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Genotypic, proteomic, and phenotypic approaches to decipher the response to caspofungin and calcineurin inhibitors in clinical isolates of echinocandin-resistant *Candida glabrata*

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Background: Echinocandin resistance represents a great concern, as these drugs are recommended as first-line therapy for invasive candidiasis. Echinocandin resistance is conferred by mutations in *FKS* genes. Nevertheless, pathways are crucial for enabling tolerance, evolution, and maintenance of resistance. Therefore, understanding the biological processes and proteins involved in the response to caspofungin may provide clues indicating new therapeutic targets.

Objectives: We determined the resistance mechanism and assessed the proteome response to caspofungin exposure. We then evaluated the phenotypic impact of calcineurin inhibition by FK506 and cephalosporine A (CsA) on caspofungin-resistant *Candida glabrata* isolates.

Methods: Twenty-five genes associated with caspofungin resistance were analysed by NGS, followed by studies of the quantitative proteomic response to caspofungin exposure. Then, susceptibility testing of caspofungin in presence of FK506 and CsA was performed. The effects of calcineurin inhibitor/caspofungin combinations on heat stress (40°C), oxidative stress (0.2 and 0.4 mM menadione) and on biofilm formation (polyurethane catheter) were analysed. Finally, a *Galleria mellonella* model using blastospores (1×10^9 cfu/mL) was developed to evaluate the impact of the combinations on larval survival.

Results: F659-del was found in the *FKS2* gene of resistant strains. Proteomics data showed some up-regulated proteins are involved in cell-wall biosynthesis, response to stress and pathogenesis, some of them being members of calmodulin–calcineurin pathway. Therefore, the impact of calmodulin inhibition was explored. Calmodulin inhibition restored caspofungin susceptibility, decreased capacity to respond to stress conditions, and reduced biofilm formation and *in vivo* pathogenicity.

Conclusions: Our findings confirm that calmodulin-calcineurin-Crz1 could provide a relevant target in life-threatening invasive candidiasis.

Introduction

The echinocandins are recommended as first-line therapy for invasive candidiasis because of their low toxicity and high efficacy, especially against azole-resistant *Candida* isolates.[1,2] Echinocandin resistance in *Candida* spp is associated with treatment failures, and is conferred by mutations in 'hot spot' regions of the target *FKS* genes that lead to amino acid substitutions in the 1,3-β-D-glucan synthase enzyme.[3,4] In *Candida albicans*, substitutions in Ser641 and Ser645 are the most frequent *FKS* mutations and cause the

most pronounced resistance phenotypes.[5] In *Candida glabrata,* the principal reported substitutions were Ser629 in Fks1, and Ser663 and Phe659 in Fks2,[3,6,7] while natural polymorphisms have been described in *Candida parapsilosis* and *Candida guilliermondii FKS1*.[8] The ultimate consequence of these mutations is a significant decrease in the echinocandin affinity for the enzyme target and high MIC values. It is important to highlight that in some resistant isolates, no *FKS* mutations were identified and isolates with the same *FKS* mutations exhibit different resistance profiles indicating that other resistance mechanisms and putative target genes may be implicated.[9]

In addition to these described resistance mechanisms, there is also a new hypothesis in which regulators of cellular stress responses could be crucial for enabling the evolution and maintenance of drug resistance.[4,10,11] Indeed, some cellular stress responses are governed by signalling pathways, the most-studied pathways being cAMP, calmodulin-calcineurin (CaM/CaL), TOR (target of rapamycin), and mitogen-activated protein kinase (MAPK).[11–14] The CaM/CaL pathway, formed by a complex of the proteins Cnb1, Cna1, Hsp90 and the transcription factor Crz1 in yeast, is involved in calcium homeostasis, cell-wall biosynthesis, protein trafficking, adaptation to environmental changes and even more importantly, in the response to antifungal drug pressure.[11,15,16] Crz1 is found downstream in the CaM/CaL pathway and is one of the main antifungal targets since Crz1 is not present in human cells. Once the transcription factor is activated by dephosphorylation, mediated by the CaM/CaL-Hsp90 complex, it moves to the cell nucleus. Crz1 contains a C2H2 zinc finger motif that binds to a specific calcineurin-dependent response element (CDRE) in the gene promoters; in *C. glabrata* Crz1 initiates activation of ∼87 genes, among which is the *FKS2* gene, which is involved in resistance to caspofungin.[9,15]

The spread of antifungal resistance and the limited number of available antifungal drugs amplifies the need to identify new fungal targets for development of novel therapeutic alternatives. Calcineurin inhibitors such as tacrolimus (FK506) and cyclosporine A (CsA), which bind to the immunophilins FKBP12 and cyclophilin A, respectively, are well recognized as immunosuppressive drugs with potential antifungal properties.[17,18]

In attempts to identify protein targets, proteomic approaches should be employed. This approach has been previously applied in *C. albicans* to study many aspects, including adaptive responses to osmotic stress, macrophage interaction, and antifungal exposure. These studies evidenced that pathways such as the MAPK signalling pathway played a significant role in several biological responses.[19–24]

To our knowledge, few proteomic studies have been done in *C. glabrata*. Among them, studies explored the implication of hyperadhesive proteins in host–pathogen interaction and biofilm formation, mechanisms of drug resistance (mainly in biofilms) and response to antifungal drugs such as clotrimazole and 5-flucytosine.[25–28] However, similar approaches to study the antifungal response to caspofungin have not been previously described.

Based on this, the first objective of the present study was to identify resistance mechanisms in echinocandin-resistant *C. glabrata* clinical isolates. We then investigated the proteomic response to caspofungin exposure and determined the impact of calcineurin inhibition on susceptibility, stress tolerance, biofilm formation, and assessed pathogenicity in the *Galleria mellonella* model.

Materials and methods

Microorganisms

One susceptible and two caspofungin-resistant *C. glabrata* isolates were studied. The first one, *C. glabrata* PUJ/HUSI 0916 was recovered from a blood culture of a haematopoietic stem cell transplant recipient admitted to the Hospital Universitario San Ignacio Bogota, Colombia. The caspofungin-resistant isolates CAGL1875 and CAGL1256 were obtained from blood and urine cultures of hospitalized patients in the ICU of the Centre Hospitalier Universitaire de Nantes, France and identified by ITS sequencing in a previous study by our research team.[11,29] Isolates were categorized as susceptible or resistant to caspofungin according to the interpretative breakpoints of CLSI M60 (MIC >0.5 mg/L indicates a resistant strain). In addition, the reference *C. glabrata* ATCC 2001, *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were used in specific experiments, as described in the results.

Sequencing and identification of molecular resistance mechanism

Twenty-five genes associated with antifungal drug resistance (Table 1) of *C. glabrata* isolates were sequenced using an Illumina paired-end sequencing platform with a read length of 300 bp and an average read depth coverage of 300×. The obtained raw read sequences were cleaned using *fastp*[30] and assembled with SPAdes v3.12.0.[31] Gene prediction was conducted with Prodigal V2.6.3[32] and the coding sequences obtained were annotated using *blastn*,[33] against the genome of *C. glabrata* ATCC 2001 reference strain. For each of these genes, multiple sequence alignment (nucleotide and amino acid) and mutation identification was performed using T-Coffee[34] and JalView[32] for visualization.

