Hypoxic conditions and iron restriction affect the cell-wall proteome of *Candida albicans* grown under vagina-simulative conditions

Grazyna J. Sosinska, Piet W. J. de Groot, M. Joost Teixeira de Mattos, Henk L. Dekker, Chris G. de Koster, Klaas J. Hellingwerf and Frans M. Klis

Correspondence

Grazyna Sosinska
sosinska@science.uva.nl

Swammerdam Institute for Life Sciences, University of Amsterdam, Nieuwe Achtergracht 166, 1018 WV, Amsterdam, The Netherlands

Proteins that are covalently linked to the skeletal polysaccharides of the cell wall of *Candida albicans* play a major role in the colonization of the vaginal mucosal surface, which may result in vaginitis. Here we report on the variability of the cell-wall proteome of *C. albicans* as a function of the ambient O₂ concentration and iron availability. For these studies, cells were cultured at 37 °C in vagina-simulative medium and aerated with a gas mixture consisting of 6% (v/v) CO₂, 0.01–7% (v/v) O₂ and N₂, reflecting the gas composition in the vaginal environment. Under these conditions, the cells grew exclusively in the non-hyphal form, with the relative growth rate being halved at ~0.02% (v/v) O₂. Using tandem MS and immunoblot analysis, we identified 15 covalently linked glycosylphosphatidylinositol (GPI) proteins in isolated walls (Als1, Als3, Cht2, Crh11, Ecm33, Hwp1, Pga4, Pga10, Phr2, Rbt5, Rhd3, Sod4, Ssr1, Ywp1, Utr2) and 4 covalently linked non-GPI proteins (MP65, Pir1, Sim1/Sun42, Tos1). Five of them (Als3, Hwp1, Sim1, Tos1, Utr2) were absent in cells grown in rich medium. Immunoblot analysis revealed that restricted O₂ availability resulted in higher levels of the non-GPI protein Pir1, a putative β -1,3-glucan cross-linking protein, and of the GPI-proteins Hwp1, an adhesion protein, and Pga10 and Rbt5, which are involved in iron acquisition. Addition of the iron chelator ferrozine at saturating levels of O₂ resulted in higher cell wall levels of Hwp1 and Rbt5, suggesting that the responses to hypoxic conditions and iron restriction are related.

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INTRODUCTION

Candida albicans is a pleomorphic pathogenic fungus that is responsible for many mucosal infections in humans, and may cause systemic, often fatal infections in immunocompromised patients (Calderone, 2002). It is also well equipped to form biofilms on abiotic surfaces such as dentures and medical devices (Blankenship & Mitchell, 2006; Ramage *et al.*, 2006). Covalently linked cell-wall proteins (CWPs) play an important role in initiating and maintaining mucosal infections and biofilms (De Groot *et al.*, 2007; Li *et al.*, 2007; Naglik *et al.*, 2006; Nobile *et al.*, 2006; Richard & Plaine, 2007; Ruiz-Herrera *et al.*, 2006; Sundstrom, 1999; Zhao *et al.*, 2006; Zupancic & Cormack, 2007). This is consistent with their cellular location because covalently linked CWPs form an external protein layer surrounding the internal skeletal polysaccharide layer of the wall, and thus come directly into contact with biotic and abiotic surfaces. As in *Saccharomyces cerevisiae*, the cell-wall proteome of *C. albicans* consists of 15 or more covalently linked CWPs and includes structural proteins,

adhesion proteins, carbohydrate-active proteins, proteases, superoxide dismutases and iron-acquisition proteins (Albrecht *et al.*, 2006; De Groot *et al.*, 2004; Garcera *et al.*, 2005; Mao *et al.*, 2003; Weissman & Kornitzer, 2004). In agreement with the outcome of similar studies carried out with *S. cerevisiae*, genomic transcript profiling studies of *C. albicans* indicate that the composition of the cell-wall proteome may vary considerably with the growth conditions of the organism. For example, both yeast and hyphal walls seem to possess specific CWPs (Sohn *et al.*, 2003; Staab *et al.*, 1999). The expression of several CWP-encoding genes changes when *Candida* cells become associated with different human epithelia or with abiotic surfaces (Garcia-Sanchez *et al.*, 2004; Sohn *et al.*, 2006), when transferred to blood (Fradin *et al.*, 2005), or when confronted with environmental stress conditions such as low or high pH (Bensen *et al.*, 2004), the presence of azoles in the medium (De Backer *et al.*, 2001; Liu *et al.*, 2005), which interferes with sterol synthesis, or the presence of caspofungin, which inhibits the synthesis of the skeletal polysaccharide β -1,3-glucan and thereby causes cell-wall stress (Liu *et al.*, 2005). Iron deprivation and hypoxic conditions are also known to affect the expression of CWP-encoding genes (Lan *et al.*, 2004; Setiadi *et al.*, 2006).

Abbreviations: CWP, cell-wall protein; GPI, glycosylphosphatidylinositol; VSM, vagina-simulative medium; YNB, yeast nitrogen base.

Collectively, these observations strongly suggest that the composition of the cell-wall proteome may vary considerably, both qualitatively and quantitatively, depending on environmental cues.

Many *Candida* infections involve colonization of the vaginal mucosal layer (Fidel & Sobel, 2002; Sobel, 2007). About three-quarters of all women suffer from vaginitis at least once during their life time. Vaginal environmental conditions are characterized by a relatively low pH, a high partial pressure of CO₂ (approx. 6%, v/v) and low to vanishing partial pressures of O₂ ($\leq 10\%$, v/v).

