Candida species are the most important fungal pathogens of humans and are collectively responsible for a vast number of infections. These range from superficial mucosal infections such as vulvovaginal candidiasis, and oropharyngeal thrush, to life-threatening infections such as disseminated hematogenous and invasive candidiasis. These latter infections have steadily increased in incidence in the last 30 years and are associated with a stubbornly high mortality rate as a result of the underlying immunodeficiency of the patients and inadequate diagnostics and treatments (1).

Of the approximately 150 species in the genus, 95% of infections are caused by just four species: C. albicans, C. tropicalis, C. parapsilosis, and C. glabrata (2–4). C. albicans is the dominant species, representing about half of disseminated disease and an even greater percentage of mucosal infections. Other clinically relevant species include Clavispora lusitaniae (anamorph: Candida lusitaniae), M. guilliermondii (anamorph: Candida guilliermondii), C. krusei, and C. dubliniensis, while D. hansenii (synonym: Candida famata) and Lodderomyces elongisporus are subjects of rare clinical reports. Because the genus Candida is polyphyletic, a better sense of evolutionary relationships comes in the “CUG clade,” a grouping of species that use an alternative genetic code in which that codon specifies serine rather than leucine (5, 6). The CUG clade encompasses all Candida species commonly isolated from patients other than C. glabrata and C. krusei.

Within the CUG clade there is great diversity in both genotype and phenotype. M. guilliermondii, C. lusitaniae, and D. hansenii are haploid, while the others are diploid. Originally classified as fungi imperfecti, sexual cycles are slowly being identified for most of these species (for a review, see reference 7). Most importantly, clinical incidence of these species is correlated, albeit imperfectly, with virulence potential in animal assays. A series of experiments testing several Candida species in mice grouped C. albicans and C. tropicalis as the most virulent (infections with high inoculums were lethal), followed by C. glabrata and C. lusitaniae (not lethal, but organisms persisted in organs), with C. parapsilosis, C. krusei, and M. guilliermondii as the least virulent, with at least some of the animals clearing even high inoculums from the kidneys (8). This is broadly consistent with a variety of other studies using subsets of these species in mouse models of disseminated or mucosal infections or gastrointestinal colonization (9–13). It is notable that in these models, C. parapsilosis is consistently less virulent than would be predicted from its clinical incidence.

A variety of phenotypes have been correlated with virulence in Candida species, primarily C. albicans, including hyphal growth, adhesion and biofilm formation, resistance to reactive oxygen and nitrogen stresses, use of nonfermentable carbon sources, modulation of macrophage functions, tolerance of a range of extracellular pH, and secreted protease and lipase activity (for a review, see reference 14). Presumably, the ability to cause disease in the mammalian host is the product of a combination of these phenotypes and others, but there are only a few instances in which virulence-related phenotypes were examined systematically across multiple CUG species; for instance, sensitivity to peroxide was assayed for eight species, finding differences that were imperfectly correlated...
TABLE 1 Fungal strains used in this study

<table>
<thead>
<tr>
<th>Candida designation</th>
<th>Teleomorph</th>
<th>Strain</th>
<th>Origin of strain</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Candida albicans</td>
<td>None</td>
<td>SC5314</td>
<td>&quot;Disseminated&quot;</td>
<td>67, 16, 68</td>
</tr>
<tr>
<td>Candida dubliniensis</td>
<td>None</td>
<td>CD36</td>
<td>Oral</td>
<td>69, 48</td>
</tr>
<tr>
<td>Candida tropicalis</td>
<td>None</td>
<td>MYA-3404</td>
<td>Blood</td>
<td>70, 17</td>
</tr>
<tr>
<td>Candida lusitaniae</td>
<td>Clavispora lusitaniae</td>
<td>ATCC 4720</td>
<td>Blood</td>
<td>71, 17</td>
</tr>
<tr>
<td>Candida guillermondii</td>
<td>Meyerozyma guillermondii</td>
<td>ATCC 6260</td>
<td>Lungs</td>
<td>72, 17</td>
</tr>
<tr>
<td>Lodderomyces elongisporus</td>
<td>None</td>
<td>NRRl YB-4239</td>
<td>Orange juice</td>
<td>73, 17</td>
</tr>
<tr>
<td>Candida famata</td>
<td>Debaryomyces hansenii</td>
<td>CBS767</td>
<td>Unknown</td>
<td>18</td>
</tr>
<tr>
<td>Candida parapsilosis</td>
<td>None</td>
<td>CDC317</td>
<td>Skin</td>
<td>47, 17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CDC173</td>
<td>Invasive</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CDC177</td>
<td>Invasive</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CLIB214</td>
<td>Feces</td>
<td>74</td>
</tr>
</tbody>
</table>