Computational transmembrane region predictions were carried out using RaptorX,[35,36] Sable,[37–40] TMHMM v.2.0,[41] TOPCONS,[42] TMpred,[43] CCTOP,[44,45] HMMTOP[46] and Phobius.[47] InterProScan5[48] was used for primary protein structure analysis.

Proteomic analysis

CAGL1875 (one of the two resistant isolates, both have the same mutation) was resuspended into both fresh yeast extract peptone dextrose (YPD) broth (10 g/L yeast extract, 20 g/L bacto peptone, 20 g/L dextrose) and YPD plus caspofungin at 5 mg/L (OD_{600nm} 0.3) and incubated at 30°C for 7 h under shaking until achieving a density of 10^7cells/mL (OD 0.8–1.0). Fifty mL of cell culture was collected and processed for sample preparation; four biological replicates were performed under each condition. Cell viability was assessed through propidium iodide staining. Cell extracts were obtained by suspending cells in lysis buffer and disrupting them by centrifugation with glass beads (0.4–0.6 mm diameter) in a Fast-Prep system (Bio101, Savant) applying five 20 s rounds at 5.5 speed with intermediate ice cooling. Cell extracts were separated from glass beads by centrifugation and the supernatant was collected and cleared by centrifugation.[49] Protein concentration was measured by Bradford protein assay. Digestion and desalting of peptides were carried out in gel with trypsin, according to Sechi and Chait.[50]

The desalted protein digest was analysed by RP-LC-ESI-MS/MS in an EASYnLC1000 System coupled to the Q-Exactive-HF mass spectrometer through the Nano-Easy spray source (Thermo Scientific, Canada). Peptide identification was carried out using the Mascotv.2 search engine through the Protein Discoverer Software. Database search was performed against SwissProt and Mascot scores were adjusted using a percolator algorithm. Acceptance criteria for protein identification were a false discovery rate (FDR) <1% and at least one peptide identified with high confidence (CI > 95%). To determine the abundances of the identified peptides and proteins, a label-free processing workflow was initiated. As an estimation of the relative protein abundances the normalized spectral abundance factor (NSAF) was used, and the average of the normalized values was

Table 1. Evaluation of genes associated with caspofungin resistance

Gene symbol	Systematic name	Mutations found	Mutation in resistant isolates (1875–1256)	Resistance-associated mutation (+/−)
CEK1	CAGL0K04169g	–	–	–
CDC55	CAGL0L06182g	–	–	–
CDC6	CAGL0K00605g	R117K, V163A, K268R, R80K	–	–
DOT6	CAGL0I05060g	P104S	–	–
FKS1	CAGL0G01034g	G14S	–	–
FKS2	CAGL0K04037g	F659-Del, T926P	F659-Del	+
FKS3	CAGL0M13827g	A42V, T1676S	–	–
MKT1	CAGL0J05566g	N512K, A643T	–	–
MOH1	CAGL0F04631g	S15N	–	–
MPH1	CAGL0F04895g	–	–	–
MRPL11	CAGL0J09724g	–	–	–
MSH2	CAGL0I07733g	–	–	–
PDR1	CAGL0A00451g	V91I, L98S, D243N	–	–
PHO4	CAGL0D05170g	S327N	–	–
SNQ2	CAGL0I04862g	P1104H	–	–
SUI2	CAGL0B03795g	–	–	–
TCB1	CAGL0J08591g	Q437E, K585R, N622K	–	–
TCB3	CAGL0L11440g	–	–	–
TOD6	CAGL0A04257g	P64S, D81N, N85D	–	–
TPK2	CAGL0M08404g	T132A, T158A	–	–
CRZ1[a]	CAGL0M06831g	–	–	–
SLT2[a]	CAGL0J00539g	–	–	–
SRP1[a]	CAGL0J11440g	–	–	–
DBP5[a]	CAGL0L110021g	–	–	–
SWI1[a]	CAGL0C01683g	–	–	–

[a]Genes encoding proteins related to the caspofungin exposure response found in this study.

calculated.[51] The recalibration of the masses was performed through a rapid search in Sequest HT against the database. Subsequently, alignment of the retention times between the different samples analysed for the quantification of the precursor ions was performed, taking into account unique peptides and razor peptides (i.e. peptides that can be assigned to more than one protein). Finally, the results were normalized to the total amount of the peptides, equalling the total abundance among the different samples. After the analyses were finalized, a final report presented the list of peptide groups and proteins with scaled abundances and selected ratios. The Proteome Discoverer application includes a feature for assessing the significance of differential expression by providing *P* values for those ratios (*P* value <0.05). The mass spectrometry proteomic data have been deposited to the ProteomeXchange Consortium via the PRIDE,[52,53] with dataset identifier PXD021578.

Gene ontology (GO) FungiDB (http://fungidb.org) was used to search for enriched GO terms in the input list of the identified *C. glabrata* gene products compared with the genes from the *C. glabrata* CBS138 genome. Terms with a *P* value <0.04 from a calculated and curated evidence list were included.

Antifungal susceptibility testing

Antifungal susceptibility testing was carried out using broth microdilution method (BMD), following the CLSI M27-A3 guidelines with slight modifications for the combination of caspofungin with the calcineurin inhibitors.[54] Briefly, isolates were subcultured on yeast YPD and grown for 24 h at 35°C. The yeast suspensions were prepared in Roswell Park Memorial Institute medium (RPMI) 1640 (Sigma–Aldrich) to a final concentration of 10^3 cells/mL. Yeast inoculum (100 μL) was added to a 96-well plate containing serial two-fold dilutions of caspofungin with or without inhibitors FK506 or CsA (15 mg/L, which was the concentration selected after screening that did not generate significant growth changes and did not show toxicity). MICs were visualized, and densitometry (530 nm, microplate reader, Thermo Scientific) was used to determine the lowest concentration of drug that caused a significant decrease (MIC/2 or ≥50%) compared with that of the drug-free growth control after 48 h of incubation. Quality control was ensured by testing the CLSI-recommended strains.[55]

Stress-related phenotypic assays

For heat-shock stress, droplet tests were performed by spotting serial dilutions of *C. glabrata* cells (10^6 to 10^3 cells/mL) onto YPD agar plates with FK506 or CsA (15 mg/L), caspofungin (1 mg/L) or both compounds. The plates were incubated at 37°C or 40°C for 24 h. For oxidative stress, YPD plates were prepared as previously, except that the medium was supplemented with the naphthoquinone menadione (0.2 and 0.4 mM). The plates were incubated at 37°C for 24 h.[11,24]

Biofilm formation

The *C. glabrata* isolates were grown on Sabouraud dextrose agar (SDA) and incubated at 30°C for 24 h. Two hundred μL of *Candida* cell suspensions (10^6 cells/mL) in RPMI-1640 with MOPS were dispensed in 96-well microdilution wells with or without GDHK-1325 250 mm Gam-polyurethane catheter pieces (Hechingen, Germany) and allowed to adhere for 24 h at 37°C. The non-adherent cells were removed by washing with 300 μL PBS.

