Candida albicans specific genes: distinct roles in host-pathogen interactions

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Abstract

Human fungal pathogens are distributed throughout their kingdom, suggesting that pathogenic potential evolved independently. *Candida albicans* is the most virulent member of the CUG clade of yeasts and a common cause of both superficial and invasive infections. We therefore hypothesised that *C. albicans* possesses distinct pathogenicity mechanisms. *In silico* genome subtraction and comparative transcriptional analysis identified a total of 65 *C. albicans* specific genes (ASGs) expressed during infection. Phenotypic characterisation of six ASG-null mutants demonstrated that these genes are dispensable for *in vitro* growth, but play defined roles in host pathogen interactions. Based on these analyses, we investigated two ASGs in greater detail. An orf19.6688Δ mutant was found to be fully virulent in a mouse model of disseminated candidiasis and to induce higher levels of the proinflammatory cytokine, IL-1β, following incubation with murine macrophages. A pga16Δ mutant, on the other hand exhibited attenuated virulence. Moreover we provide evidence that secondary filamentation events (multiple hyphae emerging from a mother cell and hyphal branching) contribute to pathogenicity: PGA16 deletion did not influence primary hypha formation or extension following contact with epithelial cells; however, multiple hyphae and hyphal branching were strongly reduced. Significantly, these hyphae failed to damage host cells as effectively as the multiple hyphae structures formed by wild type *C. albicans* cells. Together, our data show that species-specific genes of a eukaryotic pathogen can play important roles in pathogenicity.
Introduction

The fungal kingdom is predicted to consist of up to 5.1 million species (1), over 100,000 of which have been confirmed (2). Of these species, few (600) have been reported to infect humans (3) and only a fraction (~10) of those are prevalent aetiological agents (4). One such fungal species, *Candida albicans*, displays a remarkable range of interactions with humans. Primarily, *C. albicans* is a commensal member of the microbial flora of mucosal surfaces; however, it also causes infections at these sites extremely frequently: 75% of women experience at least one episode of vulvovaginal candidiasis, with *C. albicans* being the most dominant species; *C. albicans* is also a leading cause of diaper rash in infants and 90% of untreated HIV positive individuals suffer from oral candidiasis (4, 5). Importantly, *Candida* species are also the third most common cause of nosocomial bloodstream infections and these invasive infections have mortality rates of over 40% (6).

Although a number of *C. albicans* virulence factors, such as the yeast to hypha transition, secretion of extracellular hydrolases and expression of cell surface-associated adhesins have been described (7), their precise roles during the different types and stages of infection remain a hotly debated subject.

Other major fungal pathogens of humans, such as *Cryptococcus neoformans* or *Aspergillus fumigatus*, are distantly related to *C. albicans* and to each other. That is, human pathogens are distributed throughout the fungal kingdom (8). This has lead to the concept that the virulence potential of these species has evolved independently (9). This is supported by the fact that certain virulence-associated factors, such as the capsule of *C. neoformans* and gliotoxin synthesis by *A. fumigatus*, are not shared amongst pathogenic species.

This model of independent virulence evolution led us to explore the possibility that pathogenic fungal species possess unique genes which set them apart from their closer,
less-pathogenic relatives. Using comparative genomic and transcriptomic analyses, we describe 65 genes expressed during infection and unique to *C. albicans*. Further molecular analysis of six of these *C. albicans* specific genes demonstrated that they are indeed involved in host-pathogen interactions, including epithelial and endothelial damage and immune modulation. Surprisingly, all six investigated genes were required for at least one stage of infection.

**Materials and methods**

**Ethics statement**

All animal experiments were in accordance with the German animal protection law and were approved (permit no. 03-007/07) by the responsible Federal State authority (Thüringer Landesamt für Lebensmittelsicherheit und Verbraucherschutz) and ethics committee (beratende Komission nach § 15 Abs. 1 Tierschutzgesetz). The use of human primary cells in this study was conducted in compliance with the principles expressed in the Declaration of Helsinki. All protocols used were approved by the local ethics committee of the University of Jena under the permit no. 2207-01/08. Written informed consent was provided by all study participants.

**Strains and growth conditions**

*C. albicans* strains used in this study are listed in Table S2. The triple-auxotrophic strain BWP17 complemented with plasmid CIP30 was used as wild type control in all experiments. Strains were grown on YPD agar [1% yeast extract, 2% bacto-peptone, 2% D-glucose, 2% agar] or SD minimal medium agar [2% dextrose, 0.17% yeast nitrogen base, 0.5% ammonium
sulfate, 2% agar]. Liquid overnight cultures were grown in YPD or SD medium in a shaking incubator at 30°C and 180 rpm. Transformants were selected on SD agar supplemented with arginine, histidine and/or uridine (each 20 µg ml\(^{-1}\)) as required. \textit{E. coli} was grown on LB agar [1% bacto-tryptone, 0.5% yeast extract, 1% NaCl, 2% agar] and overnight \textit{E. coli} cultures were cultivated in a shaking incubator at 37°C and 210 rpm. For selection purposes 50 µg/ml ampicillin were added to solid or liquid LB medium.

