Distinct Roles of *Candida albicans*-Specific Genes in Host-Pathogen Interactions

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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 hypothesized 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 characterization 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 interleukin-1β (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 hypha 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.

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 these are prevalent etiological 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 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 led 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 among 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 (ASGs) 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 Kommission 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 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 in the supplemental material. The triple-auxotrophic

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strain BW217 complemented with plasmid Clp30 was used as a 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 cultures were grown overnight 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. *Escherichia coli* cells were grown on LB agar (1% Bacto tryptone, 0.5% yeast extract, 1% NaCl, 2% agar), and *E. coli* cultures grown overnight were cultivated in a shaking incubator at 37°C and 210 rpm. For selection purposes, 50 μg/ml ampicillin was added to solid or liquid LB medium.

**Strain construction.** For homozygous mutant construction, the BW217 genetic background was used, and alleles were sequentially deleted with PCR products based on plasmids pFA-HIS1 and pFA-ARG4 (10). Forward primers were designed to have 104 bp of 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 of homology to the immediate downstream region of the gene of interest (reverse complemented), followed by a 24-bp sequence with homology to the pFA plasmids, immediately downstream of the respective selective marker (see Table S3 in the supplemental material). By using these long primers and either pFA-HIS1 or pFA-ARG4 as the template, HIS1 and ARG4 deletion cassettes were generated for each gene of interest. Both copies of each gene were sequentially deleted by using these constructs according to the improved *C. albicans* transformation protocol described previously by Walther and Wendland (11) and selecting for either arginine or histidine prototrophy. In each case, correct integration was determined by using gene-specific upstream and downstream primers, lying outside the site of homologous recombination, to determine the absence of wild-type copies and the presence of HIS1 and ARG4 alleles as well as HIS1- and ARG4-specific internal primers to ensure the correct integration of selective markers at both 5' and 3' ends. The resulting uridine auxotrophs with homozygous deletions of the genes of interest were finally transformed with Ncol-linearized 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, the coding sequence, and either 446 bp (orf19.6688) or 339 bp (PGA16) of downstream sequence were amplified from SC5314 genomic DNA with Phusion high-fidelity DNA polymerase (Finnzymes, New England BioLabs) and cloned into MluI/Sall-digested Clp10. The resulting plasmids were sequenced, linearized with Ncol, and used to transform the respective orf19.6688 and PGA16 uridine auxotrophs. To generate plasmid Pga20/PGA16, the PGA16 sequence was excised from Clp10/PGA16 with MluI/Sall 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) by using primers PGA16-FG and PGA16-RG. The resultant product was used to transform CAF14 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/Sall, yielding a PGA16/pga16Δ+PGA16 complemented strain.

**Susceptibility to stressors.** Aliquots of SD cultures grown overnight were washed twice in phosphate-buffered saline (PBS), and 10-fold serial dilutions in 5 μl (covering a range of 10⁶ to 10⁸ cells) were spotted onto SD agar containing 0.4 mM mandelone (Sigma), 1.5 M NaCl, or 450 μg ml⁻¹ Congo red (Sigma) and incubated at 37°C for 3 to 4 days. Plates incubated at 42°C were photographed after 4 to 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 FR-146 (Cancer Research Technology, London, United Kingdom) and the human umbilical vein-derived endothelial cell line HUVEC (ATCC CRL-1730; LGC Standards) were cultured and passaged in Dulbecco modified Eagle’s medium (DMEM) with 2 mM l-glutamine (PAA Laboratories) supplemented with 10% heat-inactivated (56°C for 10 min) fetal bovine serum (FBS; PAA). For experiments, TR146 cells were used during passages 10 to 20, and HUVEC cells were used during passages 10 to 40. Both cell lines were cultured in a humidified incubator at 37°C with a 5% CO₂ atmosphere. Cultivation medium was replaced by fresh medium every second day, and Accutase (PAA) was used for detaching cells after confluence had reached approximately 80 to 100%.

**Damage assay.** Standard damage assays were performed by measuring the activity of lactate dehydrogenase (LDH) according to our previously reported protocol (13). Each experiment was performed at least three times in triplicate. For damage kinetics, 12-well plates were seeded with 2 × 10⁶ TR146 cells and incubated at 37°C in 5% CO₂ for 1 day. Monolayers were washed three times with PBS, the medium was replaced with DMEM plus 1% FBS, and cells were infected with 1 × 10⁵ *C. albicans* cells. Samples were taken at the indicated time points, and damage was assessed by measuring LDH release, normalized (fold increase in damage) against values for 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 in 5% CO₂ until they reached confluence. *C. albicans* cells were grown for at least 20 h in YPD medium 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 × 10⁵ *C. albicans* cells for 6 h.