Caspofungin was added at 1 mg/L with or without 15 mg/L of the calcineurin inhibitors for 24 h incubation at 37°C for the biofilm adhesion phase. Then wells or catheter pieces were washed twice with PBS and finally 100 μL of RPMI-1640 plus 10 μL of 700 μM resazurin (Sigma–Aldrich) was added to each well and incubated at 37°C for 4 h. The biofilm was quantified indirectly by measuring the fluorescent water-soluble resorufin product that results when resazurin is reduced by reactions associated with respiration. Fluorescence was then measured at 560 nm with emission at 590 nm. The results were expressed in arbitrary fluorescence units (AU).[11,56]

Invertebrate Galleria mellonella model

Killing assays were performed in *G. mellonella* as described by Fallon *et al.*[57] Briefly, final (sixth) instar larvae weighing approximately 300 mg were used. Suspensions of individual *Candida* isolates that had been grown on SDA for 48 h at 35°C were harvested by gently scraping colony surfaces with sterile plastic loops, washed twice in sterile phosphate-buffered saline (PBS), counted in haemocytometers and adjusted to 1×10^9 cfu/mL. Larvae received a 10 μL inoculum and 10 μL of caspofungin (1 μg/larva), FK506 and CsA (15 mg/L), or their combination by injection into the last left and right proleg using a 0.5 mL BD syringe. After inoculation, larvae were placed in Petri dishes and incubated in darkness at 37°C. To compare mortality, three biological replicates were performed with 10 larvae for each isolate evaluated. A group of 10 larvae was used for each of the controls: absolute (uncleaned, uninoculated), disinfection (cleaned with ethanol 70%), and inoculation (received 10 μL sterile PBS). The larvae were monitored for 10 days, and survival outcome was determined; larvae were considered dead when no response was observed following touch stimulation.

Statistical analysis

All experiments were performed on three independent biological replicates; survival curves were constructed using the method of Kaplan and Meier, then the curves were compared using the Log-Rank (Mantel-Cox) test. Statistical models were constructed and analysed using PRISM software version 7.

Data availability

All experimental data are provided in the manuscript and in supplementary files, or are available via ProteomeXchange with identifier PXD021578 (10.6019/PXD021578) and in the NCBI BioProject database with the accession number PRJNA692260.

Results

Next-generation sequencing (NGS) revealed FKS2 nucleotide deletion

To identify the caspofungin resistance mechanisms of *C. glabrata* isolates, 25 genes associated with antifungal drug resistance were screened for mutations. Although some mutations were identified in our resistant isolates (CAGL1256 and CAGL1875), only the *FKS2* gene exhibited a 3 nucleotide deletion (1974-CTT-1976), which has been previously reported to be associated with echinocandin-resistant phenotypes. All other observed mutations were found in both resistant and susceptible isolates (Table 1). The 3 nucleotide deletion detected (Figure 1a), which preserves the same open reading frame, explained the single amino acid deletion at Fks2 (F659-del; Figure 1b) observed in the two resistant isolates. This amino acid deletion, which confers resistance to the echinocandins, resides within the Fks2 hot spot 1 (Figure 1c).

Proteomic analysis of resistant C. glabrata treated with caspofungin revealed an increase of proteins related to stress adaptation and cell-wall organization

The CAGL1875 isolate was cultured at a concentration of caspofungin indicating resistance, then a label-free quantification proteomic method was performed to determine the number of proteins and level expression in response to caspofungin. A total of 1796 proteins were identified (Table S1, available as Supplementary data at *JAC* Online). While 1509 of them were encoded by uncharacterized ORFs, 287 were encoded by well-characterized genes. Among these proteins, 16 were identified as less abundant (i.e. downregulated) and 5 proteins were identified as more abundant (i.e. upregulated) after caspofungin exposure (>1.5-fold change and P value < 0.05). Using GO enrichment tools, several GO terms were found to be enriched from these 21 proteins. In the molecular functions, 14 terms were enriched. Among them, MAP-kinase activity, drug binding, calcium-serine/threonine, ATPase activity, and ATP-binding were over-represented. Regarding biological process categories, 18 terms were enriched, including signal transduction, pathogenesis, response to stress/drug and wall-biogenesis were over-represented (Figure 2). Following caspofungin exposure, the downregulated proteins were mainly of enzymatic groups: CAGL0I03300g (homologue to *Candida albicans* Bud16, here named as Ca.Bud16), CAGL0K07744g (Ca.Ysa1), CAGL0K05813g (Ca.Ttr1), CAGL0J06952g (Ca.Idi1), CAGL0H09218g (Cg.Sdt1). The protein with the most negative differential ratio was CAGL0M08514g (Cg.Pir5), a protein associated with β-1,3-glucan strengthening. The proteins that were more abundant after caspofungin exposure were involved in DNA binding, i.e. CAGL0M06831g (Cg.Crz1) CAGL0J11440g (Ca.Srp1), CAGL0L10021g (Ca.Dbp5) and CAGL0C01683g (Ca.Isw1). Of interest are the proteins involved in antifungal responses, CAGL0M06831g (Cg.Crz1) (CaM/CaL-pathway), CAGL0J00539g (Cg.Slt2) (PKC-pathway) and CAGL0J11440g (Ca.Srp1). The corresponding protein abundance is presented in Figure 3, and description of these proteins is provided in Table S2. An additional analysis was carried out with proteins identified as more abundant if the change in abundance ratio caspofungin: control was >1 after caspofungin exposure (Figures S1 and S2, and Table S3).

Considering the important role of the CaM/CaL in antifungal response, and the significant change of Crz1 expression (to date there are no Crz1 inhibitors) after caspofungin exposure, we focused this study on targeting upstream CaM/CaL proteins using commercial inhibitors. Additionally, the genes corresponding to the five most-abundant proteins following caspofungin exposure were evaluated for mutations, however, none was observed (Table 1).

Calcineurin inhibition restored susceptibility of caspofungin-resistant C. glabrata

Pharmacological inhibition of calcineurin by FK506 and CsA did not show any statistically significant decrease of susceptible (PUJ/HUSI0916 and ATCC 2001) or resistant (CAGL1256 and CAGL1875) *C. glabrata* growth. Otherwise, in the presence of caspofungin, the inhibitors allowed susceptibility restoration in resistant clinical isolates, with a significant reduction in MIC values from >16 mg/L to 0.25 and 0.5 mg/L, respectively (Figure 4).