**Strain construction**

For homozygous mutant construction, the BWP17 genetic background was used and alleles were sequentially deleted with PCR products based on pFA-HIS1 and pFA-ARG4 plasmids (10). Forward primers were designed to have 104 bp homology to the immediate upstream region of the gene of interest, followed by a 22 bp sequence, which has homology to the pFA plasmids, immediately upstream of the respective selective marker. Similarly, reverse primers were designed to have 104 bp homology to the immediate downstream region of the gene of interest (reverse complemented) followed by 24 bp sequence with homology to the pFA plasmids, immediately downstream of the respective selective marker (Table S3). Using these long primers, and either pFA-HIS1 or pFA-ARG4 as template, HIS1 and ARG4 deletion cassettes were generated for each gene of interest. Both copies of each gene were sequentially deleted using these constructs according to the improved \textit{C. albicans} transformation protocol of Walther and Wendland (11) and selecting for either arginine or histidine prototrophy. In each case, correct integration was determined using gene-specific upstream and downstream primers, lying outside the site of homologous recombination to determine absence of wild type copy and presence of ::\textit{HIS1} and ::\textit{ARG4} alleles, as well as \textit{HIS1} and \textit{ARG4} specific internal primers to ensure correct integration of selective markers at
both 5’ and 3’. Resulting uridine auxotrophs with homozygous deletions of the genes of interest were finally transformed with Ncol-linearised Clp10 (12) to restore URA3 to the RP10 locus.

In the cases of orf19.6688 and PGA16, wild type alleles containing the entire upstream intergenic region, coding sequence and either 446 bp (orf19.6688) or 339 bp (PGA16) downstream sequence were amplified from SC5314 genomic DNA with PHUSION high fidelity DNA polymerase (FINNZYMES, New England Biolabs) and cloned into MluI / SalI – digested Clp10. Resulting plasmids were sequenced, linearised with Ncol and used to transform the respective of19.6688Δ and pga16Δ uridine auxotrophs. To generate the Clp20/PGA16 plasmid, the PGA16 sequence was excised from Clp10/PGA16 with MluI / SalI and cloned into Clp20 cut with the same restriction enzymes.

**PGA16 Heterozygote Reconstruction and Complementation**

A PGA16-deletion cassette was generated by amplifying URA3 from pFA-URA3 (10) using primers PGA16-FG and PGA16-RG. The resultant product was used to transform CAI4 and uridine prototrophs selected on SD medium. A resulting PGA16/pga16::URA3 heterozygote was transformed with a wild type PGA16 fragment excised from plasmid Clp10/PGA16 via digestion with MluI / SalI, yielding a PGA16/pga16Δ+PGA16 complemented strain.

**Susceptibility to stressors**

Aliquots of overnight SD cultures were washed twice in phosphate buffered saline (PBS) and 10-fold serial dilutions in 5 µl (covering a range of $10^6 – 10^1$ cells) were spotted onto SD agar containing 0.4 mM menadione (Sigma), 1.5 M NaCl or 450 µg ml$^{-1}$ Congo red (Sigma) and
incubated at 37°C for 3-4 days. Plates incubated at 42°C were photographed after 4-6 days. Each experiment was performed at least twice. Representative pictures are shown.

**Endothelial and oral epithelial cells**

The human buccal carcinoma derived epithelial cell line TR-146 (Cancer Research Technology, London) and the human umbilical vein derived endothelial cell line HUVEC (ATCC CRL-1730, LGC Standards, Promocell) were cultured and passaged in Dulbecco Modified Eagles Medium (DMEM) with 2 mM L-glutamine (PAA) supplemented with 10% heat inactivated (56°C for 10 min) fetal bovine serum (FBS, PAA). For experiments, TR146 cells were used during passage 10-20 and HUVEC cells during passage 10-40. Both cell lines were cultured in a humidified incubator at 37°C with 5% CO₂ atmosphere. Cultivation medium was replaced by fresh medium every second day and accutase (PAA) was used for detaching cells after confluency had reached approximately 80-100%.

**Damage assay**

Standard damage assays were performed by measuring the activity of lactate dehydrogenase (LDH) following our previously published protocol (13). Each experiment was performed at least three times in triplicate. For damage kinetics, 12 well plates were seeded with 2 × 10⁵ TR146 cells and incubated at 37°C, 5% CO₂ for 1 day. Monolayers were washed 3 × with PBS, the medium replaced with DMEM + 1% FBS and infected with 1 × 10⁵ *C. albicans* cells. Samples were taken at indicated time points and damage assessed by LDH, normalised (fold increase in damage) against time-matched uninfected control wells. The damage kinetics experiment was performed twice in quadruplicate.
Hyphal ramification

To generate *C. albicans* microcolonies, TR146 cells were seeded onto glass coverslips in 12 well plates and incubated at 37°C, 5% CO₂ until confluence. *C. albicans* cells were grown for at least 20 h in YPD to generate predominantly single yeast cells, 50 cells per well were used to infect the monolayers for 16 h. Monolayers were subsequently fixed and fluorescently stained as described previously (14). To quantify earlier hyphal ramification events (multiple hyphae per mother cell and hyphal branching), monolayers were infected in the same manner, but with $1 \times 10^4$ *C. albicans* cells for 6 h.

Macrophage killing assay

To analyse killing of *C. albicans* strains by macrophages, human acute monocytic leukemia cells (THP-1) were cultured in RPMI 1640 medium supplemented with 10% foetal bovine serum (PAA). Monocytes were differentiated into macrophages by addition of 5 µl phorbol 12-myristate 13-acetate (PMA; Enzo Life Sciences) for 24 hours at 37°C and 5% CO₂. Subsequently, a 24-well plate was seeded with $4 \times 10^5$ macrophages per well and incubated for 24 hours at 37°C and 5% CO₂. Twenty *C. albicans* cells per well were then added to the macrophages and incubated for two days. Experiments were performed in dodecaplicate on three independent occasions. *C. albicans* cells in medium only served as positive control.