**Macrophage killing assay.** To analyze killing of *C. albicans* strains by macrophages, human acute monocytic leukemia cells (THP-1) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (PAA). Monocytes were differentiated into macrophages by the addition of 5 μl phorbol 12-myristate 13-acetate (PMA; Enzo Life Sciences) for 24 h at 37°C in 5% CO₂. Subsequently, a 24-well plate was seeded with 4 × 10⁵ macrophages per well and incubated for 24 h at 37°C in 5% CO₂. Twenty *C. albicans* cells per well were then added to the macrophages and incubated for 2 days. Experiments were performed 12 times on three independent occasions. *C. albicans* cells in medium only served as the positive control.

**Cytokine measurements.** To determine cytokine stimulation by *C. albicans*, the murine peritoneal macrophage J774.2 cell line was used. A total of 1 × 10⁶ J774.2 cells were seeded into 6-well plates in DMEM plus 10% heat-inactivated FBS and incubated at 37°C in 5% CO₂ for 1 day. The cells were then washed twice with PBS, and the medium was replaced with DMEM plus 1% FBS. The macrophages were then infected with 1 × 10⁸ *C. albicans* cells per well and incubated for a further 24 h, and supernatants were used for determining cytokine release. Cytokine (IL-1β, tumor necrosis factor alpha [TNF-α], and granulocyte-macrophage colony-stimulating factor [GM-CSF]) measurements were performed by using an enzyme-linked immunosorbent assay (ELISA) (eBioScience). The experiment was performed three times in triplicate.

**Mouse model of hematogenously disseminated candidiasis.** Six- to eight-week-old female BALB/c mice (*Mus musculus*) (18 to 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 guidelines outlined by 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 × 10⁶ 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 anesthetized 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) stain according to standard protocols.

Statistical differences in damage of host cells by the different C. albicans strains were compared by two-tailed, type 3 Student’s t test. Statistical analysis of the susceptibility of C. albicans strains to killing by macrophages was performed by using one-way analysis of variance (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-Willcoxon tests. P values of ≤ 0.05 were considered to be statistically significant. All statistical tests were performed by 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 pathogen (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. Candida 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 by using Find-Target software at the CandidaDB website (http://genolist.pasteur.fr/CandidaDB/) (15). C. albicans SC5314 was set as the query genome, and C. albicans WO-1 was set 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 to 40 (score). The resulting genome subtraction yielded 254 genes without sequence similarity in the non-C. albicans Candida species. The predicted protein sequences of these genes were batch downloaded from the Candida Genome Database (CGD) (http://www.candidagenome.org/) (16) and subsequently compared to fungal genomes held by the Broad Institute (http://www.broadinstitute.org/annotation/fungi/fgit/). 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 analyzed by using BLASTp at the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi), yielding no hits in other species. We term these genes ASGs, for C. albicans-specific genes. It should be noted that this set of genes may share orthologues in species that 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 in models of oral (18) and liver (19) infections was therefore analyzed by using GeneSpring software. Expression data were 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 misannotated open reading frames and did not represent bone fide genes. We therefore focused our analysis on the 65 ASGs that were expressed. Interestingly, one-third of the ASGs exhibited statistically significant (P < 0.05) induction (>1.5-fold upregulation) during infection; among these, 14 genes were upregulated ≥2-fold (see Table S1 in the supplemental material). This suggested that these ASGs may play roles during C. albicans-host interactions.

In silico prediction of subcellular localization. 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 scrutinized with a range of bioinformatic tools. Initial subcellular localization prediction using WoLF PSORT (http://www.genscript.com/psort/wolf_psort.html) suggested the presence of two cytosol-, 14 extracellular-, 1 Golgi apparatus-, 11 mitochondrial-, 29 nucleus-, and 6 plasma membrane-localized proteins encoded among the C. albicans-specific genes (Table 1). Only one gene had a predicted functional motif, orf19.3738 (PGA22), which possesses a putative aspartyl protease motif.