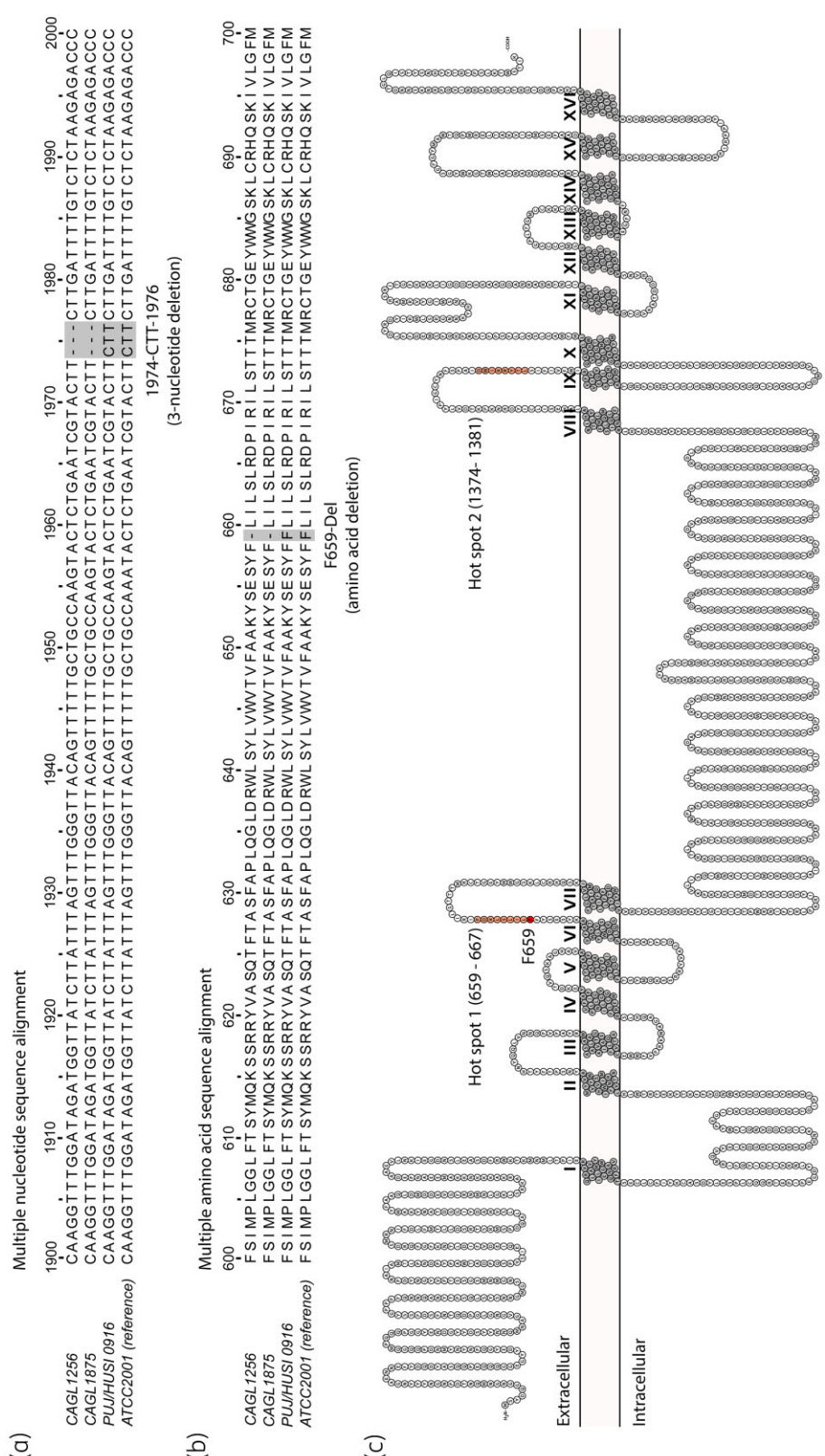

Figure 1. Multiple alignments of Fks2 (β-1,3-glucan synthase catalytic subunit 2) nucleotide and amino acid sequences from *C. glabrata* resistant and susceptible strains. Multiple nucleotide (a) and amino acid sequence alignments (b) that show a 3 nucleotide deletion (1974-CTT-1976) and a single amino acid deletion (F659-Del), respectively, occurring only in resistant strains of *C. glabrata*. (c) A consensus *C. glabrata* Fks2 membrane protein structure and topology model predicted by seven different membrane protein secondary structure prediction servers and visualized with Protter.[88] Hot spots 1 and 2 are showed in red, as well the position F659 at which the single amino acid deletion associated with resistance to echinocandins occurs. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

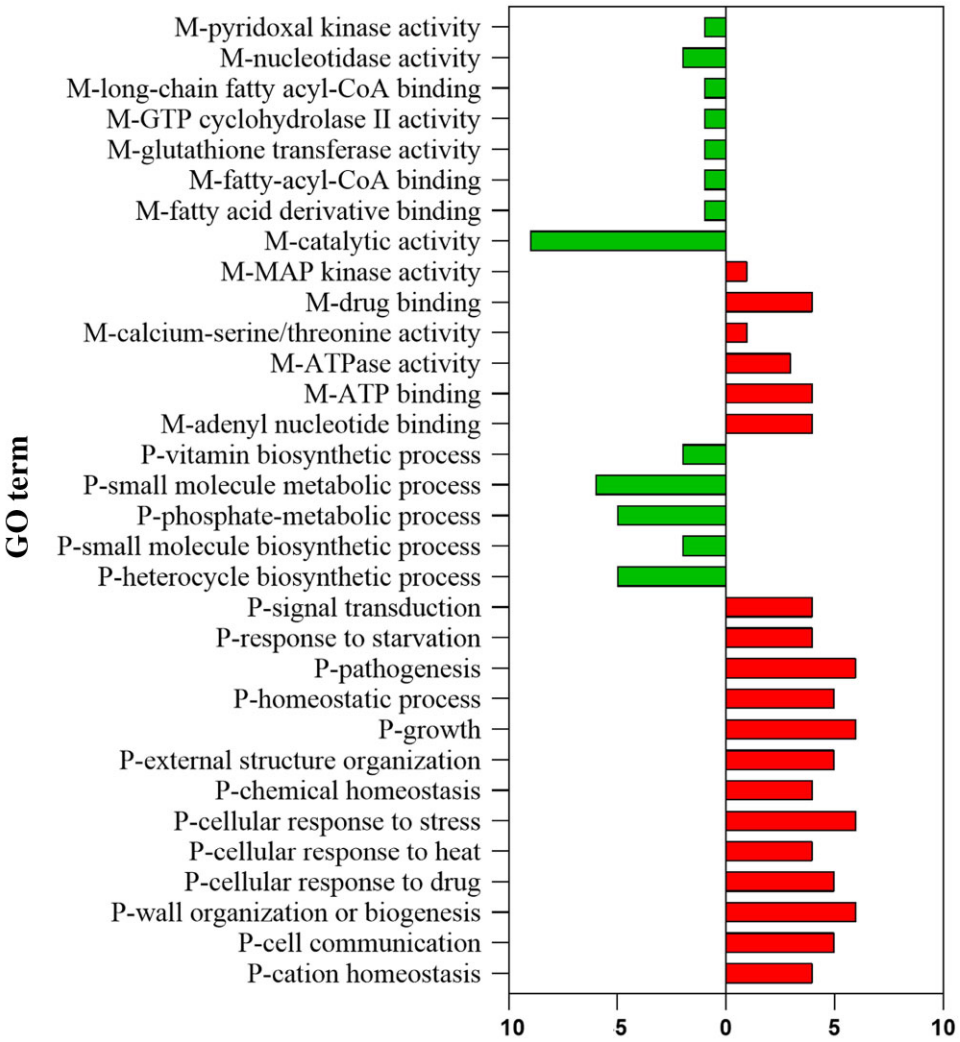

Figure 2. Gene Ontology (GO) analysis of the proteins considered differentially abundant after caspofungin treatment (>1.5-fold change and p-value <0.05). M indicates a molecular function; P indicates a biological process; the *x*-axis indicates the number of associated proteins. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Response to oxidative stress is independent of caspofungin resistance phenotype and not correlated with calcineurin signalling