Cytokine measurements

To determine cytokine stimulation by *C. albicans*, the murine peritoneal macrophage J774.2 cell line was used. $1 \times 10^6$ J774.2 cells were seeded into 6 well plates in DMEM + 10% heat-inactivated FBS and incubated at 37°C, 5% CO₂ for 1 day. The cells were then washed 2 ×
with PBS and the medium replaced with DMEM + 1% FBS. The macrophages were then infected with $1 \times 10^6$ *C. albicans* cells per well, incubated for a further 24 h and supernatants used for determining cytokine release. Cytokine (IL-1β, TNF-α and GM-CSF) measurements were performed by ELISA (eBioScience). The experiment was performed three times in triplicate.

Mouse model of hematogenously disseminated candidiasis

Six to eight weeks old female Balb/C mice (*Mus musculus*) (18-20 g; Charles River, Germany) were used for infection experiments. The mice were housed in groups of five in individually ventilated cages and cared for in strict accordance with the principles outlined in the *European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes* (http://conventions.coe.int/Treaty/en/Treaties/Html/123.htm). Animals were challenged intravenously on day 0 with $2.5 \times 10^4$ cfu/g body weight in 200 μl PBS via the lateral tail vein. The health status of the mice was examined at least twice a day by a veterinarian. Body surface temperature and body weight were recorded daily. Mice displaying severe signs of illness such as isolation from the group, apathy, hypothermia and drastic weight loss, were anaesthetized by application of 200 μl ketamine hydrochloride (50 mg ml$^{-1}$) prior to blood collection by heart puncture. Gross pathological alterations were recorded during necropsy. Left kidneys were collected for histology and fixed with buffered formalin. Paraffin-embedded sections were stained with Periodic acid-Schiff (PAS) according to standard protocols.

Statistics
Differences in damage of host cells by the different *C. albicans* strains were compared by two-tailed, type three Student’s t-test. The statistical analysis for the susceptibility of *C. albicans* strains to killing by macrophages was performed using one-way ANOVA followed by Tukey’s test. Differences in survival of mice infected with the different *C. albicans* strains were evaluated by Log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon tests. *P*-values ≤ 0.05 were considered to be statistically significant. All statistical tests were performed using GraphPad Prism version 5.00.

**Results**

Identification of *C. albicans* specific genes by comparative genome subtraction

Based on the hypothesis that pathogenic potential evolved independently in fungal pathogens, we reasoned that genes unique to the major human pathogenic yeast, *C. albicans* may be important for infection. Although *C. albicans* belongs to the CUG clade, which comprises several other human pathogens (*Candida tropicalis*, *Candida parapsilosis*, *Candida guilliermondii* and *Candida lusitaniae*), the generally lower pathogenic potential of these other species suggests that *C. albicans*-specific factors may contribute to its higher virulence. *C. dubliniensis* on the other hand, although less virulent than *C. albicans* in murine infection models, is, genetically, very closely related, and the two species share many important phenotypic attributes, such as the ability to undergo the yeast-to-hypha transition. Due to the fundamental importance of morphogenesis in *C. albicans* biology, we decided to define *C. albicans* specific genes as those lacking orthologues in any other sequenced organism, with the exception of *C. dubliniensis*. 
“Orthologous genes” were strictly defined as those with a BLASTp score of >40 in any other sequenced organism. First, a comparative genomic subtraction was performed using FindTarget software at CandidaDB (15) http://genodb.pasteur.fr/cgi-bin/WebObjects/CandidaDB. *C. albicans* SC5314 was set as the query genome and *C. albicans* WO-1 as a reference genome. *Candida tropicalis, Candida lusitaniae, Candida guilliermondii, Lodderomyces elongisporus, Debaryomyces hansenii, Pichia stipitis* and *Saccharomyces cerevisiae* were set as the exclusion genome list. Both the selection and exclusion criteria were set as 40 (score). The resulting genome subtraction yielded 254 genes without sequence similarity in the non-albicans species. The predicted protein sequences of these genes were batch downloaded from the *Candida* Genome Database (16) CGD - http://www.candidagenome.org/) and subsequently compared to fungal genomes held by the BROAD Institute (http://www.broadinstitute.org/annotation/fungi/fgi/). Eleven of the 254 genes displayed a BLASTp score of >40 in other fungal species and were manually removed. The remaining 243 genes were then analysed using BLASTp at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi), yielding no hits to other species. We term these ASGs for *C. albicans* specific genes. It should be noted that this set of genes may share orthologues in species which have not yet been sequenced.

*C. albicans* specific genes are expressed during infection

According to Molecular Koch’s postulates, virulence factors must be expressed during infection (17). The expression profile of the 243 ASGs during models of oral (18) and liver (19) infection was therefore analysed using GeneSpring software. Expression data was present for only 65 of the 243 ASGs. Although it is likely that some of the ASGs with no detectable expression were simply not expressed in these models or were absent for
technical reasons, we could not rule out that some of them may be mis-annotated open reading frames and did not represent *bone fide* genes. We therefore focused our analysis on the 65 ASGs which were expressed. Interestingly, one third of the ASGs exhibited statistically significant (*P* < 0.05) induction (>1.5-fold up-regulation) during infection; amongst these, 14 genes were up-regulated > 2-fold (Table S1). This suggested that these ASGs may play roles during *C. albicans*-host interactions.

**In silico** prediction of sub-cellular localisation

Because of the very nature of their identification, we did not expect to identify conserved functional domains in the ASG sequences. Nevertheless the predicted protein sequences of the 65 expressed ASGs were scrutinised with a range of bioinformatic tools. Initial sub-cellular localisation prediction using WoLF PSORT (http://wolfpsort.org/) suggested the presence of two cytosolic-, 14 extracellular-, one golgi-, 11 mitochondrial-, 29 nuclear- and six plasma membrane- localised proteins encoded amongst the *C. albicans* specific genes (Table 1). Only one gene had a predicted functional motif; this was orf19.3738/PGA22 which possesses a putative aspartyl protease motif.