Among the 14 extracellular proteins, 5 possessed predicted glycosylphosphatidylinositol (GPI) anchors. As the C. albicans genome (6,524 genes) encodes 104 predicted GPI-anchored proteins (20), this represents a significant enrichment of GPI-anchored proteins among the ASG set (P = 0.0054 by Fisher’s exact test). This enrichment of cell surface-localized protein-encoding genes among 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 subtelomeric 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 were 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 that 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 alignments by 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; and (iii) orf19.1266 and orf19.5246. These seven genes were individually analyzed by BLAST at the Candida Genome Database and aligned by using ClustalW2 (Fig. 2), confirming sequence similarity. Groups 1 and 2 encode predicted extracellular proteins, and group 3 encodes predicted nuclear (orf19.5246) and cytoplasmic (orf19.1266) proteins (Table 1). The presence of these paralogues...
| Gene    | Common name | Chromosome | Motif(s) (no. of transmembrane helices) | Localization(s)* |
|---------|-------------|------------|----------------------------------------|------------------|
| orf19.3712 | R           |            | Transmembrane helices (4)              | nucl             |
| orf19.3713 | R           |            |                                        | mito             |
| orf19.1677 | 3           |            |                                        | nucl             |
| orf19.3738 | PGA22       | R          | Signal peptide; aspartyl protease motif; GPI anchor | extr             |
| orf19.6302 | PGA39       | R          | Signal peptide; GPI anchor             | extr             |
| orf19.6350 | 1           |            |                                        | cyto             |
| orf19.2475 | PGA26       | 1          | Signal peptide; GPI anchor             | extr             |
| orf19.5057 | 1           |            |                                        | nucl             |
| orf19.4439 | 1           |            | Signal peptide; transmembrane helices (2) | extr             |
| orf19.1109 | 5           |            | Bipartite nuclear localization signal | mito             |
| orf19.1116 | 5           |            |                                        | nucl             |
| orf19.6920 | 7           |            | Transmembrane helices (2)             | mito             |
| orf19.7028 |            |            |                                        | nucl             |
| orf19.3851 | R           |            |                                        | nucl             |
| orf19.4241 | 5           |            |                                        | nucl             |
| orf19.3134 | 4           |            |                                        | nucl             |
| orf19.6493 | 7           |            |                                        | nucl             |
| orf19.1958 | 5           |            | Transmembrane helices (2)             | mito             |
| orf19.5190 | 7           |            |                                        | nucl             |
| orf19.1999 | 2           |            |                                        | nucl             |
| orf19.848  | PGA16       | 2          | Signal peptide; GPI anchor             | extr             |
| orf19.3906 | 5           |            | Signal peptide; transmembrane helices (4) | plas             |
| orf19.3908 | 5           |            | Transmembrane helices (4)             | extr             |
| orf19.1258 | 4           |            | EF-hand calcium-binding domain         | nucl             |
| orf19.1266 | 4           |            |                                        | cyto             |
| orf19.7170 | 7           |            | Signal peptide                        | extr             |
| orf19.6534 | 7           |            | Transmembrane helices (3)             | extr             |
| orf19.3210 | 5           |            |                                        | cyto, nucl       |
| orf19.4280 | 5           |            |                                        | nucl             |
| orf19.5246 | 1           |            | Leucine zipper pattern                | nucl             |
| orf19.5262 | 1           |            |                                        | nucl             |
| orf19.937  | 5           |            | Transmembrane helices (3)             | mito             |
| orf19.951  | 5           |            |                                        | nucl             |
| orf19.994  | 1           |            |                                        | nucl             |
| orf19.4691 | 4           |            | Transmembrane helices (4)             | extr             |
| orf19.3336 | 1           |            | Transmembrane helices (3)             | plas             |
| orf19.6688 | 7           |            | Bipartite nuclear localization signal | nucl             |
| orf19.3376 | 4           |            | Signal peptide                        | extr             |
| orf19.3378 | 4           |            | Signal peptide                        | extr             |
| orf19.5306 | 4           |            | Signal peptide                        | nucl             |
| orf19.7356 | 3           |            | Signal peptide                        | extr             |
| orf19.322  | 3           |            | Transmembrane helices (3)             | plas             |
| orf19.344  | 3           |            |                                        | mito             |
| orf19.4055 | 5           |            |                                        | nucl             |
| orf19.4069 | 2           |            |                                        | nucl             |
| orf19.3413 | FGR37       | 6          | Transmembrane helices (1)             | mito             |
| orf19.3427 | 6           |            | Transmembrane helices (1)             | mito             |
| orf19.4085 | 2           |            | Transmembrane helices (1)             | nucl             |
| orf19.6777 | 3           |            | Signal peptide                        | golg             |
| orf19.3435 | 6           |            |                                        | nucl             |
| orf19.6021 | IHD2        | 1          |                                        | nucl             |
| orf19.6030 | 1           |            |                                        | nucl             |
| orf19.2833 | PGA34       | R          | Signal peptide; GPI anchor             | extr             |
| orf19.4149 | 3           |            | Transmembrane helices (3)             | plas             |
| orf19.3543 | 2           |            |                                        | mito             |
| orf19.5549 | 6           |            | Transmembrane helices (1)             | mito             |
| orf19.4936 | 1           |            | Transmembrane helices (2)             | plas             |
| orf19.69   | 1           |            |                                        | nucl             |
| orf19.7608 | R           |            | Signal peptide                        | extr             |
| orf19.1724 | 3           |            |                                        | cyto, nucl       |
| orf19.1735 | R           |            |                                        | nucl             |
| orf19.7553 | R           |            |                                        | nucl             |
| orf19.4321 | 5           |            | Transmembrane helices (2)             | plas             |
| orf19.635  | R           |            |                                        | nucl             |
| orf19.4214 | 6           |            | Transmembrane helices (3)             | mito             |