To understand the oxidative stress response in resistant *C. glabrata*, growth was assessed in the presence of menadione, a cytotoxic quinone that generates superoxide. The four isolates grew in up to 0.2 mM menadione and addition of FK506 and CsA did not show any significant modification. The significant growth reduction of the resistant isolate CAGL1875 suggested higher susceptibility to caspofungin in presence of 0.2 mM menadione. Since combinations of caspofungin and calcineurin inhibitors lead to complete growth inhibition without any additional stress, the impact of 0.2 mM menadione addition could not be interpreted. In the presence of 0.4 mM menadione, PUJ/HUSI0916 and CAGL1256 strains maintained a similar growth rate, independent of the caspofungin resistance phenotype (Figure 5a).

Calcineurin inhibitors compromised growth of caspofungin-resistant C. glabrata in heat-shock conditions

The spot test at 37°C confirmed previous BMD CLSI results concerning the effect of calcineurin inhibitors on caspofungin-resistant isolates (Figure 4). Heat shock at 40°C did not have an impact on isolate growth for all but ATCC 2001. Interestingly, at this temperature, the growth of caspofungin-resistant isolates was noticeably compromised by calcineurin inhibitors. At both 37°C and 40°C the inhibitor/caspofungin combination strongly affected growth (Figure 5b).

Calcineurin inhibitors significantly reduced biofilm-forming capacity

The four isolates had the capacity to form biofilm, but biofilm formation was lower in the catheter model. Caspofungin treatment

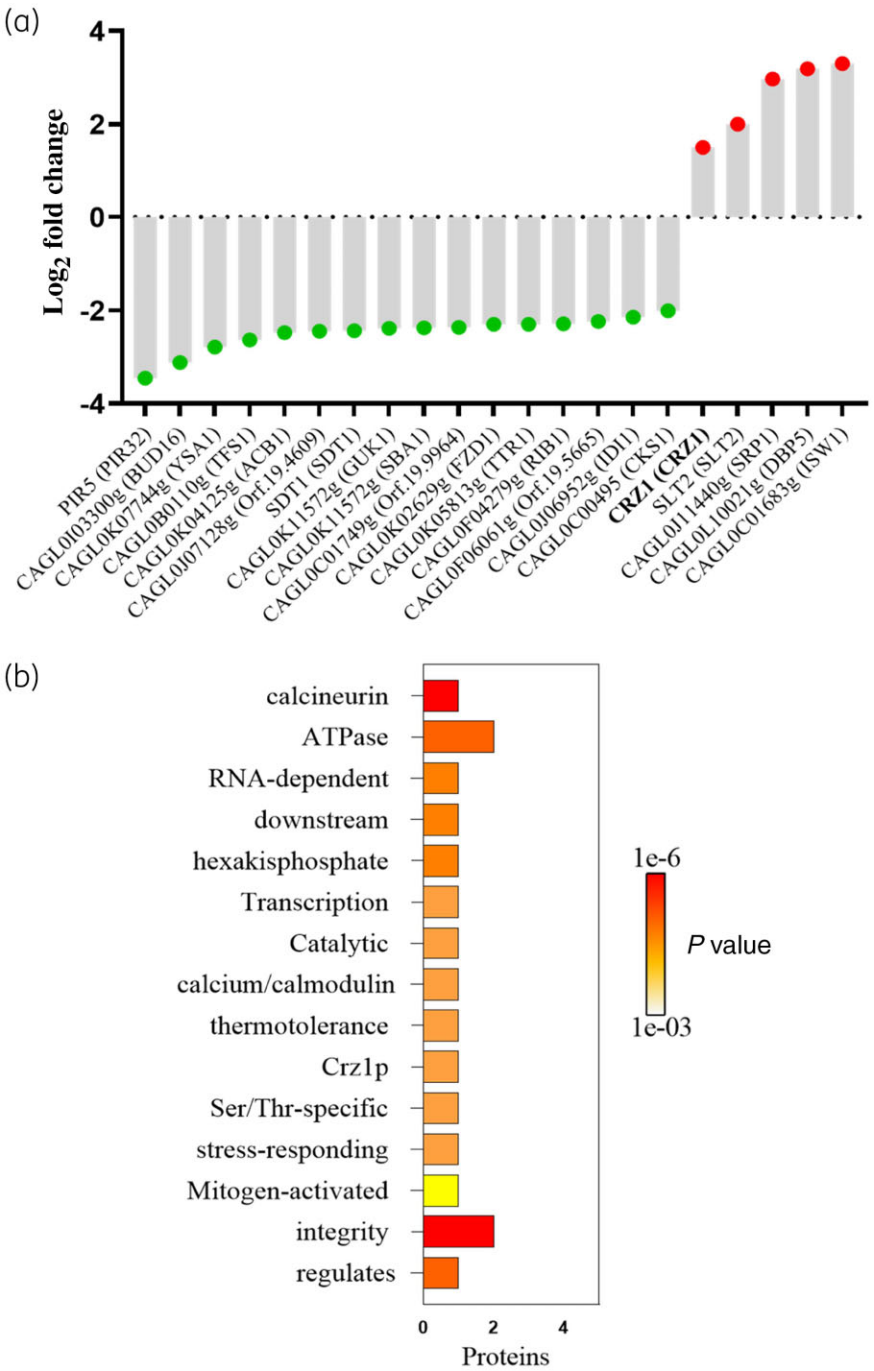

Figure 3. Label-free quantitative proteomics results. (a) Protein abundance profile. Down-regulated proteins are marked with a green spot and up-regulated are marked with a red spot (*Candida albicans* orthologue names are given inside the parentheses). (b) Word enrichment that was created using the *P* values (Fisher's exact test) and the full terms from the enrichment analysis via a program called GO summaries available at the FungiDB website (https://fungidb.org/fungidb/app/). This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

reduced biofilm formation in susceptible isolates especially on catheter, whereas no significant activity was detected for resistant isolates (Figure 6a and b). In contrast, addition of calcineurin inhibitors to caspofungin significantly reduced the biofilm-formation capacity of resistant isolates, regardless of the model used ($P < 0.05$).