Amongst the 14 extracellular proteins, five possessed predicted GPI-anchors. As the *C. albicans* genome (6524 genes) encodes 104 predicted GPI-anchored proteins (20), this represents a significant enrichment of GPI-anchored proteins amongst the ASG set (*P* = 0.0054 by Fisher’s exact test). This enrichment in cell surface-localised protein encoding genes amongst the ASGs fits with the concept of the microbial cell surface as a more rapidly evolving cellular structure and may promote cell surface diversity (21).
Genomic location

In *A. fumigatus*, infection-associated genes have been shown to cluster at sub-telomeric regions of the genome (22). All 65 *C. albicans* specific genes were therefore plotted as a function of genomic location. As shown in Fig. 1, ASGs were not restricted to any particular genomic locale, but distributed throughout the eight chromosomes with evidence of four clusters (indicated by asterisks). Interestingly, 13 (20%) were situated on chromosome 5, representing a significant enrichment of ASGs on this chromosome (*P* = 0.0085 by Fisher’s exact test).

*C. albicans* specific gene families

Many *C. albicans* genes which are associated with infection belong to families, such as the ALS (agglutinin like sequence), SAP (secreted aspartic protease) and LIP (lipase) gene families (7, 23). We reasoned that if *C. albicans* specific genes were under positive selection in the ecological niche of the warm-blooded host, they may be amplified by gene duplication events. All 65 predicted protein sequences were subjected to multiple alignment using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Seven genes yielded alignment scores of >25 and clustered into three groups: (i) orf19.3908, orf19.4691 and orf19.3906; (ii) orf19.3376 and orf19.3378; (iii) orf19.1266 and orf19.5246. These seven genes were individually analysed by BLAST at the *Candida* Genome Database and aligned using ClustalW2 (Fig. 2), confirming sequence similarity. Groups (i) and (ii) encode predicted extracellular proteins and group (iii) encodes a predicted nuclear (orf19.5246) and cytoplasmic (orf19.1266) protein (*Table 1*). The presence of these paralogues suggests that some of the *C. albicans* specific genes have undergone duplication and may therefore be under positive selection.
Six *C. albicans* specific genes are dispensable for *in vitro* growth

In order to characterise the ASGs in greater detail, 8 genes, which displayed differential expression patterns during infection (see above and Table S1) were selected for further molecular analysis. These were: orf19.3908, orf19.4055, orf19.4691, orf19.5057, orf19.6534, orf19.6688, orf19.7170 and orf19.848 (*PGA16*).

Homzygous mutants lacking six genes (attempts to delete the second copy of orf19.4055 and orf19.5057 were unsuccessful) were successfully generated and tested under a variety of *in vitro* growth conditions on agar-containing media. All strains grew at rates similar to the wild type control (BWP17 + Clp30) on minimal (SD) and rich (YPD) media at 37°C. All mutants also exhibited wild type levels of growth under oxidative, osmotic, cell wall and thermal stress, with the exception of *pga16*Δ which was moderately more sensitive to cell wall and thermal stress. Furthermore, all mutants formed normal filamentous colonies following growth at 37°C on agar containing 10% foetal bovine serum, Spider medium, Lee’s medium and embedded in YP-sucrose agar and in RPMI in contact with a plastic surface (data not shown). These data suggest that in general, ASGs are not required for normal growth under standard *in vitro* conditions.

*C. albicans* specific genes are required for host-interactions

As far as it is known, *C. albicans* spends its entire natural lifecycle in association with warm-blooded animals, normally as a member of the commensal microbial flora. Under certain pre-disposing conditions however, the fungus is able to overgrow the microbiota, causing superficial infections on skin or mucosal surfaces and damaging the local tissue. Furthermore, if *C. albicans* reaches the bloodstream of susceptible individuals, life-
threatening disseminated candidiasis can occur. During both superficial and invasive
infections, the fungus must also be capable of resisting local immune responses. As an initial
assessment of the roles of *C. albicans* specific genes during these different stages of
candidiasis, wild type and ASG-null mutant strains were tested using *in vitro* infection models
reflecting oral infection, endothelial disruption (representing a key stage of disseminated
candidiasis) and immune cell-interaction.

As a superficial infection model, $2 \times 10^4$ wild type or ASG-null mutant cells were used to infect
monolayers of human oral epithelial cells (TR146) in 96 well plates and epithelial damage
was assessed following 15 h infection as compared to 100% lysis (uninfected monolayer
treated with 0.1% Triton X-100). Following infection, wild type *C. albicans* caused damage
equivalent to lysis of 44.2% of the epithelial monolayer. Deletion of the paralogous genes,
orf19.3908 or orf19.4691, encoding predicted integral membrane proteins, caused a
moderate but significant reduction in damage. Deletion of orf19.6688 (encoding the
predicted nuclear protein) and orf19.848 (*PGA16*) (encoding a predicted cell surface protein)
elicited even stronger reductions in epithelial damage (**Fig. 3A**).

To investigate the epithelial damage potential of orf19.6688$\Delta$ and *pga16Δ* in more detail, a
time course infection was performed. For these experiments, TR146 cells were grown in 12
well plates and infected with $10^5$ *C. albicans* cells; samples were taken at 2 hour intervals
and damage assessed by measuring LDH release. As shown in **Fig. 3B**, infection with wild
type cells resulted in an effectively linear increase in epithelial damage during this time
course. Strikingly, both orf19.6688$\Delta$ and *pga16Δ* caused substantially less epithelial damage,
even after extended (24 h) infection.
The ability of *C. albicans* to grow as filamentous hyphae is thought to contribute to pathogenesis, as mutants with morphological defects often display attenuated virulence. Epithelial monolayers were infected with low numbers of wild type, orf19.6688Δ or pga16Δ cells (< 100 cells per 12 well plate monolayer (24)) and microcolony development visualised after 15 h incubation by fluorescent microscopy. Both wild type and orf19.6688Δ formed symmetrical microcolonies with radial hyphal growth patterns of similar appearance. Moreover, the radial hyphae of both wild type and orf19.6688Δ strains invaded the epithelium, as demonstrated by a differential staining protocol (14). pga16Δ cells, on the other hand formed aberrant, asymmetrical microcolonies (Fig. 3C).