* That is, the reference for the report of the isolation of the strain.
* That is, the reference for the report(s) of the complete genome sequence.
* In some older literature, the teleomorph is named Pichia guillermondii.
* L. elongisporus has never been classified as a Candida species.
* Isolation source per Centraalbureau voor Schimmelcultures for Schimmelcultures with the alias CBS 2065.

With virulence (15). The phenotypic diversity both in vitro and in animal models comes from a combination of genomic (gene content) and regulatory (expression) variations among these species. The substantial genomic differences between these species have been analyzed primarily in silico (16–18), with interspecies comparisons at a molecular level only beginning to appear.

We assayed eight species of the CUG clade for a variety of host-relevant phenotypes, including interactions with phagocytes, morphology in multiple conditions, nutritional flexibility, and stress resistance. Although there is not a perfect correlation between these in vitro phenotypes and virulence, we found in general that the most pathogenic species have the highest growth rates in a variety of conditions, are most resistant to relevant stresses, and are the most robust when confronted by phagocytes. These findings are an important contribution to the dissection of virulence within this genus and will inform future molecular, genomic, and proteomic studies within this clade.

MATERIALS AND METHODS

Strains and media. The fungal strains used are listed in Table 1. For each species, the strain chosen was the one used for the respective genome sequencing project, while several additional strains of *C. parapsilosis* were obtained from G. Butler. Strains were propagated on standard yeast media (19), including YPD (1% yeast extract, 2% peptone, 2% dextrose) and YNB (0.17% yeast nitrogen base, 0.5% ammonium sulfate, 2% dextrose). Media were adjusted as indicated in the text with various stress-inducing agents. SLAD medium (0.17% yeast nitrogen base, 10 mM ammonium sulfate, 2% dextrose, 2% agar) was prepared as described previously (21). Strains were grown at 37°C except for *D. hansenii*, which grows poorly at that temperature and was propagated at room temperature (25°C) instead.

Cell culture experiments used the RAW264.7 murine macrophage-like cell line (American Type Culture Collection), which was propagated in RPMI with glutamine and HEPES (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Fisher/HiClone) and grown in a 5% CO2 environment.

Stress sensitivity assays. To assess growth on various carbon sources, strains were grown overnight in YPD at 30°C and then diluted to an optical density at 600 nm of 0.1 in 8 ml of YPD or in YNB with 2% glucose, ethanol, lactate or Casamino Acids (CAA), or 1% acetate, present as the sole carbon source. Each medium was set to pH 6, except for the CAA media, which was adjusted to pH 4. Cultures were grown with aeration at 37°C for 24 h and monitored by obtaining the optical density at the indicated times. Aliquots of the CAA cultures were also used for pH measurements to assess alkalinization of the media, as we have reported previously (21, 22). Sensitivities to other stresses were assayed using a BioTek SynergyMX automated plate reader in a 96-well plate format. Strains were grown overnight in YPD at 30°C, washed with water, and resuspended in 200 µl of YPD medium containing the relevant stressor. The plate was incubated at 30°C with periodic agitation, and the optical density was measured every 10 min for up to 16 h. Each experiment had three replicates per condition, and this was repeated at least three times. Doubling times were calculated for rolling 200-min periods every 10 min from 1 to 12 h. The time at which the peak growth rate was achieved differed somewhat by species and condition; the maximal division rate is reported.