In this study, we have analysed the cell-wall proteome of *C. albicans* cells grown in vagina-simulative medium (VSM) at 6% CO₂ (v/v) and at O₂ levels ranging from 7 to 0.01% (v/v). We also studied the effect of iron availability by growing the cells at 7% O₂ with an iron chelator. Our results show that under these conditions a new set of CWPs is incorporated into the walls compared to cells grown in rich medium at pH 5–5.6 (De Groot *et al.*, 2004). At restrictive O₂ concentrations the levels of at least four CWPs increase, i.e. the structural protein Pir1, the adhesion protein Hwp1 and, in particular, Pga10 and Rbt5, two iron-acquisition proteins. These observations are consistent with the notion that hypoxic conditions may lead to a cellular response that involves an increased scavenging capacity for iron.

METHODS

Strains and growth conditions. The *C. albicans* strains used in this study are listed in Table 1. VSM was modified from Moosa *et al.* (2004) and Owen & Katz (1999) and consisted of 58 mM NaCl, 18 mM KOH, 2 mM Ca(OH)₂, 1.75 mM glycerol, 6.7 mM urea, 33 mM glucose, and 6.7 g yeast nitrogen base (YNB) l⁻¹ (Difco). In addition, lactic acid (22 mM; pK_a=3.85) and acetic acid (17 mM; pK_a=4.76), which are natural compounds in the vaginal fluid, were added to maintain the pH at 4.2. Strains were pre-cultured to saturation in YPD medium [1% (w/v) Bacto-yeast extract; 2% (w/v) Bacto-peptone; 2% glucose (w/v)] at 30 °C and 200 r.p.m. The cells were then inoculated in VSM in batch fermenters (working volume 0.5 l) at 37 °C with an aeration rate of 0.5 l min⁻¹ and stirred at 200 r.p.m. The cells were cultured overnight under ambient air to an OD₆₀₀ of ~0.1; aeration was subsequently switched to gas mixtures, consisting of N₂, 0.01–7% (v/v) O₂ and 6% (v/v) CO₂, which leads to dissolved O₂ concentrations that correspond to values measured in the human vagina (Wagner & Ottesen, 1982). The cells were harvested at an OD₆₀₀ of ~1. To induce iron restriction, 1 mM ferrozine [5,6-diphenyl-3-(2-pyridyl)-1,2,4-triazine-4',4''-disulfonic

acid; Sigma-Aldrich] was added to overnight cultures at OD₆₀₀ ~0.1. Culturing was continued until an OD₆₀₀ ~1 was reached and the cells were collected for cell-wall analysis. The OD₆₀₀ of cultures was measured using a Shimadzu model UV mini 1240 spectrophotometer (OD₆₀₀=1 corresponds to 1.5×10^7 cells ml⁻¹). To determine the percentage of budded cells (the budding index), 200 cells were taken from two independent cultures for each condition and counted. Relative growth rates were determined by following the OD₆₀₀ of three separate cultures in the range OD₆₀₀ ~0.1 to ~1. A reference cell culture grown in rich medium (pH 5.5) (Table 2) was obtained as follows. The medium consisted of 20 g glucose l⁻¹, 10 g Casamino acids l⁻¹ (Becton Dickinson), 6.7 g YNB l⁻¹ (Difco), 110 µg leucine ml⁻¹, 55 µg tyrosine ml⁻¹, 55 µg tryptophan ml⁻¹ and 55 µg adenine sulfate ml⁻¹, pH 5.5, as described by De Groot *et al.* (2004). The cells were cultured overnight in a batch fermenter aerated with atmospheric air with stirring at 200 r.p.m. at 30 °C and were harvested at mid-exponential phase.

Cell-wall isolation. Cell walls were isolated as described previously (De Groot *et al.*, 2004). Cultures were harvested in the exponential phase of growth, washed with cold demineralized water and with 10 mM Tris/HCl buffer, pH 7.5, and disintegrated in a Bio-Savant Fast Prep 120 machine (Qbiogene), using 0.25–0.50 mm diameter glass beads (Emergo BV). A protease inhibitor mixture (Sigma-Aldrich) was added to protect CWPs from degradation by intracellular proteases. To remove non-covalently linked proteins associated with the wall preparation, crude cell walls were washed with 1 M NaCl and boiled twice for 5 min in 2% (w/v) SDS, 150 mM NaCl, 100 mM Na-EDTA, 100 mM β-mercaptoethanol, 50 mM Tris/HCl, at pH 7.8. SDS-extracted walls were washed three times with demineralized water and freeze-dried.

Sample preparation for MS analysis. For MS analysis, CWPs were modified by reduction and S-alkylation as follows. Cell walls were treated with the reducing reagent 10 mM dithiothreitol in 100 mM NH₄HCO₃ for 1 h at 55 °C. The samples were then cooled and the walls were alkylated by incubating them for 45 min at room temperature in the dark in 65 mM iodoacetamide in 100 mM NH₄HCO₃. The reaction was quenched by incubating the walls in 55 mM dithiothreitol in 100 mM NH₄HCO₃ for 5 min. Prior to digestion with trypsin, the walls were washed three times with 50 mM NH₄HCO₃. Trypsin digestion of S-alkylated CWPs was carried out as described by Yin *et al.* (2005). Cell walls were incubated overnight at 37 °C in the presence of sequencing-grade trypsin (Roche Applied Science) using a CWP/enzyme ratio of 50:1.