Following fungal access to, and dispersal throughout the bloodstream, *C. albicans* must next traverse the endothelial lining of blood vessels to infect deep-seated organs. To assess the roles of the ASGs in this stage of disseminated candidiasis, wild type and mutant cells were used to infect HUVEC endothelial cells and endothelial damage was assayed following 24 h infection. Deletion of either orf19.3908 or orf19.7170 caused a moderate but significant reduction in endothelial damage (Fig. 4). As was the case for oral epithelial cells, both orf19.6688Δ and pga16Δ caused substantially less endothelial damage compared to the wild type.

Taken together, these data demonstrate an important role for ASGs for damage of host cells: with the exception of orf19.6534, all analysed genes were required for optimal epithelial and/or endothelial damage. Deletion of orf19.6688 and *PGA16* resulted in particularly strong defects in host cell damage.
Although sometimes associated with immune deficiencies (e.g. neutropenia), the majority (80%) of patients who develop disseminated candidiasis are not immuno-suppressed in the classical sense (6). In vivo therefore, *C. albicans* must be capable of resisting attack by immune cells such as macrophages and neutrophils. Therefore, the ability of mutants to survive interaction with THP-1 macrophages was determined. The killing potential of THP-1 macrophages was found to be relatively low and variable (up to 30% killing for wild type). Despite this, both orf19.6534Δ and orf19.6688Δ exhibited reproducibly reduced survival following co-incubation with macrophages compared to the wild type control (Fig. 5) and survival of orf19.6688Δ was significantly lower (*P* = 0.0224).

Together these data suggest that the *C. albicans* specific genes tested here are not generally required for growth under many standard laboratory conditions, but may play key roles during host pathogen interactions. Indeed, all six analysed genes were required for wild type behaviour in at least one of the infection models used here. Notably, deletion of orf19.6688 and *PGA16* resulted in particularly strong alterations in host-pathogen interactions.

orf19.6688 and *PGA16* play differential roles during disseminated candidiasis

Because of their mutant phenotypes during host pathogen interactions, we decided to further investigate the roles of orf19.6688 and *PGA16* during systemic infection. orf19.6688Δ+orf19.6688 and *pga16*+*PGA16* complemented strains were therefore constructed by reinserting a single copy of the gene of interest to the RP10 locus of the respective homozygous mutants using Clp10 (12).
The virulence of both orf19.6688Δ and pga16Δ mutants was assessed in an intravenous murine model of acute haematogenous disseminated candidiasis. As disseminated candidiasis can result in a febrile state, growth at 39.1°C in YPD was first tested to ensure that all strains were capable of growing at potentially elevated *in vivo* temperatures. All strains had a similar generation time at this temperature (data not shown).

Ten female BALB/c mice (5-6 week old; 18-20 g; Charles River, Germany) per strain were infected intravenously with $2.5 \times 10^4$ colony forming units (cfu)/g body weight as described in Materials and Methods and survival monitored. Surprisingly, despite exhibiting reduced *in vitro* damage of epithelium and endothelium, and reduced survival in the presence of macrophages, the orf19.6688Δ mutant was fully virulent in the mouse model of disseminated candidiasis. Indeed, although the mean survival times of mice infected with wild type and orf19.6688Δ strains were similar (Fig. 6A), post-mortem examination revealed more severe kidney pathologies in mice infected with orf19.6688Δ (Fig. 6B) and the orf19.6688Δ foci of infection were associated with high levels of granulocyte infiltration. These pathological observations suggest that orf19.6688 may actually have a negative impact on virulence during systemic candidiasis, possibly by dampening inflammation.

In contrast, mice infected with *pga16Δ* cells survived for longer than those infected with wild type cells. Indeed, 50% of *pga16Δ*-infected mice survived to the end of the experiment (21 days post infection). Complementation of *pga16Δ* with a single wild type allele significantly increased virulence, but not to wild type levels (Fig. 7A). These data indicate that *C. albicans* requires both copies of *PGA16* for full virulence. Histological analysis of the kidneys of *pga16Δ*-infected mice indicted the presence of aberrant filamentous morphologies (Fig. 7B).
orf19.6688Δ modulates macrophage IL-1β expression

In vivo, orf19.6688Δ cells attracted high levels of granulocyte infiltrates (Fig. 6B). As polymorphonuclear cells are known to play an important role during C. albicans infection, we sought to determine whether orf19.6688 influenced C. albicans interaction with neutrophils. Fungal survival was therefore assessed following 3 h co-incubation with human neutrophils (25). However, wild type, orf19.6688Δ and orf19.6688Δ+ orf19.6688 strains all exhibited similar survival (data not shown).

Following intravenous infection with virulent C. albicans strains, mice die of progressive sepsis (26), a condition associated with a severe proinflammatory cytokine response (27). Monocyte-dependent cytokines, such as IL-1 and TNFα, are predominantly responsible for inflammation during disseminated candidiasis (28). Similarly, in vitro macrophage recognition and subsequent killing of C. albicans is mediated by proinflammatory cytokine production (29). orf19.6688Δ exhibited reduced survival following co-incubation with a human macrophage cell line (Fig. 5) and appeared to cause more severe kidney pathology following intravenous murine infection (Fig. 6B).