Morphological characterization. For determination of cellular morphology in liquid inducing conditions, strains were grown overnight in YPD at 30°C, washed with water, and diluted into control YPD medium, RPMI (pH 7.4), or 10% FBS in water. After 1 to 4 h at 37°C, the cultures were centrifuged briefly to concentrate the cells before photographing them at ×400 with an Olympus IX-81 inverted microscope.

Cellular and colony morphologies were assessed on solid medium under nitrogen starvation and embedded conditions. Colony morphology was observed on solid SLAD medium in petri dishes. For imaging of cellular morphology, agar pads of the same media were prepared on standard microscope slides. Highly dilute cultures were spotted to these pads, grown for 48 h at 37°C, and imaged using a Zeiss Axiostar microscope fitted with a trinocular camera mount. Cells were embedded in an agar matrix by mixing an average of 100 to 250 cells in RPMI top agar (0.5% agar) at 42°C in petri dishes. After solidifying, they were grown 4 days at 37°C and photographed at two resolutions using an Olympus IX-81 inverted microscope.

Fungal-macrophage coculture experiments. (i) Morphology. RAW264.7 macrophages were seeded on glass coverslips in 12-well plates at a density of 105 cells/well in 1 ml of RPMI plus 10% FBS and allowed to adhere for 2 h at 37°C. Fungal strains were grown overnight in YPD, washed with water, resuspended in phosphate-buffered saline (PBS), and counted using a hemocytometer. Fungal cells were added at ratios of 1:1 to 2:5:1 (with higher ratios used for the weaker pathogens) incubated for 1 h. Cocultures were washed twice with PBS and then treated with 350 ng of calcifluor

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Priest and Lorenz

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white/ml for 10 s to stain nonphagocytosed cells. After two washes with PBS, the cells were fixed with paraformaldehyde and permeabilized with 0.1% Triton X-100. A rabbit polyclonal anti-\textit{Candida}-FITC antibody (LS-Bio) was then used to stain and visualize both intracellular and extracellular fungi. The proportion of filamentous cells (hyphal or pseudohyphal) was ascertained by scoring morphology in photomicrographs.

(ii) Cytotoxicity. The ability of the fungal species to kill macrophages was assessed through detection of lactate dehydrogenase (LDH) in the culture supernatant using the CytoTox 96 kit (Promega) as described previously (22). RAW264.7 cells were seeded in 96-well plates at 2.5 x 10^5 cells/well and allowed to adhere overnight. Fungal overnight cultures were washed and diluted in PBS, added to the macrophages at a 3:1 ratio, and incubated for 5 h before the supernatant was removed and assayed for LDH activity according to the manufacturer’s protocol. The ability of each species to induce macrophage damage was expressed as a percentage of the total LDH released from chemically lysed cells.

(iii) NO suppression assay. RAW264.7 macrophages were seeded at 6 x 10^5 cells/well in 12-well plates. Fungal species were prepared as described above and added to the wells at fungal cell/macrophage ratios of 1:10 or 1:100. Concurrently, 100 ng of lipopolysaccharide (LPS)/ml and 100 U of gamma interferon (IFN-\gamma)/ml were added, and the cocultures were incubated at 37°C in 5% CO_2 for 24 h. Cell-free culture supernatants were assayed for the presence of nitrite, which spontaneously forms from nitric oxide in aqueous solutions, using Griess reagent, as described previously (23). Assays were performed in triplicate.