Calcineurin inhibition reduced C. glabrata pathogenicity in the invertebrate G. mellonella

C. glabrata isolates typically lead to complete mortality of *G. mellonella* by 4–6 days post-infection. Treatment with caspofungin (1 μg/larva) increased the larval survival when infected with susceptible isolates but did not exhibit, as expected, any statistically

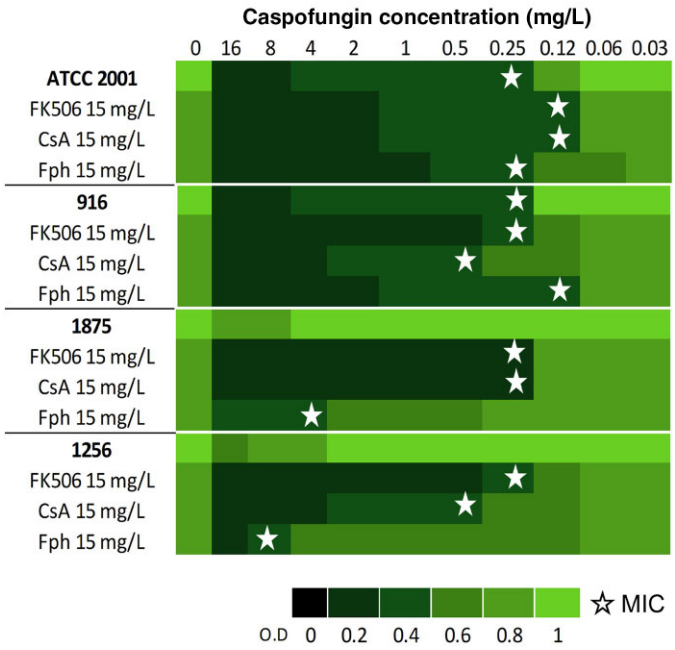

Figure 4. Caspofungin MICs (indicated by stars) under basal and calcineurin inhibition (FK506, CsA, Fph). Resistant strains have MICs >0.5 mg/L (1875 and 1256). The green bars indicate relative fold growth.

significant change for caspofungin-resistant isolates. However, addition of calcineurin inhibitors to caspofungin proved to be effective in prolonging survival ($P < 0.05$). No larval mortality was observed in control larvae injected with an equivalent volume of PBS (Figure 7).

Discussion

C. glabrata is one of the most prominent *Candida* species detected in bloodstream isolates worldwide, typically exhibiting intrinsic resistance to azoles.[58–61] Moreover, echinocandin resistance in *C. glabrata* has increased, causing a serious clinical challenge.[62] Different mechanisms of resistance to echinocandins have been described, mainly associated with *FKS* gene alterations.[6,9] In this work, we employed NGS to provide a view of mutations involved in clinical caspofungin-resistant isolates targeting genes previously associated with echinocandin resistance.[9,63,64] To date, only a single *FKS2* gene deletion associated with caspofungin resistance has been found; however, a larger comprehensive comparative analysis is ongoing.

Herein, we describe the first proteome description of resistant *C. glabrata* after caspofungin exposure. Considering that caspofungin causes osmotic disruption of the fungal cell, enrichment of molecular functions and biological processes, as expected, were associated with antifungal response, cell wall biogenesis,

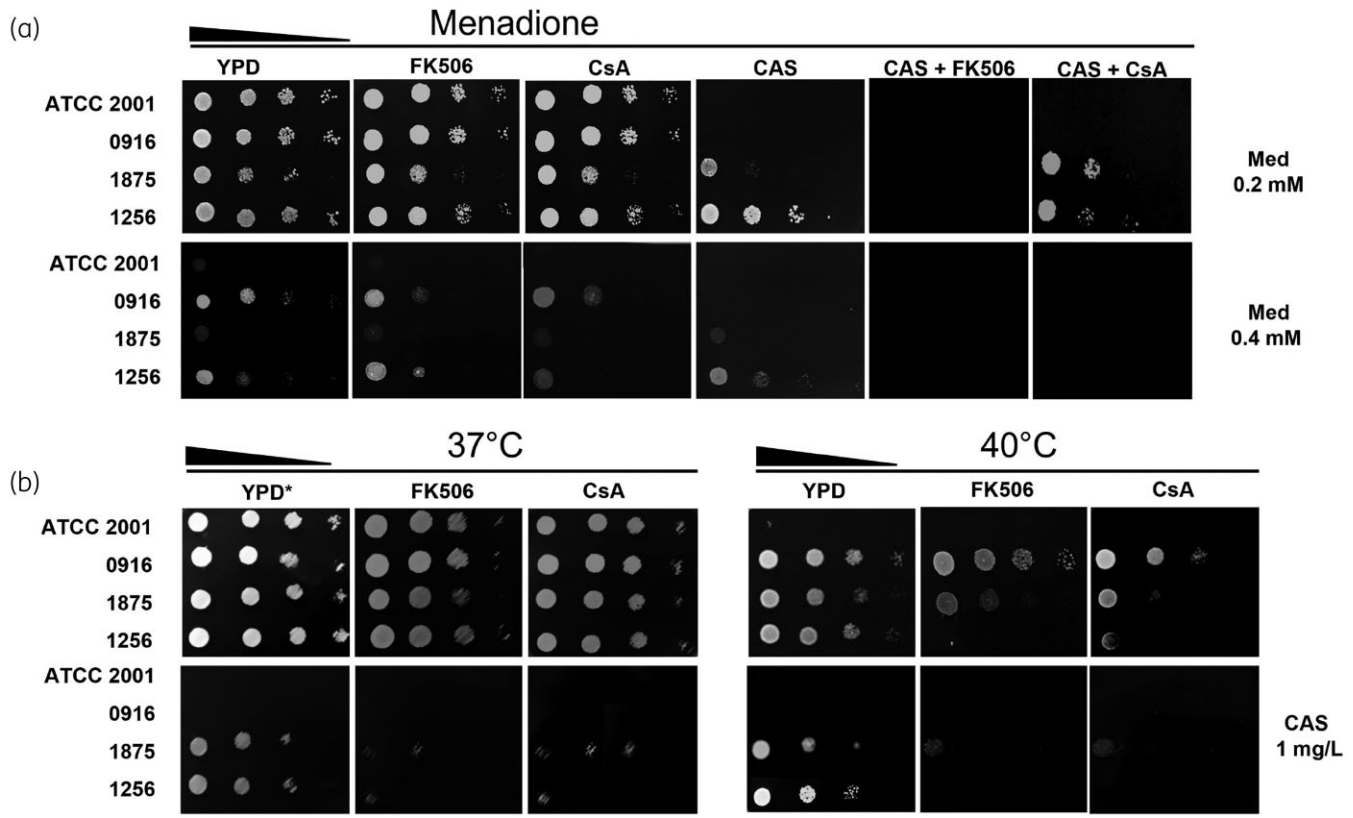

Figure 5. Stress responses under CaM/CaL inhibition. (a) Strains grown in the presence of 0.2 and 0.4 mM menadione (Med). (b) Strains were streaked onto YPD and incubated at 37°C and 40°C, with or without 1 mg/L caspofungin (CAS), with or without 15 mg/L Fk506 and CsA. YPD at 37°C was used as no drug growth control (see the asterisk).