We therefore hypothesised that orf19.6688 may play a role in immune interaction. Specifically, we predicted that deletion of orf19.6688 may result in elevated production of proinflammatory cytokines. A murine macrophage-like cell line was chosen to investigate this hypothesis to reflect the situation during experimental systemic candidiasis. Three cytokines were selected for analysis. These were TNFα, IL-1β and GM-CSF.

All strains induced high levels of TNFα, similar to an LPS positive control. GM-CSF stimulation by all three strains, on the other hand, was very low (around the detection limit). This was
not due to an inability of the macrophage-like cells to produce GM-CSF because stimulation with 1 µg LPS resulted in the release of 23.2±3.2 pg/ml GM-CSF (data not shown).

Fig. 8 shows that while wild type C. albicans elicited release of 2.8 pg/ml IL-1β, orf19.6688Δ stimulated the release of 9.0 pg/ml. Complementation of orf19.6688Δ with a single wild type copy of orf19.6688 reduced IL-1β production to 6.5 pg/ml.

Therefore, orf19.6688 deletion results in significantly higher activation of IL-1β, but does not appear to elicit a universal, non-specific up-regulation of proinflammatory cytokines and does not influence killing by neutrophils. Taken together, our data suggests that orf19.6688 may be involve in immune modulation.

PGA16 mediates hyphal ramification

As PGA16 was required for damage of human cells in vitro and virulence in mice, we sought to characterise the pga16Δ mutant in greater detail. Although dispensable for initial hypha formation (data not shown), pga16Δ formed aberrant hyphal microcolonies on epithelial monolayers (Fig. 3C). Indeed, the microcolonies formed by pga16Δ were asymmetrical and the progenitor mother cell was often visible, distal from the colony centre. Given these microcolony characteristics, we hypothesised that Pga16 may be involved in the ramification of microcolonies. In order to assess this quantitatively, we incubated wild type or pga16Δ cells on epithelial monolayers (or under identical conditions without human cells) for 6 h and assessed the numbers of hyphae emerging from each mother cell (2° hyphae) and the numbers of hyphae emerging from each primary hypha (branches). Under control conditions (without epithelial cells), the majority of wild type and pga16Δ cells formed a single primary hypha with very few branches (Fig. 9A,B). Interestingly, incubation of wild type C. albicans in the presence of epithelial cells stimulated the production of secondary and tertiary hyphae
from mother cells and increased the degree of hyphal branching. In contrast, the presence of epithelium did not stimulate such secondary filamentation events by \textit{pga16Δ} cells. These cells grew predominantly as a single primary hypha, with low numbers of secondary hyphae emerging from mother cells and a lesser degree of hyphal branching (Fig. 9A,B).

We propose that reduced ramification events (secondary hyphae and branches) account for the aberrant colony morphology and reduced epithelial damage caused by the \textit{pga16Δ} mutant (Fig. 3). However, analysis of the \textit{pga16Δ+PGA16} revertant strain revealed similar low levels of secondary hyphae and branches. Indeed, \textit{pga16Δ+PGA16} also caused the same (low) level of epithelial damage as the \textit{pga16Δ} homozygous mutant (data not shown). We therefore transformed the \textit{PGA16/pga16Δ} heterozygous mutant with plasmid Clp20 (to restore outstanding uridine and histidine auxotrophies) and tested the epithelial damage potential of this heterozygous strain. \textit{PGA16/pga16Δ+Clp20} failed to form secondary hyphae and exhibited the same epithelial damage as the \textit{pga16Δ} mutant (data not shown), indicating that \textit{C. albicans} may require two copies of \textit{PGA16} to damage epithelial cells. We therefore transformed \textit{PGA16/pga16Δ} with the Clp10/\textit{PGA16} complementation plasmid and assessed epithelial interactions. Again, this strain failed to form secondary hyphae and caused the same degree of epithelial damage as the \textit{pga16Δ} homozygous strain. We postulated that the outstanding histidine auxotrophy of \textit{PGA16/pga16Δ+Clp10/PGA16} may influence interactions with epithelial cells. We therefore sub-cloned \textit{PGA16} into Clp20 (which restores both uridine and histidine auxotrophies) and complemented the \textit{PGA16/pga16Δ} heterozygous strain. Again, this heterozygous complemented strain exhibited similar low levels of secondary hyphae formation and epithelial damage (data not shown).
Ectopic integration of a gene may impair transcriptional regulation and therefore function. We therefore designed a strategy to generate a heterozygous complemented strain with both copies of PGA16 expressed from its native loci. We deleted a single copy of PGA16 in the CAI-4 (uridine auxotrophic) genetic background with a URA3-based deletion cassette. Reassuringly, this strain exhibited defective hyphal ramification and reduced epithelial damage. We then replaced the pga16::URA3 allele via transformation with a wild type copy of PGA16 and selection with 5-fluoroorotic acid (5-FOA). Reconstitution with a second copy of PGA16 to the pga16Δ locus restored epithelial damage to wild type levels (Fig. 10), indicating that two copies of PGA16 are indeed required for epithelial damage and that these two copies are required to be at the native locus for this phenotype.

Discussion

In this study we hypothesised that C. albicans possesses distinct pathogenicity mechanisms represented by the presence of genes unique to this fungus. We describe 65 such species-specific genes, which are expressed during infection (Table 1 and S1). Of the subset we analysed, several of these genes were required for optimal host pathogen interactions, but dispensable for in vitro growth. For orf19.6688, which is predicted to encode a nuclear-localised protein, we demonstrate a role in modulating the proinflammatory response of macrophages. For Pga16, a predicted GPI-anchored protein, we demonstrate roles in secondary filamentation events, microcolony ramification, epithelial damage and virulence in vivo.