MS identification of covalently linked CWPs. Instrument and analysis settings were as described by Yin *et al.* (2005). Tryptic peptides (5–10 µg) were desalted and concentrated on a C18-ZipTip pre-column (Millipore) and fractionated using a nano-LC system (PepMap C18; LC Packings, Dionex). Eluted peptides were directly ionized by electrospray in a Micromass quadrupole time-of-flight mass spectrometer (Waters). The ions from the survey spectrum were

Table 1. *C. albicans* strains used in this study

Strain	Description	References
SC5314	Clinical isolate	Fonzi & Irwin (1993)
CAF 2-1	<i>ura3Δ::imm434/URA3</i>	Fonzi & Irwin (1993)
BCa18-2	<i>ura3Δ::imm434/ura3Δ::imm434 rbt5Δ::hisG/rbt5Δ::hisG-URA3-hisG</i>	Braun <i>et al.</i> (2000)
KC85	<i>ura3Δ::imm434/ura3Δ::imm434; pga10Δ::hisG pga10Δ::hisG-URA3-hisG</i>	Weissman & Kornitzer (2004)
KC100	<i>ura3Δ::imm434/ura3Δ::imm434; rbt5Δ::hisG/rbt5Δ::hisG; pga10Δ::hisG pg⁻a10Δ::hisG-URA3-hisG</i>	Weissman & Kornitzer (2004)

Table 2. Proteins identified by LC/MS/MS or immunoblot analysis in cell walls isolated from cultures grown in VSM in the presence of 7–0.01 % O₂/6 % CO₂

For comparison, the CWP's identified in cells from a reference culture grown in rich medium and aerated with atmospheric air are also presented. +, Detected by MS/MS; –, not detected by MS/MS; ++, detected by immunoblot; --, not detected by immunoblot.

Name	Function and features	Sequence of identified peptides	Position	VSM	Reference
GPI-modified proteins					
Als1	1260 aa; adhesion protein (Klotz <i>et al.</i> , 2007)	STVDPSPGYLYASR	176–188	+	–
		GLNDWNYPVSSSEFSYTK*	237–254	+	–
		SQSKPFTLR	303–311	+	+
Als3	1155 aa; hypha-induced adhesion protein (Argimon <i>et al.</i> , 2007); highly expressed during vaginitis (Cheng <i>et al.</i> , 2005)	FTTSQTSVDLTAHGVK	77–92	+	–
		CFTAGTNTVTFNDGGK	150–165	+	–
		CFTAGTNTVTFNDGGKK	150–166	+	–
		KISINVDFER	166–175	+	–
		ISINVDFER	167–175	+	–
		GLNDWNYPVSSSEFSYTK*	237–254	+	–
		TCSSNGIFITYK	255–266	+	–
Cht2	583 aa; chitinase (McCreath <i>et al.</i> , 1995)	SNQVALYWGQNGAGGQER†	22–39	+	+
		TVLLSLGGVGVDYGFSDVASATK	99–121	+	–
		FADTLWNK	122–129	+	+
		FADSAPNK	229–236	+	+
		FADSAPNKNK	229–239	+	+
		LFVGVPAATSNAGYVDTSK	240–258	+	–
		GENFVVQVK	291–299	+	–
Crh11	453 aa; putative transglycosidase (Pardini <i>et al.</i> , 2006)	SSDCSPVPALGSSFLEK	29–45	+	–
		RFDNPSFK	77–84	+	–
		FDNPSFK	78–84	+	+
		DAVTWSVDGVSIR	170–182	+	–
		SVLVADYSSGK	236–246	+	+
		QYSYSDQSGSWESIK	247–261	+	+
		YDQAQDDIK	271–279	+	–
		YDQAQDDIKK	271–280	–	+
Ecm33	423 aa; required for cell wall integrity (Martinez-Lopez <i>et al.</i> , 2006); induced by fluconazole (Copping <i>et al.</i> , 2005) and hypoxia (Setiadi <i>et al.</i> , 2006)	NDDLTELDFPK	263–273	+	–
		TIGGALQISDNSELK	276–290	+	–
		SFSGFPK	291–297	+	+
		VSGGFILK	322–329	+	–
		VSGGFILKTDGK	322–333	+	–
		LSCSAFNK	334–341	+	–
Hwp1	634 aa; hypha-specific protein (Staab <i>et al.</i> , 1999); induced by hypoxia (Setiadi <i>et al.</i> , 2006; this work)	Immunological identification only		++	--
Pga4	451 aa; transglucosidase	GVDYQPGGSSELEDPLADTNVCER	42–68	+	–
		YFQELGINTIR	69–79	+	–
		LPSGLYFNCGDDDMAR	199–214	+	–
		GDSVTTNDDDFDNLK	302–315	+	–
		TKNPSGDGGYK	321–332	+	–
		GLAEPGTGHGFDAYVQGNCNAK	365–385	+	–
Pga10	250 aa; iron-acquisition protein (Weissman & Kornitzer, 2004); induced by ketoconazole (Liu <i>et al.</i> , 2005) and hypoxia (this work)	IYDQLPECAK‡	47–56	+	+
		IYDQLPECAKECVK‡	47–60	+	+
		Immunological identification (this work)		++	Not checked
Phr2	544 aa; transglucosidase expressed at pH <5.5; induced by fluconazole (Copping <i>et al.</i> , 2005)	EDLPAIEIVGNK†	23–34	+	–
		DIPYLEAVDTNVIR	75–88	+	–
		PSWDLDLFER	128–137	+	–
		KSNTDASAFVK	164–174	–	–
		SNTDASAFVK\$	165–174	+	+
		SIPVGYSANDDSAIR	191–205	+	+
		ASGYESATNDYK	239–250	+	–
		NLGIPIFFSEYGCNEVRPR	251–269	+	–

Table 2. cont.