Biofilm formation. To assess biofilm formation, we used a modification of an established assay to measure adherence to polystyrene plates (24). Fungal strains were grown to log phase in YNB plus 2% glucose, washed with PBS, and inoculated to 96-well plates at 10^5 cells/well. The plate was incubated for 1.5 h with gentle shaking at 37°C. Subsequently, nonadherent cells were removed by aspiration and washing twice with PBS. Biofilms were allowed to develop in YNB plus 2% glucose for 24 h at 37°C. Then the wells were washed twice with PBS and air dried for 45 min. After extensive washing, the wells were destained with 95% ethanol for 45 min. The destain was transferred with 0.4% crystal violet for 45 min. After extensive washing, the biofilm was incubated for 1.5 h with gentle shaking at 37°C. Subsequently, the wells were washed and diluted in PBS, added to the macrophages at a 3:1 ratio, and incubated for 5 h before the supernatant was removed and assayed for LDH activity according to the manufacturer’s protocol. The ability of each species to induce macrophage damage was expressed as a percentage of the total LDH released from chemically lysed cells.

RESULTS

The CUG clade contains highly virulent (and intensively studied) species such as \textit{C. albicans} and much less virulent and virtually uncharacterized species such as \textit{M. guilliermondii} and \textit{L. elongisporus}. The phylogenetic relationships between these species are depicted in Fig. 1 in which the uneven link between phylogeny and virulence is apparent: \textit{C. albicans} and \textit{C. tropicalis} are significant pathogens, while \textit{C. dubliniensis} is not. \textit{C. parapsilosis} is frequently isolated clinically, \textit{L. elongisporus} is not. Among the haploid species, \textit{C. lusitaniae}, although relatively rare clinically, is nonetheless much more common than \textit{M. guilliermondii}, which in turn is more common than \textit{D. hansenii} (25). Thus, we sought to add to the existing bioinformatics comparisons based on genome sequence and predicted protein content (17, 26, 27) by understanding the phenotypic differences that may contribute to virulence in these species.

Utilization of different carbon sources. We began the phenotypic assessment of these species by measuring growth rates in standard media. In aerated cultures at 37°C in either rich YPD or minimal YNB medium, both with 2% glucose as the primary (YPD) or sole (YNB) carbon source, each species grew rapidly (Fig. 2A), with the exception of \textit{D. hansenii}, which grows very poorly at 37°C and was omitted from most of the remaining assays. \textit{C. tropicalis} had the fastest doubling times in both conditions (53.6 and 59.0 min in YPD and YNB-glucose, respectively), growing slightly more quickly than \textit{C. albicans} and \textit{C. lusitaniae}. \textit{C. parapsilosis} was consistently the slowest growing species, with a cell division time ca. 50% longer than that for \textit{C. tropicalis} under optimal conditions.

The acquisition and utilization of alternative nonfermentable carbon sources such as lactate or amino acids has been proposed to be important in some host niches (21, 22, 28–30); \textit{C. albicans} mutants lacking the ability to metabolize nonsugar compounds are attenuated in mouse models (22, 31–34). Thus, we assayed growth rates in minimal YNB medium with lactate, acetate, or amino acids (in the form of Casamino Acids) as the sole carbon source. \textit{C. albicans}, \textit{C. tropicalis}, and \textit{C. lusitaniae} utilized amino acids effectively as the carbon source (doubling times of 78.8 to 88.5 min), while the growth of the other species was much slower (Fig. 2B). Growth was uniformly slower in the presence of lactate and acetate (Fig. 2B), with significant variations between species; the doubling times for \textit{C. tropicalis}, for instance, were far faster in acetate-containing media than other species, but among the slowest in the presence of lactate. Although all species metabolized acetate, there was significant variability between experiments in the lag time before growth began, particularly for \textit{C. lusitaniae} and \textit{M. guilliermondii}. \textit{C. parapsilosis} was the slowest growing species under all of these conditions.