With the advent of genome sequencing of numerous pathogenic and, importantly, non-pathogenic fungal species, comparative genomics can now be employed to identify
pathogen-specific factors (30, 31). In the current study we have taken advantage of these recently released genomes to characterise the role of \textit{C. albicans} specific genes during infection.

Here we defined \textit{C. albicans} specific genes using both comparative genomic and transcriptomics criteria as: (1) genes without sequence similarity in any other species, with the exception of \textit{C. dubliniensis}; (2) expressed during intraperitoneal mouse infection or oral infection (RHE and/or samples from patients suffering from oral candidiasis) (18, 19). \textit{C. dubliniensis} was not included in the exclusion criteria because this species can, like \textit{C. albicans}, form hyphae. \textit{C. albicans} hyphae are the dominant invasive morphology and hypha formation is widely believed to be an important virulence attribute (32, 33).

On the sequence level, our \textit{C. albicans} specific gene set displayed a number of interesting features. The entire \textit{C. albicans} genome (>6500 genes) encodes 283 predicted soluble secreted proteins (34) and 104 predicted GPI proteins (20). Of the \textit{C. albicans} specific gene set, fourteen (21.5%) encode proteins with predicted signal peptides. Five of these likely remain attached to the cell surface via a GPI anchor and two may be retained in the membrane as they possess more than one transmembrane helix. The remaining seven are likely secreted to the extracellular space as they contain no retention motifs (Table 1). An additional four genes encode predicted plasma membrane proteins, but lack signal peptides. This indicates that a large proportion of these unique genes encode proteins which may directly interact with the [host] environment of \textit{C. albicans}. This hypothesis is supported by our functional analysis: of the six mutants analysed in this study, five lacked genes encoding predicted secreted or cell surface proteins: all five mutants displayed defective interactions in at least one infection model.
Interestingly, almost half (29) of the ASGs encode predicted nuclear proteins, suggesting that *C. albicans* may possess a number of unique proteins with regulatory roles, possibly fine-tuning transcriptional responses. In the current study, one such predicted nuclear protein-encoding gene was identified and characterised (orf19.6688). Initial infection model screening suggested a role for orf19.6688 in both tissue damage (Fig. 3 and 4) and immune evasion (Fig. 5). Despite these *in vitro* phenotypes, *in vivo*, deletion of orf19.6688 did not attenuate virulence in a murine model of systemic infection. Further dissection revealed that although orf19.6688Δ was killed by neutrophils normally, the mutant elicited significantly higher release of the proinflammatory cytokine, IL-1β from murine macrophages.

Recognition of *C. albicans* by macrophages is mediated by an array of receptors (29) including Dectin-1 (35) and Dectin-2 receptors (36), as well as TLR-2 and TLR-4 (37, 38), which recognise fungal pattern associated molecular patterns (PAMPs) such as β-1,3-glucan (35) – although the exact roles of Toll-like receptors remains debatable (39). Following recognition, IL-1β is synthesised via the NLRP3 inflammasome (40). Although β-1,3-glucan represents a major PAMP, other fungal factors also stimulate cytokine production – for example, the secreted aspartic proteases, Sap1,2,3 and 6 have recently been demonstrated to induce IL-1β production by human monocytes (41). The observed up-regulation of IL-1β by macrophages exposed to orf19.6688Δ indicates that orf19.6688 may play a role in down-regulating the expression, exposure or secretion of antigenic fungal components. Such a strategy would be advantageous for a commensal organism and its host, where neither party would benefit from potentially destructive inflammation. In this respect, it will be intriguing to evaluate the role of unique genes, such as orf19.6688, during commensal carriage of *C. albicans*. 
Pga16, on the other hand was required for epithelial and endothelial damage, as well as virulence in a mouse model of systemic candidiasis. Although the molecular function of Pga16 remains unclear at this stage and will require further investigation, we show that this protein is required for epithelial-induced hyphal ramification.

We have recently reported that the presence of an epithelial monolayer stimulates *C. albicans* to form multiple hyphae per mother cell (42). Here we provide further evidence that epithelial cells promote the emergence of multiple hyphae per mother cell and hyphal branching (processes which we collectively term hyphal ramification).

We propose that coordinated hyphal ramification by *C. albicans* is necessary for the foraging behaviour of this fungus on one of its natural substrates (human epithelia) and that such behaviour contributes to pathogenicity. This hypothesis is supported by molecular data: deletion of *PGA16* strongly reduced multiple filamentation events at earlier time points (6 h) and this led to both aberrant microcolony development and epithelial damage potential at later time points (16-24 h). Whether the hyphal ramification defect of *pga16Δ* was also responsible for the attenuated virulence of this strain *in vivo* remains unclear; however, histological analysis did reveal atypical filamentous growth of *pga16Δ* in the kidneys of infected mice. In this context, we note that Vam3/Pep12 is required for normal hyphal branching (43) and virulence (44). In contrast, *rsr1Δ* and *bud2Δ* mutants, which exhibit defective hyper-branching phenotypes (45) also exhibit defective epithelial damage and attenuated virulence (46). Therefore, it would appear that the ability of *C. albicans* to correctly coordinate hyphal branching and mycelial ramification is an important element of its pathogenic lifestyle.
Our molecular analysis of PGA16 revealed some potentially interesting aspects of C. albicans genetics. Firstly, complementation of the pga16Δ homozygous mutant with a single copy of PGA16 to the RP10 locus was sufficient to increase virulence in the mouse model of disseminated candidiasis, but did not increase pathogenicity in an in vitro epithelial infection model. The adhesion molecule, Als3, has been shown to be absolutely required for biofilm formation in vitro, yet the als3Δ mutant can form biofilms in vivo (47). These authors suggested that [additional, or stronger] in vivo signals are sufficient to activate the expression of compensatory adhesins, thus bypassing the need for ALS3. In our case, in vivo signals, which might be absent, or reduced in vitro, may drive sufficient expression of PGA16 in the pga16Δ+PGA16 strain, to restore virulence.