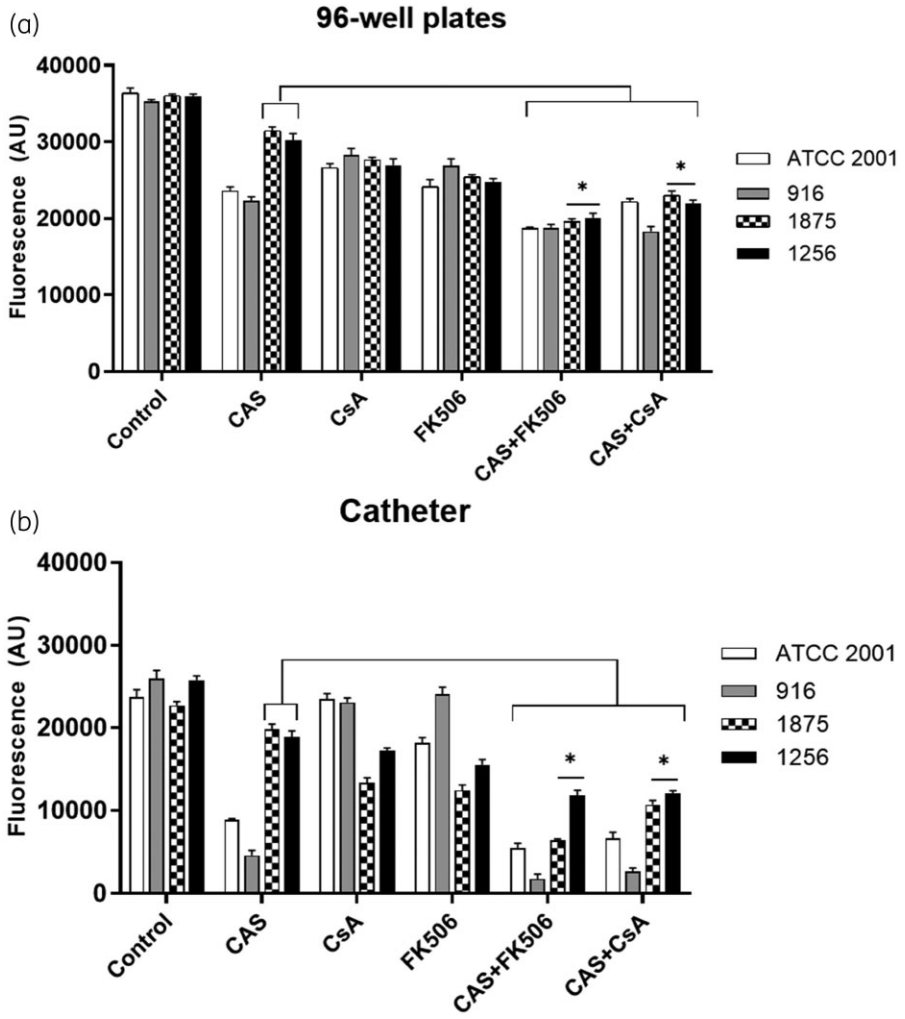

Figure 6. Biofilm formation by *C. glabrata* isolates grown (a) in microplate wells and (b) on catheter pieces, exposed to CsA, FK506, caspofungin and their combinations. Fluorescence was measured at 560 nm with emission at 590 nm. Data are expressed in arbitrary fluorescence unit (AU). An asterisk indicates a *P* value <0.05.

and modulation of PKC and CaM/CaL pathways. These results, similar to those previously reported in *C. albicans*, confirm the association of these processes in the growth of *C. glabrata* with echinocandin exposure.[13,65] Furthermore, we observed that caspofungin exposure resulted in increased GO annotations related to stress adaptation, such as chemical and cation homeostasis, cell wall organization or biogenesis, and response to heat. These features have been described by Hoehamer *et al.*[19] as important changes in the *C. albicans* proteome in response to ketoconazole, amphotericin B, and caspofungin treatments.

C. glabrata exposure to caspofungin resulted in an increased abundance of MAP kinase Slt2 and Crz1 proteins which, being part of PKC and CaM/CaL pathways, respectively, have been implicated in cell-wall biogenesis and integrity. This compensation phenomenon, also observed in *Saccharomyces cerevisiae, C. albicans* (Mkc1), and recently published in *C. glabrata*, constitutes a mechanism of tolerance to caspofungin.[13,14,66–68] Mutants lacking *SLT2/MKC1* and *CRZ1* are both susceptible to echinocandins in *in vitro* assays.[14,69] Nevertheless, Slt2 overexpression leads to hypervirulence.[70] Another three proteins (CAGL0C01683g, CAGL0L10021g, CAGL0J11440g) were found with higher abundance after exposure to caspofungin, however, these proteins have not been characterized in *C. glabrata* to date. The protein CAGL0C01683g (Isw1) homologue in *C. albicans* and *S. cerevisiae* has been described as a chromatin remodelling factor involved in the repression of the initiation of transcription. *ISW1* also works in parallel with the NuA4 and Swr1 complexes in the repression of stress-induced genes.[71] Inhibitors of DNA methyltransferases are attractive compounds for epigenetic drug discovery. Therefore, the Isw1 function and its role in the antifungal drug response need further studies. The protein CAGL0L10021g (C.a, S.c Dpb5) is an ATP-dependent cytoplasmic RNA helicase involved in translation termination along with Sup45p (eRF1); it also has a role in the cellular response to heat stress.[72] Finally, the protein CAGL0J11440g (C.a, Sc Srp1) (importin-α) has nuclear import signal receptor activity and is involved in the degradation of proteins. Loss of Srp1 is lethal, although several temperature-sensitive mutants have been described.[73,74] To date, there are no drugs against these