Name	Function and features	Sequence of identified peptides	Position	VSM	Reference
Rbt5	241 aa; iron-acquisition protein; induced by ketoconazole (Liu <i>et al.</i> , 2005) and hypoxia (Setiadi <i>et al.</i> , 2006; this work)	IYDQLPECAK‡	47–56	+	+
		IYDQLPECAKECVK‡	47–60	+	+
		QSTSSTPCPYWDTGCLCVMPQFAGAVGNCVAK	61–92	+	–
		MPQFAGAVGNCVAK	79–92	+	–
		QFAGAVGNCVAK	81–92	+	–
		Immunological identification (this work)		++	Not checked
Rhd3	204 aa; unknown function	KTDDSAPIITIVAK	138–150	+	+
		TDDSAPIITIVAK	139–150	–	+
Sod4	232 aa; putative Cu/Zn superoxide dismutase (Martchenko <i>et al.</i> , 2004)	GDSPISTDSK†	16–25	+	+
		GDSPISTDSKGK†	16–27	–	+
		GDSPISTDSKGKAPLVAR†	16–33	+	–
		TPAALELGDLSGR	105–117	+	+
Ssr1	234 aa; structural CWP (Garcera <i>et al.</i> , 2003)	APPACLLACVAK†	23–34	+	+
		APPACLLACVAKVEK†	23–37	+	–
		CSGLNDLSCICTTK	41–54	+	+
		CSGLNDLSCICTTKNSDVEK	41–60	+	–
		EICPNGDADTAISAFK	64–79	+	+
Utr2	470 aa; possible role in cross-linking β -1,3-glucan and chitin (Pardini <i>et al.</i> , 2006)	MSTFQESFDSKDKVK	75–89	+	–
		IQFSLWPGDSSNAK	254–268	+	–
		GTIEWAGGLINWSEDIKK	269–287	+	–
Ywp1	533 aa; unknown function; expression higher in yeast (Granger <i>et al.</i> , 2005)	VITVVACDEHK	329–339	+	–
Non-GPI proteins					
MP65	378 aa; endo- β -1,3-glucanase (Sandini <i>et al.</i> , 2007)	GITYSPYSDNGGCK	128–141	+	–
		SESQIASEIAQLSGFDVIR	142–160	+	–
		LYGVDCSQVEAVLK	161–174	+	–
		AYVDEGRK	234–241	–	+
		VWTACSGKK	302–310	+	–
		SNQQAIISSIK	334–344	+	+
Pir1	346 aa; putative β -1,3-glucan cross-linking protein; induced by fluconazole (Copping <i>et al.</i> , 2005) and hypoxia (this work)	ACSSANNLEMTLHDSVLK	249–266	+	–
		Immunological identification (this work)		++	Not checked
Sim1	372 aa; unknown function; induced by hypoxia (Setiadi <i>et al.</i> , 2006)	DGYCYSYACQAGMSK	162–176	+	–
		APCSVVDGDTYFK	253–265	+	–
Tos1	468 aa; unduced by fluconazole (Copping <i>et al.</i> , 2005)	SGEYIIFSGSK	288–299	+	–
		TLQYGEATCSCWK	358–370	+	–

*Common peptide of Als1 and Als3.

†Predicted N-terminal peptide of the mature protein.

‡Common peptide of Pga10 and Rbt5.

§Common peptide of Phr1 and Phr2.

automatically selected for fragmentation in a collision chamber using Masslynx software. Tandem MS spectra of ionized peptide fragments were analysed with Biolynx and Masslynx Pepseq software. Proteins were identified by comparison of the identified amino acid peptide sequences with *in silico* digests of the proteins translated from Assembly 19 of the *C. albicans* SC5314 genome sequence (www.candidagenome.org/) using Mascot software. Several precautions were taken to optimize the reproducibility of the results over time and to monitor drift of the equipment. (1) The nano-LC column was cleaned daily by thoroughly washing it with 100% solvent B (acetonitrile + 0.1% formic acid). (2) To prevent carry over from previous samples, at least one empty injection was run between each sample. (3) The amount of sample loaded in the LC step of each run was measured using an A₂₁₄ chromatogram. (4) To control the efficiency of ionization

and to verify that the run-to-run variation of the ion intensity was <20%, the total ion count (TIC) profile of each LC/MS/MS run was determined. (5) To verify the accuracy of MS selection, the instrument was calibrated daily with 1 pmol cytochrome *c* (Dionex). (6) Each sample (containing ~1 pmol protein) was run at least three times, and in two of them the exclusion list was used to ensure complete coverage. (7) The general performance of the LC/MS/MS system was checked monthly with a cytochrome *c* digest to verify that a similar number of peptides of similar Mascot score were identified.