Tolerance to common stresses. To assess sensitivity to various host-relevant stresses, including reactive oxygen and nitrogen species (hydrogen peroxide and the nitric oxide donor Deta-NONOate), osmotic stress (sorbitol and sodium chloride), pH (pH 2 to 9), and arsenate, we used a 96-well plate format with an automated plate reader (Fig. 2C, see also Fig. S1 in the supplemental material). Growth was generally slower than in the broth cultures but \textit{C. tropicalis} (111.5 min), \textit{C. albicans} (117.3 min), and \textit{C. lusitaniae} (129.4 min) were again the fastest-growing species, followed by \textit{C. dubliniensis} (159.9 min). The other three species, in contrast, grew less well in the 96-well plates, perhaps due to the more limited aeration in this format. The doubling time for \textit{M. guilliermondii} was more than eight times longer in the plates compared to broth culture (572.9 versus 68.7 min).

When stressors were added to YPD in the 96-well plate assays, the growth patterns changed markedly. \textit{C. albicans} and \textit{C. dubliniensis} were relatively resistant to hydrogen peroxide, NONOate, arsenate (which can induce oxidative stress), and pH extremes (Fig. 2C and D; additional concentrations of stress agents are shown in Fig. S1 in the supplemental material), with doubling times within 2-fold of the control. The addition of 1 M sodium chloride greatly slowed the growth of all species (Fig. 2C and D), although this was not due to osmotic stress, because none of the species were sensitive to 1 M sorbitol (see Fig. S1 in the supple-
mental material). While *C. albicans* was fairly resilient under these stress conditions, the other species were sensitive to specific stressors; *C. tropicalis*, for instance, failed to grow in 10 mM peroxide or 1 mM arsenate and grew slowly at pH 2. In contrast, *C. lusitaniae* and *C. parapsilosis* were quite resistant to peroxide but acutely sensitive to reactive nitrogen species. Growth patterns in stress conditions are summarized in the heat map in Fig. 2D.

**Modulation of extracellular pH.** We have previously described a phenomenon in which *C. albicans* neutralizes acidic media through the excretion of ammonia derived from the catabolism of amino acids as a carbon source, and we observed this occurring in other *Candida* species as well, including *C. glabrata* (21, 35). Alkalination occurs optimally in minimal medium with Casamino Acids as the sole source of carbon, a condition in which all species grew fairly well (Fig. 2B); thus, we tested their ability to neutralize the medium (see Fig. S2 in the supplemental material).

All seven species rapidly raised the extracellular pH from 4 to about 7.5 in about 12 h and, while there were some differences in the kinetics, this largely correlated with growth rates in this media.

**Morphology.** A hallmark of *C. albicans* is its polymorphic na-
ture and ample evidence indicates that the transition between morphological forms is required for virulence (36, 37). Of the other species, only C. tropicalis and C. dubliniensis form true hyphae, and they do this far less readily than C. albicans (38, 39). We grew each species in standard hyphal-inducing conditions, including in serum or in RPMI (at pH 7.4). Cellular morphology was consistent with previously published results (39), with abundant hyphae seen in C. albicans, some hyphae in C. dubliniensis, and a mix of pseudohyphae and rarer true hyphae in C. tropicalis (see Fig. S3 in the supplemental material). All other species remained in the yeast form.

Nitrogen limitation induces pseudohyphal formation in many yeast species, including C. albicans, C. tropicalis and S. cerevisiae (20, 39–41), so we tested each of the CUG species on solid low ammonia SLAD medium. As previously reported, C. albicans forms pseudohyphae on this medium as observed using a low-magnification stereomicroscope (Fig. 3). The filamentous growth of C. tropicalis and C. lusitaniae was surprisingly robust on this medium. To assess cellular morphology, cells were incubated on thin films of SLAD prepared on microscope slides for 24 to 48 h and then analyzed by higher-resolution differential interference contrast imaging. Although the clarity is compromised by the agar substrate, pseudohyphal cells were seen at least occasionally for all species except M. guilliermondii (Fig. 3). Interestingly, the florid filamentous growth of C. tropicalis cells was largely composed of true hyphae, as evidenced by the parallel cell walls, branched hyphae, and absence of constrictions at septae. It even appeared to form aerial hyphae on SLAD (see Video S1 in the supplemental material). C. tropicalis hyphal forms were far more common under nitrogen limitation than in other reported hyphal-inducing conditions.