*Abbreviations: nucl, nuclear; mito, mitochondrial; extr, extracellular; cyto, cytosol; plas, plasma membrane; golg, Golgi apparatus.
Damage the local tissue. Furthermore, if ota, causing superficial infections on skin or mucosal surfaces and conditions, however, the fungus is able to overgrow the microbiome in association with warm-blooded animals, normally as a member of the immune responses. As an initial assessment of the roles of infections, the fungus must also be capable of resisting local immune mechanisms. As far as it is known, the ASGs in this stage of disseminated candidiasis, wild-type and ASG-null mutant strains were tested by using in vitro infection models reflecting oral infection, endothelial disruption (representing a key stage of disseminated candidiasis), and immune cell interactions.

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 of infection, compared to 100% lysis (uninfected monolayer treated with 0.1% Triton X-100). Following infection, wild-type \(C.\ albicans\) caused damage equivalent to the lysis of 44.2% of the epithelial monolayer. Deletion of the paralogous genes orf19.3908 and orf19.4591, encoding predicted integral membrane proteins, caused a moderate but significant reduction in damage. Deletion of orf19.6688 (encoding the predicted nuclear protein) and orf19.8481 (PGA16) (encoding a predicted cell surface protein) elicited even stronger reductions in epithelial damage (Fig. 3A).

To investigate the epithelial damage potential of the orf19.6688Δ and pga16Δ mutants in more detail, a time course infection was performed. For these experiments, TR146 cells were grown in 12-well plates and infected with \(10^7\) \(C.\ albicans\) cells, samples were taken at 2-h intervals, and damage was 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 orf19.6688Δ and pga16Δ cells 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 was visualized after 15 h of incubation by fluorescence microscopy. Both the wild-type and orf19.6688Δ strains formed symmetrical microcolonies with radial hyphal growth patterns with similar appearances. Moreover, the radial hyphae of both the 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 of 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 the orf19.6688Δ and pga16Δ strains caused substantially less endothelial damage than the wild type.

Taken together, these data demonstrate an important role for ASGs in damage of host cells: with the exception of orf19.6534, all analyzed 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 immunosuppressed in the clas-
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 interactions 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 the wild type). Despite this, both the orf19.6534/H9004 and orf19.6688/H9004 strains exhibited reproducibly reduced survival following coincubation with macrophages compared to the wild-type control (Fig. 5), and the survival of the orf19.6688Δ strain 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 analyzed genes were
required for wild-type behavior 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 into the RP10 locus of the respective homozygous mutants by using Cip10 (12).

The virulence of both the orf19.6688Δ and pga16Δ mutants was assessed in an intravenous murine model of acute hematogenous disseminated candidiasis. As disseminated candidiasis can result in a febrile state, growth at 39.1°C in YPD medium was first tested to ensure that all strains were capable of growing at potentially elevated in vivo temperatures. All strains had similar generation times at this temperature (data not shown).