Isolation of CWPs for immunoblot analysis. Glycosylphosphatidylinositol (GPI)-modified CWPs were released by treating cell-wall material with recombinant *Trichoderma harzianum* endo- β -1,6-glucanase (Bom *et al.*, 1998) as described by Kapteyn *et al.* (2001).

Freeze-dried cell walls (4 mg) were incubated overnight with 2.5 μ l [0.16 U (mg cell walls)⁻¹] enzyme and 2 μ l of a protease inhibitor mixture (Sigma-Aldrich) in 200 μ l 50 mM sodium phosphate buffer, pH 5.5, at 37 °C overnight. To release mild alkali-extractable CWPs, cell walls were incubated with 30 mM NaOH at 4 °C for 17 h with gentle shaking; the reaction was stopped by neutralization with 30 mM acetic acid (Mrša *et al.*, 1997). In each lane, the equivalent of 0.15 mg dried walls (corresponding to ~1 mg dried biomass) was applied.

Immunoblot analysis of CWPs. CWPs were separated by electrophoresis using linear 3–8% polyacrylamide gradient gels in Tris/acetate (Invitrogen). The separated proteins were transferred onto an Immobilon polyvinylidene difluoride (PVDF) membrane (Millipore). To reduce non-specific staining by the antisera, the membrane-blotted proteins were first incubated with 50 mM periodic acid in 100 mM sodium acetate (pH 4.5) prior to the blocking step. Immunoblot analysis was performed with polyclonal Hwp1 (Staab *et al.*, 1996) or Pga10 antiserum (Weissman & Kornitzer, 2004), diluted 1:10 000 in 5% (w/v) milk powder in PBS buffer, pH 7.4, for 2 h. *S. cerevisiae* Pir2 antiserum (Russo *et al.*, 1992) was diluted 1:50 000 and incubated with the blots overnight to enhance interaction with the probed proteins. After washing with PBS, the membranes were incubated with goat anti-rabbit antiserum, conjugated with peroxidase (GARPO) at a dilution of 1:10 000 in 5% (w/v) milk powder in PBS buffer, pH 7.4. Proteins were visualized using Enhanced Chemiluminescence (ECL) (Amersham Biosciences).

Quantazyme sensitivity. Quantazyme sensitivity of intact cells was measured as described by Kapteyn *et al.* (2001). Exponentially growing cells were centrifuged and washed twice with 50 mM Tris/HCl, pH 7.5. The OD₆₀₀ of the cell suspensions was adjusted to 1 and the cells were pre-incubated for 1 h with 40 mM β -mercaptoethanol in 50 mM Tris/HCl buffer, pH 7.5. Quantazyme (from *Oerskovia xanthineolytica*; Quantum Biotechnologies) was added at 20 U (ml cell suspension)⁻¹. Incubation was carried out at 30 °C, and the decrease in OD₆₀₀ was measured at 5 min intervals.

Determination of the polypeptide and chitin content of isolated cell walls. Cell walls (4 mg) were suspended in 100 μ l 1 M NaOH. The suspension was incubated at 100 °C for 10 min, cooled and neutralized with 100 μ l 1 M HCl. Insoluble material was pelleted by centrifugation and the supernatant was used for protein determination using the bicinchoninic acid protein assay (Pierce). A calibration curve was prepared by using BSA treated in the same way as the cell wall samples.

Chitin content was measured using the method described by Kapteyn *et al.* (2001). NaOH-extracted (4 mg; see protein determination above) cell walls were hydrolysed for 17 h in 1 ml 6 M HCl at 100 °C. Samples were evaporated under a stream of air and resuspended in 1 ml de-mineralized water. To 0.1 ml sample, 0.1 ml 1.5 M Na₂CO₃ in 4% acetylacetone was added and the mixture was boiled for 20 min. After cooling, 0.7 ml 96% ethanol and 0.1 ml 1.6 g *p*-dimethylaminobenzaldehyde in 30 ml concentrated HCl and 30 ml 96% (v/v) ethanol were added before incubation for 1 h at room temperature. A calibration curve was prepared by measuring A₅₂₀ in a concentration range of 0–40 μ g glucosamine ml⁻¹.

RESULTS

The effect of changes in the gas-phase composition on the relative growth rate and cell-wall composition of *C. albicans* grown in VSM

For the studies reported in this communication we used a growth medium consisting of YNB with modified

concentrations of calcium, chloride, potassium and sodium ions, conforming to the concentrations in the vaginal fluid of healthy pre-menopausal women during the non-menstrual phase (Owen & Katz, 1999) (Methods). The medium further contained urea and glycerol and was buffered at pH 4.2 by making use of the physiological buffers found in the vaginal fluid, i.e. 22 mM lactic acid (pK_a 3.85) and 17 mM acetic acid (pK_a 4.76) (Owen & Katz, 1999). Glucose, which is present in the vaginal fluid at a concentration of ~33 mM, served as the main carbon source. The cells were grown at 37 °C. The microenvironment of the human vagina is characterized by lower O₂ and higher CO₂ levels compared to atmospheric values; (Wagner & Ottesen, 1982). The O₂ concentrations measured in the vaginal fluid vary from almost 0 to maximally 0.11 mM [equivalent to 10% (v/v) partial O₂ pressure], depending on the day of the menstrual cycle and the method used for measuring gas tensions. Vaginal CO₂ levels, however, vary only slightly during the menstrual cycle (6–8%, v/v) (Wagner & Ottesen, 1982). Therefore, in this study we supplied O₂ to cells in concentrations equivalent to a partial O₂ pressure between 0.01 and 7% (v/v), in combination with 6% (v/v) CO₂, using batch fermenters with controlled aeration.