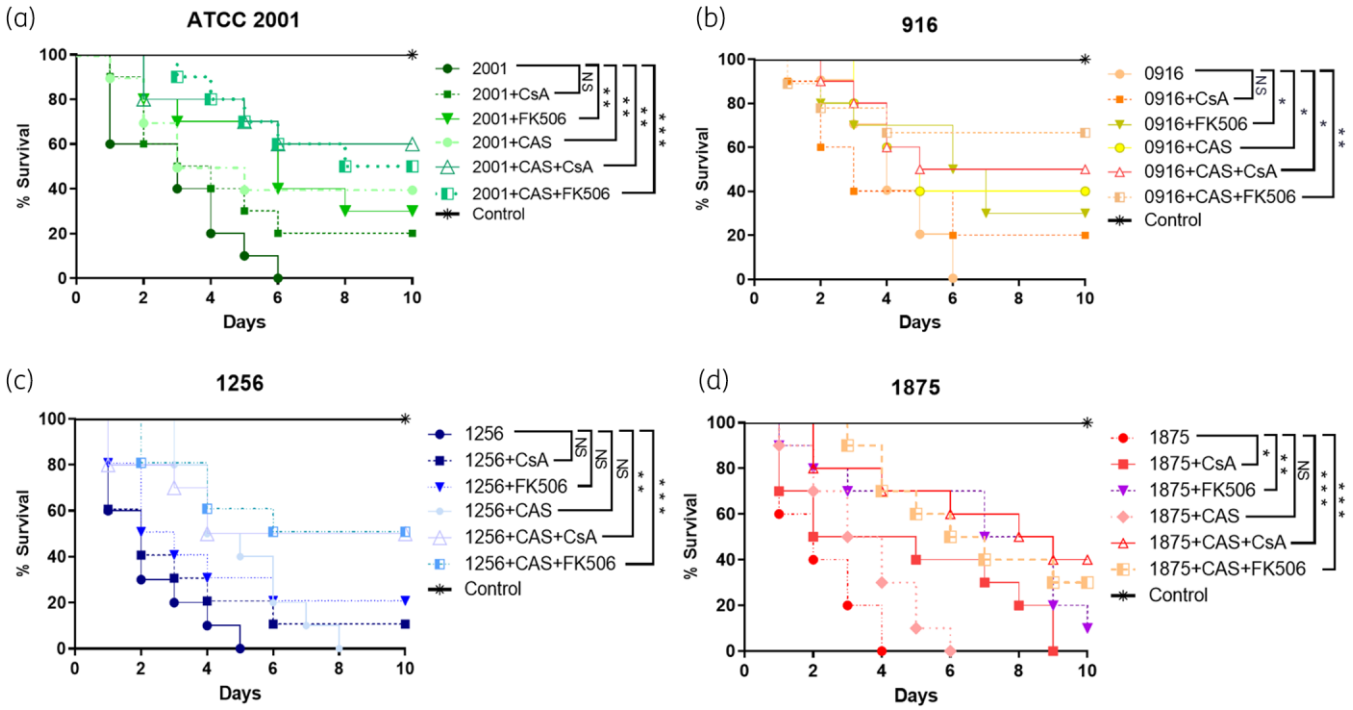

Figure 7. *Galleria* time–kill curves of caspofungin-susceptible (a, b) and resistant (c, d) *C. glabrata* isolates exposed to, caspofungin (1 μg/larva), calcineurin inhibitors (15 mg/L) and their combinations. The data are expressed as the percentages of survival. Log-rank (Mantel–Cox) test with *P* values of <0.05 was used to indicate statistical significance as follows: $^{*}P < 0.05$, $^{**}P < 0.02$ and $^{***}P < 0.001$.

proteins, however, as some host and yeast enzymes are not identical, traditional medicinal chemistry and structure-based drug design can exploit these differences to synthesize drugs with high specificity for the yeast. Conversely, Pir5 protein was decreased in abundance in response to caspofungin exposure. Pir proteins are a structural constituent of the cell wall and are associated with cell-wall organization owing to linkage to multiple β-1,3-glucan chains. The changes in the Pir proteins are a consequence of the activation of the cell-wall integrity pathway.[75–77] This decrease of Pir5 abundance appears to be part of a general compensatory mechanism in response to cell-wall weakening caused by caspofungin; consequently, the cell increases chitin and/or mannan production, a phenomenon reported in *S. cerevisiae* and *Candida* spp.[75,78]

Given the importance of the CaM/CaL-Crz1 pathway in several biological processes, the impact of FK506 and CsA calcineurin inhibitors was studied in temperature and oxidative stress conditions. Similar to previous studies, we confirm that the CaM/CaL pathway is involved in thermotolerance, mainly at higher temperatures.[79] By contrast, according to our results, the inhibition of calcineurin does not appear to affect the growth of *C. glabrata* in oxidative stress. The antioxidant capacity of *C. glabrata,* mainly associated with the catalase Cta1, is higher than that of *S. cerevisiae* and *C. albicans*. Cta1 is controlled by the transcription factors Yap1, Msn2, and Msn4 and modulated by pathways other than CaM/CaL.[80]

Biofilm formation is another important factor in the understanding of cellular disruption. Biofilms are thought to provide ecologic advantages such as protection from the environment, nutrient availability, metabolic cooperation, and acquisition of new traits. In general, *C. glabrata* biofilms possessed a higher density of cells comparatively to *C. tropicalis* and *C. parapsilosis* biofilms. This may be implicated in the typical high degree of resistance of *C. glabrata* biofilms to azole antifungals and amphotericin B.[81] Biofilm eradication as a therapeutic approach is generally effective using echinocandins, as long as the isolate is drug susceptible.[82] In our study, planktonic cells of caspofungin-resistant isolates maintain this characteristic in biofilm community state, even in the presence of high doses of caspofungin. Nevertheless, this situation can be reversed by addition of CaM/CaL inhibitors, as we demonstrated in the clinical-relevant model using polyurethane catheter pieces.

On the other hand, we believe that the effect of FK506 and CsA on heat-shock tolerance or susceptibility restoration to caspofungin could contribute to their *in vivo* activity. Indeed, in *Galleria* the use of CaM/CaL inhibitors reduces the mortality caused by all isolates, as well the addition of inhibitors to caspofungin enhances its efficacy, allowing a significant increase in larval survival. We believe that treatment with the inhibitors plus the immune response of *G. mellonella* (antimicrobial peptides, lytic enzymes, and melanin) enhances the defence of the larvae to the *C. glabrata* infection. Meaning that the antifungal activity of the inhibitors and the immune system work together, resulting in greater larvae survival. This is in concordance with previous data showing the role of CaM/CaL pathway in virulence of fungal species.[79,83]

Despite these promising findings, non-immunosuppressive analogs of both FK506 and CsA with no cross-activity with calcineurin in human cells must be developed.[17,84,85] With regards to the CaM/CaL pathway, the challenge also will lie in focusing on the transcription factor Crz1, as recently explored for *Rhizoctonia solani*.[86] Transcription factors are now attractive as antifungal

drug targets since they are evolutionarily divergent between fungi and humans and therefore can be exploited as selective targets.[87]

In conclusion, our study provides proteomic evidence that proteins of CaM/CaL pathway, such as Crz1, are more abundant after caspofungin exposure. In addition, inhibition of this pathway in the clinical isolates with an *FKS2* gene mutation changed their planktonic and biofilm susceptibility, thermotolerance, and finally pathogenicity. Synthesis of more specific antifungal compounds targeting this stress response pathway could be a successful therapeutic strategy for fighting life-threatening fungal diseases.

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Author contributions

A.C.-G. performed experiments and wrote the main manuscript, A.C.-G., L.M. and C.G. designed proteomic experiments and analysed proteomic data, C.A.-M. analysed clinical implications, N.E.V.-V. conducted bioinformatics analysis of genomic data, and prepared Figure 1. D.M.E. and J.B. sequenced isolates. A.C., P.L.P. and C.M.P.-G. designed the experiments and wrote the final manuscript, and P.L.P., C.M.P.-G. conceived the experiments and managed the resources. All authors have read and agreed to the published version of the manuscript.

Supplementary data

Figures S1 and S2 and Tables S1 to S3 are available as Supplementary data at *JAC* Online.

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