Ten female BALB/c mice (5 to 6 weeks old and 18 to 20 g; Charles River, Germany) per strain were infected intravenously
with \(2.5 \times 10^4\) CFU/g body weight as described in Materials and Methods, and survival was monitored. Surprisingly, despite exhibiting reduced in vitro damage of epithelium and endothelium and reduced survival in the presence of macrophages, the orf19.6688\(\Delta\) mutant was fully virulent in the mouse model of disseminated candidiasis. Indeed, although the mean survival times of mice infected with the wild-type and orf19.6688\(\Delta\) strains were similar (Fig. 6A), postmortem examination revealed more severe kidney pathologies in mice infected with the orf19.6688\(\Delta\) mutant (Fig. 6B), and the orf19.6688\(\Delta\) 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 pga16 cells survived longer than did those infected with wild-type cells. Indeed, 50% of pga16\(\Delta\) mutant-infected mice survived to the end of the experiment (21 days postinfection). Complementation of the pga16\(\Delta\) mutant 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 pga16\(\Delta\) mutant-infected mice indicated the presence of aberrant filamentous morphologies (Fig. 7B).

The orf19.6688\(\Delta\) mutant modulates macrophage IL-1\(\beta\) expression. In vivo, orf19.6688\(\Delta\) 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 interactions with neutrophils. Fungal survival was therefore assessed following 3 h of coincubation with human neutrophils (25). However, the wild-type, orf19.6688\(\Delta\), and orf19.6688\(\Delta\)+orf19.6688 strains all exhibited similar survival rates (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 TNF-\(\alpha\) are predominantly responsible for inflammation during disseminated candidiasis (28). Similarly, in vitro macrophage recognition and subsequent killing of C. albicans are mediated by proinflammatory cytokine production (29). The orf19.6688\(\Delta\) strain exhibited

FIG 6 The orf19.6688\(\Delta\) mutant is hypervirulent in a mouse model of hematogenously disseminated candidiasis. (A) Immunocompetent female BALB/c mice (10 per strain) were infected with the wild-type (Wt), orf19.6688\(\Delta\) mutant, or orf19.6688\(\Delta\)+orf19.6688 complemented strain, and survival was monitored. (B) Histological analysis of kidney sections.
reduced survival following coincubation with a human macrophage cell line (Fig. 5) and appeared to cause more severe kidney pathology following intravenous murine infection (Fig. 6B).

We therefore hypothesized that orf19.6688 may play a role in immune interactions. Specifically, we predicted that deletion of orf19.6688 may result in increased 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 cytokines were TNF-α, IL-1β, and GM-CSF.

All strains induced high levels of TNF-α, similar to a lipopolysaccharide (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).

Figure 8 shows that while wild-type C. albicans elicited the release of 2.8 pg/ml IL-1β, the orf19.6688Δ strain stimulated the release of 9.0 pg/ml. Complementation of the orf19.6688Δ strain

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

FIG 8 The orf19.6688Δ mutant elicits hyperactivation of IL-1β by murine macrophages. The indicated C. albicans strains were incubated with the J774.2 murine macrophage-like cell line for 24 h, and IL-1β release was measured by using an ELISA. n.s., not significant. *, P < 0.05; **, P < 0.01.
with a single wild-type copy of orf19.6688 reduced IL-1β production to 6.5 pg/ml.

Therefore, orf19.6688 deletion results in a significantly higher level of activation of IL-1β but does not appear to elicit a universal, nonspecific upregulation of proinflammatory cytokines and does not influence killing by neutrophils. Taken together, our data suggest that orf19.6688 may be involved 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 characterize the pga16Δ mutant in greater detail. Although dispensable for initial hypha formation (data not shown), the pga16Δ mutant formed aberrant hyphal microcolonies on epithelial monolayers (Fig. 3C). Indeed, the microcolonies formed by the pga16Δ mutant were asymmetrical, and the progenitor mother cell was often visible, distal from the colony center. Given these microcolony characteristics, we hypothesized 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 and 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 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 and B).