The growth rate of *C. albicans* on the medium selected and under an atmosphere of 7% O₂/6% CO₂ equals 0.60 h⁻¹ (corresponding to a generation time of ~70 min; see Fig. 1), which is considerably faster than under atmospheric conditions in the same medium (0.45 h⁻¹, corresponding to a generation time of ~90 min). As *S. cerevisiae* can fix CO₂ via pyruvate carboxylase to form the citric acid cycle intermediate oxaloacetate, and as higher CO₂ concentrations up to ~6% result in increased CO₂ fixation (Cazzulo *et al.*, 1968; Liener & Buchanan, 1951), the increased

Fig. 1. The effect of lowering the O₂ concentration on the relative growth rate of *C. albicans* cells growing in VSM at 37 °C and at 6% CO₂. The data shown represent the means of three cultures. The error bars represent SD.

growth rate might be caused by the elevated CO₂ concentration. Fig. 1 further shows that at 7 % (v/v), O₂ is present at a saturating concentration with respect to the growth rate. The maximal growth rate of *C. albicans* in batch culture decreases significantly, however, when the O₂ level is lowered to less than 0.5 % (v/v) O₂ and is halved at ~0.02 % (v/v), which corresponds to a dissolved O₂ concentration of approximately 0.23 µM. Further experiments were carried out using an O₂ level of 0.02 % (v/v).

Cells cultured in VSM at low O₂ levels in combination with elevated CO₂ levels grew exclusively in the yeast and pseudohyphal form, under all O₂ concentrations tested; a similar morphology was observed when the cells were grown in VSM and aerated with an atmospheric gas mixture (data not shown). The lower growth rates at low O₂ concentrations were accompanied by a decrease in the budding index, which dropped from 91 % at 7 % O₂ to 33 % at 0.01 % O₂, indicating that the G1 phase of the cell cycle is extended when cells are deprived of O₂. Interestingly, lower O₂ levels resulted in increased resistance of intact cells to Quantazyme, a recombinant β-1,3-glucanase, indicating that the structure or composition of the cell wall had changed (Fig. 2). This was not reflected in significant changes in the total polypeptide content of the walls, which was 2.4 % at both 7 % O₂ (v/v) and 0.02 % O₂ (v/v). The respective chitin contents of these wall preparations were 3.5 and 2.9 %. The small differences in these cell-wall components may imply that the increased Quantazyme resistance of *C. albicans* is due to an altered protein composition of its wall (see next section).

Fig. 2. Increased resistance of cells, grown under a low partial pressure of O₂, to the endo-β-1,3-glucanase Quantazyme. The cells were grown at 7 % O₂/6 % CO₂ (●) or at 0.02 % O₂/6 % CO₂ (○). The values shown are the means of two replicates.

Effects of O₂ deprivation on the cell-wall proteome

For a qualitative comparison of the covalently linked proteins in the cell wall of wild-type cells grown at O₂ concentrations ≤ 7 % (v/v) and at 6 % CO₂ (v/v) in VSM, we applied LC followed by tandem MS (LC/MS/MS) of CWP-derived peptides. In combination with immunoblot analysis we detected 19 proteins, including 15 known or predicted GPI proteins, and four non-GPI proteins (Table 2, Fig. 3). For comparison, cell walls obtained from a fermenter-grown reference culture grown in rich medium were also analysed. Importantly, the GPI-proteins Als3, Hwp1 and Utr2, and the non-GPI proteins Sim1 and Tos1, were only found in VSM cultures. Additionally, in VSM cultures a specific peptide confirming the presence of Rbt5 was detected, which was not identified previously (De

Fig. 3. O₂ limitation results in increased cell-wall levels of Pir1 (a), Hwp1 (b), and Pga10 and Rbt5 (c). Cells were grown overnight in a batch fermenter under air to an OD₆₀₀ of ~0.1. The cells were then cultured with controlled aeration using a gas mixture consisting of nitrogen, 6 % CO₂ and 0.01–7 % O₂ until an OD₆₀₀ of ~1 was reached. Covalently linked CWPs were released from cell walls with mild alkali extraction (Pir1) or β-1,6-glucanase (Hwp1, Pga10, Rbt5). For each condition, proteins isolated from equal amounts of cell walls (~0.15 mg dried walls, which corresponds to ~1 mg dried biomass) were subjected to immunoblot analysis. Sizes of marker proteins are indicated on the left. The diffuse nature of the Pir1 band is probably due to allelic variability (*Candida* Genome Database – www.candidagenome.org) and the high degree of glycosylation of Pir1.