C. albicans cells embedded in an agar matrix also form abundant hyphae, and this is largely independent of other inducing stimuli, occurring in rich YPD medium even at 25 to 30°C. These conditions also bypass the Cph1p and Efg1p transcription factors classically associated with hyphal growth in vitro and in vivo in certain animal models (42–44). We examined the morphology of each CUG clade species when grown embedded in 0.5% agar in YPD (Fig. 3). Although the three-dimensional nature of these structures presents a challenge to clear photography, matrix embedding stimulated filamentous growth in most of the species, with M. guilliermondii again excepted. C. albicans exhibited the classic “beads-on-a-string” morphology with yeast cells budding from the septa of hyphae that could extend for hundreds of microns. Hyphal growth was also florid in C. tropicalis, but yeast cells were rarely seen budding from hyphae; rather, extensive angular branches produced a dense hyphal network. Pseudohyphal projections were observed at various frequencies in the other species.

**Interactions with macrophages.** Avid hyphal growth of C. albicans is also common after phagocytosis by macrophages. We investigated morphology after phagocytosis under standard conditions using cells labeled with fluorescein isothiocyanate (FITC)-concanavalin A (Fig. 4). One hour after initiation of the coculture, germ tube formation was apparent in a majority of C. albicans cells, whereas they were more rarely seen (and were shorter) in C. dubliniensis. Pseudohyphae, but not true hyphae, were sometimes seen in phagocytosed C. tropicalis cells and swollen and/or elongated cells of C. parapsilosis were observed rarely. The remaining species remained exclusively in the yeast form.

We quantitated the cellular morphology in phagocytosed ver-

**FIG 3** Morphology of CUG species. To assess colony morphology under nitrogen limitation, strains were grown on SLAD medium in standard petri dishes for 4 days at 37°C before imaging with a stereomicroscope at ×20 magnification. To determine cellular morphology, strains were grown at 37°C for 48 h on SLAD agar pads on microscope slides before imaging at ×400. To assess colony morphology under embedded conditions, cells were diluted in YPD-top agar (0.5% agar) at ~100 cells/plate, followed by incubation at 37°C for 5 days before imaging on the stereomicroscope at ×40.
sus nonphagocytosed cells, which were distinguished by staining fixed, nonpermeabilized cells with calcofluor white, which binds to the cell wall of only nonphagocytosed fungal cells. As seen in Fig. 5, the proportion of hyphal or pseudohyphal cells were significantly higher in cells that remained in the media than those that were phagocytosed for *C. albicans*, *C. tropicalis*, and *C. parapsilosis*, indicating that the phagolysosomal environment inhibited some filamentation (the difference was not statistically significant for *C. dubliniensis*). Filamentous forms were not observed for the other species.

The robust filamentous growth of *C. albicans* has been proposed to damage macrophages through physical disruption of the membrane, although recent reports ascribe some of the lysis to fungus-induced pyroptosis (45, 46). To assess the ability of the CUG species to lyse macrophages, we utilized a standard assay that measures release of host LDH into the medium. The fungal species were incubated with macrophages at a 3:1 ratio for 5 h before supernatants were assayed for LDH activity (Fig. 6). *C. albicans*, *C. dubliniensis*, and *C. tropicalis* induced nearly as much LDH release as chemically lysed macrophages (the positive control), while macrophage integrity remained high in cocultures with the other species. The species that induce lysis are the only species to filament to a significant degree inside macrophages (Fig. 4 and 5), reinforcing a link between morphogenesis and macrophage damage. However, the substantial variation in the proportion of phagocytosed cells in the hyphal or pseudohyphal form was not reflected in differences in LDH release, suggesting that nonmorphogenetic factors also contribute to lysis.