Our second observation was that reintroduction of a second copy of PGA16 to the RP10 locus in a pga16Δ/PGA16 heterozygous mutant did not restore pathogenicity in our in vitro epithelial model. In contrast, reintroduction of a second copy of PGA16 to its native locus restored the wild type phenotype. C. albicans is a diploid organism, and examples of haploinsufficiency are numerous (48-50). Similarly, integration of genes at ectopic chromosomal locations can result in altered expression patterns and can have serious phenotypic consequences – the most famous (or infamous) example, in C. albicans, being URA3 (51-54). Our own study on PGA16 suggests that both haploinsufficiency and positional effects can occur for the same gene.

In summary, the current study suggests that the C. albicans specific genes analysed here are largely dispensable for growth in standard laboratory media, but can play distinct roles in
pathogen-host interactions. Our findings support the view that the pathogenic potential of human fungal pathogens has arisen independently, multiple times during evolution.

Acknowledgments

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Figures Legends

Figure 1. Chromosomal map of C. albicans specific genes. Chromosomal coordinates for all 65 expressed C. albicans specific genes were downloaded from CGD and plotted as a function of chromosome location. Asterisks denote clusters of genes at the same loci; ~ indicates the presence of a gene at the telomere. Note that a higher than expected
percentage of genes clusters on chromosome 5 ($P = 0.0085$ by Fisher’s exact test). Chr., chromosome.

Figure 2. Three families of \textit{C. albicans} specific genes. Alignments of the predicted protein sequences of the three unique gene families and $P$ values of sequence similarity across the entire predicted protein length.

Figure 3. \textit{C. albicans} specific genes are required for epithelial damage. Epithelial cell (TR146) monolayers were infected with indicated \textit{C. albicans} strains for 15 h (A) or as indicated (B) and damage determined by measuring lactate dehydrogenase (LDH) release as a percentage of 100% lysis (A) or in ng/ml (B); $^*P < 0.05$ and $^{**}P < 0.01$ compared to the wild type (Wt) strain. (C) \textit{C. albicans} microcolony formation on epithelial monolayers.

Figure 4. \textit{C. albicans} specific genes are required for endothelial damage. Endothelial (HUVEC) monolayers were infected with indicated \textit{C. albicans} strains for 24 h and damage determined by measuring lactate dehydrogenase (LDH) release as a percentage of 100% lysis. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to the wild type (Wt) strain.

Figure 5. orf19.6688 is required for resisting killing by macrophages. Indicated \textit{C. albicans} strains were incubated in the presence or absence of PMA-activated THP-1 macrophages for 48 h. Wild type (Wt), $^*P < 0.05$.

Figure 6. orf19.6688Δ is hypervirulent in a mouse model of haematogenously disseminated candidiasis. Immune competent female BALB/c mice (10 per strain) were infected with the
wild type (Wt), orf19.6688Δ mutant or orf19.6688Δ+orf19.6688 complemented strain and survival monitored (A). Histological analysis of kidney sections (B).

Figure 7. PGA16 is required for full virulence in a mouse model of haematogenously disseminated candidiasis. Immune competent female BALB/c mice (10 per strain) were infected with the wild type (Wt), pga16Δ mutant or pga16Δ+PGA16 complemented strain and survival monitored (A). Histological analysis of kidney sections (B). The lower panels depict magnified views of the white-boxed areas shown in the upper panels. *P < 0.05 compared with mice either infected with the Wt or pga16Δ+PGA16 complemented strain.

Figure 8. orf19.6688Δ elicits hyper activation of IL-1β by murine macrophages. Indicated C. albicans strains were incubated with the J774.2 murine macrophage-like cell line for 24 h and IL-1β release measured by ELISA. IL-1β, interleukin-1β; n.s., not significant. *P < 0.05 and **P < 0.01.

Figure 9. PGA16 mediates hyphal ramification. The number of hyphae per mother cell (A) and the number of branches per primary (1°) hypha (B) were quantified for the indicated C. albicans strains without (-) or following exposure to epithelial cells (+TR146) for 6 h. Data shown are the results of two independent experiments. In each experiment 100 cells per condition were analysed. Wild type (Wt).

Figure 10. Reintroduction of PGA16 to the pga16Δ locus in a PGA16/pga16Δ-heterozygote restores epithelial damage. Epithelial cell (TR146) monolayers were infected with indicated C. albicans strains for 24 h and damage determined by measuring lactate dehydrogenase
(LDH) release as a percentage of 100% lysis. **$P < 0.01$** compared to the wild type (Wt) strain and $PGA16$-complemented heterozygote.
### Table 1. In silico analysis of C. albicans specific genes

Abbreviations: nucl, nuclear; mito, mitochondria; extr, extracellular; cyto, cytosol; plas, plasma membrane; golg, Golgi apparatus

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References


**Figure 1.**
Figure 2.
Figure 3.
Figure 4.

![Bar graph showing % lysis for HUVEC with various conditions](image)

Figure 5.

![Box plot showing % killing for various conditions](image)
Figure 6.
Figure 7.

A

B

Wt

pga16\n
pga16\n+PGA16

pga16\n
Figure 8.
Figure 9.

A  Hyphae per mother cell

B  Branches per 1st hypha

Figure 10.

TR146

% Lysis

Wt  PGA16/pga16Δ  PGA16/pga16Δ+PGA16