Groot *et al.*, 2004). The transglucosylase Phr2 was identified in the walls at all tested O₂ concentrations, consistent with its function at acidic pHs (De Bernardis *et al.*, 1998; Fonzi, 1999; Mühlshlegel & Fonzi, 1997). Phr2 peptides were also identified in the reference culture, probably due to the fact that during culture the pH of the reference culture dropped from 5.5 to 4.9. This is consistent with the known pH-dependency of *PHR2* expression (Mühlshlegel & Fonzi, 1997). Als3, an adhesion protein that mimics mammalian cadherins (Phan *et al.*, 2007), was also identified in all VSM samples (20 identifications of Als3 peptides; data not shown), whereas no Als3 peptides were found in the reference culture. This is consistent with the frequent occurrence of *ALS3* transcripts in vaginal samples during *C. albicans* infection (Cheng *et al.*, 2005). Als3 has been described as a hypha-specific protein (Argimon *et al.*, 2007; Hoyer *et al.*, 1998); however, as our samples did not contain hyphae, our observations demonstrate that the synthesis of Als3 may also occur in non-hyphal cells under selective environmental conditions. Table 2 also shows that the peptides released by trypsin from GPI-modified CWPs and identified by tandem MS were predominantly found in the N-terminal region of these proteins, consistent with the observation that this region generally is less serine- and threonine-rich and therefore less glycosylated (Chen *et al.*, 1995). No such tendency was observed for the non-GPI proteins.

Because our mass spectrometric approach identified the same set of covalently linked CWPs at all O₂ levels tested (data not shown), we then used an immunological approach to detect quantitative changes in the cell-wall proteome. Using a cross-reactive antiserum raised against *S. cerevisiae* Pir2 (Russo *et al.*, 1992), we found increased levels of Pir1 protein in cell-wall extracts obtained with mild alkali, when the O₂ level decreased below 0.02 % (v/v) (Fig. 3a). As Pir1 protein is a potential β -1,3-glucan cross-linking protein and essential for cell-wall integrity (Klis *et al.*, 2007; Martinez *et al.*, 2004), this might explain the increased Quantazyme resistance of intact cells grown at low O₂ levels.

As the expression of *HWP1* is increased in non-hyphal cells grown under hypoxic conditions (Setiadi *et al.*, 2006), we also quantitatively analysed Hwp1 levels. Fig. 3(b) shows that Hwp1 was indeed present in the walls isolated from cells grown under vagina-simulative conditions at all O₂ concentrations tested and that its level increased at hypoxic O₂ levels. Nevertheless, the highest levels observed under these conditions were still significantly lower than the levels found in hyphal walls; Hwp1 was not found in walls of cells grown in rich medium even after overexposure of the immunoblots (data not shown).

The transcript levels of *RBT5*, which encodes a predicted GPI protein involved in iron acquisition (De Groot *et al.*, 2003; Weissman & Kornitzer, 2004), also showed a considerable increase under hypoxic conditions (Setiadi

et al., 2006). Fig. 3(c) shows that an antiserum raised against Pga10, a homologue of Rbt5 and also involved in iron acquisition (Weissman & Kornitzer, 2004), identified two bands with molecular masses of about 100 and 130 kDa at an O₂ concentration of ≤ 0.5 % (v/v). To investigate if these bands represented Rbt5 and Pga10 or other members of the family (Weissman & Kornitzer, 2004), we analysed the homozygous single-deletion mutants and the double deletion. Fig. 4 shows that the two bands incorporated in the cell wall of the control strain (CAF2-1) were not present in the double-deletion strain and that only a single band is detected in each of the single mutant strains grown under low-O₂ conditions. This is consistent with the notion that the 100 kDa band corresponds to Rbt5 and the 130 kDa band to Pga10. Interestingly, *PGA10* and *RBT5* belong to the same transcriptional module as *HWP1* (level 16, module 16; see also the *Candida* Genome Database – www.candidagenome.org; Ihmels *et al.*, 2005), showing that these three genes are co-regulated under several other test conditions as well.

Because the high-affinity iron-uptake system of *C. albicans* requires molecular O₂ (Kosman, 2003), hypoxic conditions are expected to lead to iron deprivation, raising the question whether iron deprivation alone might induce similar changes in the cell-wall proteome as O₂ limitation. Fig. 5 shows that the changes in the cell-wall proteome of cells treated with the ferrous iron chelator ferrozine (1 mM, resulting in a limited decrease in relative growth rate from 0.60 to 0.55 h⁻¹) partially overlapped with the changes observed in the cell-wall proteome of cells grown under hypoxic conditions. The levels of Rbt5 and Hwp1 in cell walls increased in both ferrozine-treated cells and in

Fig. 4. Immunoblot analysis of the Rbt5 family of proteins in *rbt5Δ* and *pga10Δ* mutant strains using a Pga10 antiserum. The cells were grown in VSM at 0.02 % O₂/6 % CO₂ (v/v). CWPs were released using β -1,6-glucanase.

cells grown under hypoxic conditions, but in ferrozine-treated cells the levels of Pir1 and Pga10 were not increased.

DISCUSSION

CWPs of *C. albicans* play a major role in the infection of mucosal surfaces in the human body, including the vaginal mucosal surface. Vaginal environmental conditions are characterized, among others, by a growth temperature of 37 °C, an acidic pH and by an increased concentration of CO₂ and low concentrations of O₂ compared to atmospheric values. Here we have studied the effect of vaginal growth conditions on the cell-wall proteome and, in particular, the effect of low O₂ concentrations. In the VSM that we developed for our studies, no hyphal growth was observed under any of the conditions tested. It is important to realize that the medium used in this study and the way in which the cells were grown do not fully mimic vaginal conditions. For example, VSM contains YNB, but no sterols or fatty acids, both of which may be expected to be present under *in vivo* conditions. In addition, *Candida* cells might grow in the vagina in the form of biomats or biofilms, whereas cells in our experiments were cultured in a rapidly stirred and fully aerated fermenter.