We propose that reduced ramification events (secondary hyphae and branches) account for the aberrant colony morphology and reduced epithelial damage caused by the pga16Δ mutant (Fig. 3). However, analysis of the pga16Δ+PGA16 revertant strain revealed similarly low levels of secondary hyphae and branches. Indeed, the pga16Δ+PGA16 strain also caused the same (low) level of epithelial damage as that caused by the pga16Δ homozygous mutant (data not shown). We therefore transformed the PGA16/pga16Δ heterozygous mutant with plasmid Clp20 (to restore outstanding uridine and histidine auxotrophies) and tested the epithelial damage potential of this heterozygous strain. The PGA16/pga16Δ+Clp20 strain failed to form secondary hyphae and exhibited the same epithelial damage as that exhibited by the pga16Δ mutant (data not shown), indicating that C. albicans may require two copies of PGA16 to damage epithelial cells. We therefore transformed the PGA16/pga16Δ strain with the Clp10/PGA16 complementation plasmid and assessed epithelial interactions. Again, this strain failed to form secondary hyphae and caused the same degree of epithelial damage as that caused by the pga16Δ homozygous strain. We postulated that the outstanding histidine auxotrophy of the PGA16/pga16Δ+Clp10/PGA16 strain may influence interactions with epithelial cells. We therefore subcloned PGA16 into Clp20 (which restores both uridine and histidine auxotrophies) and complemented the PGA16/pga16Δ heterozygous strain. Again, this heterozygous complemented strain exhibited similarly low levels of secondary hypha 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 pga16Δ: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 at 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 hypothesized that C. albicans possesses distinct pathogenicity mechanisms represented by the presence of genes...
unique to this fungus. We describe 65 such species-specific genes that are expressed during infection (Table 1; see also Table S1 in the supplemental material). Of the subset that we analyzed, 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 nucleus-localized 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, nonpathogenic fungal species, comparative genomics can now be employed to identify pathogen-specific factors (30, 31). In this study, we have taken advantage of these recently released genomes to characterize the role of C. albicans-specific genes during infection.

Here we defined C. albicans-specific genes, using both comparative genomic and transcriptional criteria, as (i) genes without sequence similarity to genes of any other species, with the exception of C. dubliniensis, and (ii) genes expressed during intraperitoneal mouse infection or oral infection (reconstituted human epithelium and/or samples from patients suffering from oral candidiasis) (18, 19). C. dubliniensis was not included in the exclusion criteria because this species, like C. albicans, can form hyphae. 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 C. albicans-specific gene set displayed a number of interesting features. The entire C. albicans genome (>6,500 genes) encodes 283 predicted soluble secreted proteins (34) and 104 predicted GPI proteins (20). Of the C. albicans-specific gene set, 14 (21.5%) encode proteins with predicted signal peptides. Five of these proteins 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 proteins 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 these lack signal peptides.

This indicates that a large proportion of these unique genes encode proteins that may interact directly with the (host) environment of C. albicans. This hypothesis is supported by our functional analysis: of the six mutants analyzed in this study, five lacked genes encoding predicted secreted or cell surface proteins, and 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 this study, one such predicted nuclear protein-encoding gene was identified and characterized (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 the orf19.6688Δ mutant was killed by neutrophils normally, it elicited a 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 (36) receptors as well as Toll-like receptor 2 (TLR-2) and TLR-4 (37, 38), which recognize fungal pattern-associated molecular patterns (PAMPs) such as β-1,3-glucan (35), although the exact roles of Toll-like receptors remain debatable (39). Following recognition, IL-1β is synthesized 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 were recently demonstrated to induce IL-1β production by human monocytes (41). The observed upregulation of IL-1β by macrophages exposed to the orf19.6688Δ mutant indicates that orf19.6688 may play a role in downregulating 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 cell-induced hyphal ramification.

We 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 behavior of this fungus on one of its natural substrates (human epithelia) and that such behavior 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 to 24 h). Whether the hyphal ramification defect of the pga16Δ mutant was also responsible for the attenuated virulence of this strain in vivo remains unclear; however, histological analysis did reveal atypical filamentous growth of the pga16Δ mutant 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, the rsr1Δ and bud2Δ mutants, which exhibit defective hyperbranching phenotypes (45), also exhibit defective epithelial damage and attenuated virulence (46). Therefore, it appears that the ability of C. albicans to correctly coordinate hyphal branching and mycelial ramification is an important element of its pathogenic life-style.

Our molecular analysis of PGA16 revealed some potentially interesting aspects of C. albicans genetics. First, complementation of the pga16Δ homozygous mutant with a single copy of PGA16 at 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). The authors of that study 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 the reintroduction of a second copy of PGA16 into 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 at 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 of PGA16 suggests that both haploinsufficiency and positional effects can occur for the same gene.

In summary, this study suggests that the C. albicans-specific genes analyzed 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.

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