6457	Protein folding	6.7E-03			
6461	Protein complex assembly	2.9E-02			
30163	Protein catabolism			7.5E-03	1.7E-02
6950	Response to stress			4.1E-03	
9408	Response to heat			1.4E-05	
45454	Cell redox homeostasis		9.2E-06		
7059	Chromosome segregation		6.5E-02		
7569	Cell aging		1.2E-02		
226	Microtubule cytoskeleton organisation and biogenesis		3.7E-02		
30036	Actin cytoskeleton organisation and biogenesis				8.6E-03

synthesis and degradation functions. In *C. albicans*, protein synthesis genes are generally up-regulated at pH 8, and protein degradation genes are down-regulated under these conditions [29]. However, we found that *C. glabrata* proteins involved in protein synthesis were expressed at lower levels at pH 8.0, and protein catabolic enzymes were expressed at higher levels in an alkaline environment (Table 6). Also, the functional category "response to stress" was significantly enriched in the subset of *C. glabrata* proteins that were up-regulated at higher pH (Table 6). At face value, these data might suggest that, in contrast to *C. albicans*, C. *glabrata* perceives low pH as less stressful than the more basic conditions. This is consistent with the idea that *C. glabrata* is an opportunistic pathogen of humans that has evolved primarily in relatively acidic environmental niches such as rotting fruit. However, it should be borne in mind that, as mentioned above, mild alkaline conditions stimulate hyphal development in *C. albicans*, but not in *C. glabrata* [17,18]. This is likely to account for at least some of the observed differences in gene regulation between these species at pH 8.0. Furthermore, caution should be exercised in comparing the effects of ambient pH upon the *C. glabrata* proteome (this study) and the *C. albicans* transcriptome [29] because it is well known that the correlation between the transcriptome and proteome can be poor, even in the same organism.

In conclusion, this study, which is one of very few proteomic studies in *C. glabrata* [19, 38], reveals fundamental differences between the global pH responses of major fungal pathogens of humans (*C. glabrata* and *C. albicans*), and provides a valuable platform for the molecular analysis of pH adaptation in this model pathogen.

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