Using a combination of tandem MS and immunoblot analysis, we identified 19 CWPs, five of which (Als3, Hwp1, Sim1, Tos1 and Utr2) were not found in a reference culture grown in rich medium. This is in agreement with earlier results (De Groot *et al.*, 2004). This supports the notion that the composition of the cell-wall proteome of *C. albicans* is tightly controlled. The presence of Phr2 in the cell walls is in agreement with earlier observations showing that *PHR2* is specifically expressed at pHs below pH 5.5 and that the resulting protein is directly involved in cell wall assembly and is essential for colonization of the vagina (De Bernardis *et al.*, 1998; Fonzi, 1999; Mühlischlegel & Fonzi, 1997). Immunoblot analysis identified the presence of an additional GPI protein (Hwp1), which was missed by our MS analysis due to the lack of suitable tryptic peptides (i.e. not heavily glycosylated and with a mass that does not exceed the detection limit of the mass spectrometer). Both

Als3 and Hwp1 have been described as hypha-specific adhesion proteins (Argimon *et al.*, 2007; Hoyer *et al.*, 1998; Staab *et al.*, 1999). However, as our cultures did only contain yeast and pseudohyphal cells, our results show that both proteins can also be expressed in non-hyphal cells, depending on the specific growth conditions. This is consistent with the observation that *HWPI* is moderately expressed in pseudohyphal cells (Snide & Sundstrom, 2006). Interestingly, *ALS3* transcripts have been frequently detected in clinical vaginal fluid specimens, suggesting that the adhesion protein Als3 is important for colonization of this environment (Cheng *et al.*, 2005).

O₂ limitation affects multiple processes in eukaryotic cells, such as iron uptake, mitochondrial respiration, sterol synthesis and the synthesis of unsaturated fatty acids, all of which at some stage require molecular O₂ (Berg *et al.*, 2007; Kosman, 2003; Schweizer, 2004). There are several indications that the hypoxic conditions used in our study result in a molecular response that resembles the response observed when cells grow under iron-restricted conditions. First, when the O₂ concentrations were lowered, the levels of the iron-acquisition proteins Pga10 and Rbt5 in the cell wall increased. Second, intact cells became more resistant to the endo- β -1,3-glucanase Quantazyme (Fig. 2) and to the cell-wall-degrading enzyme preparation Zymolyase (data not shown). This is in agreement with the observation that iron limitation in *C. albicans* results in increased resistance of intact cells to Zymolyase (Sweet & Douglas, 1991). Third, genomic transcript analyses have revealed that iron-acquisition and iron-uptake genes, including *RBT5*, are not only upregulated in cells grown at low-iron conditions (Lan *et al.*, 2004; Weissman & Kornitzer, 2004), but also under hypoxic conditions (Setiadi *et al.*, 2006). Finally, addition of the iron-chelating compound ferrozine to the medium, at saturating O₂ levels, resulted in an increased level of Rbt5 in the wall (Fig. 5). This is in agreement with the observation that the antifungal agent ciclopirox, which is believed to possess iron-chelating properties as well, strongly increases the transcript level of *RBT5* (Lee *et al.*, 2005; Sigle *et al.*, 2005). Interestingly, the presence of ferrozine in the medium also caused an increase in the cell wall of the adhesion protein

Fig. 5. Western blot analysis of CWPs from cells grown at 7% O₂/6% CO₂ (control), 0.02% O₂/6% CO₂ (low-O₂ conditions) or 7% O₂/6% CO₂ in the presence of 1 mM ferrozine (low-iron conditions). Covalently linked CWPs were released from cell walls with β -1,6-glucanase (Pga10, Rbt5 and Hwp1) or by mild alkali extraction (Pir1) as described in Methods.

Hwp1, suggesting an additional control mechanism for *HWP1* expression. We propose that hypoxic conditions result in reduced iron uptake and competition for iron by iron-containing enzymes and that this might lead in various ways to increased expression of specific CWP-encoding genes. Another explanation for the response to hypoxic conditions may reside in the fact that numerous O₂-dependent reactions in the cell are carried out by iron-containing enzymes [e.g. haems in respiration and Fe-dependent sterol synthesis (Kaplan *et al.*, 2006)]. Maintaining a sufficiently high rate of O₂-dependent enzymic reactions could therefore be achieved by an increased synthesis of iron-containing enzymes. To be able to do so, an increase in iron-scavenging proteins like *PGA10* and *RBT5* would indeed be expected. Our results do not exclude that processes such as sterol synthesis and the synthesis of unsaturated fatty acids, which are affected by the low O₂ concentrations used in our study, might also cause major changes in (plasma) membrane properties indirectly affecting the cell wall as well. Indeed, several CWPs (Ecm33, Phr2, Pga10, Pir1 and Rbt5; see Table 2) are induced when the cells are treated with azoles, which are known to affect ergosterol synthesis. We conclude that the cell-wall proteome of *C. albicans* sensitively reflects the environmental conditions and helps the cell to adjust to stress conditions encountered during the infection process. Our results also show that the composition of the cell-wall proteome is tightly controlled, and that multiple signalling pathways are involved in the regulation of its composition.

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