We recently reported that *C. albicans* cells modulate macrophage function by suppressing the production of reactive nitrogen species via an unknown soluble, secreted factor (23). We tested how broadly this activity was conserved in the CUG clade by assaying NO production in 24-h cocultures. Supernatants were analyzed using the Griess reagent, which detects nitrite, a spontaneous breakdown product of NO in aqueous cultures at neutral pH. As seen in Fig. 7, even at a fungus/macrophage ratio of 1:100, *C. albicans* and *C. dubliniensis* effectively suppressed NO production. The other species inhibited NO release modestly at the higher fungus/macrophage ratio, with *C. tropicalis* and, to a lesser extent, *C. lusitaniae* being the most effective of this second group.

### Biofilm formation

The formation of biofilms on medical implants, such as venous catheters, is an important risk factor in

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**FIG 4** Morphology of phagocytosed cells. RAW264.7 macrophages were allowed to adhere to glass coverslips in 12-well plates and then cocultured with each CUG species for 1 h at 37°C in 5% CO₂. After washing, fixing, and permeabilization, the fungal cells were incubated with an α-*Candida* polyclonal antibody conjugated to FITC before imaging. Scale bar, 10 μm.

**FIG 5** Quantitation of the morphology of phagocytosed cells. Photomicrographs of fungi cocultured with RAW264.7 cells (representative examples are shown in Fig. 4) were scored as filamentous (pseudohyphal or hyphal) or yeast. At least three replicate experiments were performed, from which an aggregate total of at least 100 cells were counted. Asterisks indicate a *P* value of <0.01 relative to nonphagocytosed cells.

**FIG 6** Macrophage damage induced by CUG species. Macrophage membrane integrity was estimated by assaying release of LDH from the cells as described in Materials and Methods and expressed relative to the maximum amount of LDH activity released from chemically lysed cells.
disseminated candidiasis and poses significant obstacles to effective therapy. *C. albicans* biofilms are highly polymorphic, and hyphal morphogenesis is required for optimal biofilm formation. To examine biofilms formed by CUG clade species, we used a standard assay that measures adherence to a polystyrene substrate in which biomass is estimated by the binding of the dye crystal violet, using two medium conditions: YNB (Fig. 8A) or RPMI (Fig. 8B). There was a clear distinction between three species that robustly adhered to the polystyrene in both conditions as measured by the retention of crystal violet (*C. albicans*, *C. dubliniensis*, and *C. tropicalis*) and the others, which did not (Fig. 8). Curiously, *C. lusitaniae* adhered to the plastic surface well when grown in YNB but not when grown in RPMI (Fig. 8).

**Strain variation in *C. parapsilosis***. Our data clearly group *C. parapsilosis* with the less virulent species, a significant discrepancy relative to its clinical incidence. The sequenced reference isolate used here, CDC317, was isolated from the skin of a health care worker and, despite the implication of this strain in a hospital outbreak of invasive infection (47), it is possible that it is less virulent species, a significant discrepancy not when grown in RPMI (Fig. 8).

**Discussion**

In this study, we have assessed the fitness of eight species of the CUG clade in a variety of *in vitro* and *ex vivo* assays often used as proxies for virulence, including assays for resistance to host-associated stresses, hyphal morphogenesis, and interactions with phagocytes. Given the clinical significance of these species and the potential for them to be used to understand the evolution and/or mechanisms of fungal pathogenesis, the lack of comparative studies has been a hindrance. Taken together, our results correlate well with expectations based on animal models of virulence (8, 10, 12, 13), with *C. albicans* and *C. tropicalis* as the most robust species, though *C. tropicalis* was notably sensitive to oxidative stress. Although *C. tropicalis* is usually cited as the third or fourth most common cause of invasive infections (2, 4, 25), this species performs about as well as *C. albicans* in animal models, including those of disseminated hematogenous infections, gastrointestinal colonization, and dissemination from the gastrointestinal tract (8, 10, 12, 13). At the other end of the spectrum, *M. guilliermondii*, *L. elongisporus* and *D. hansenii* (when we were able to test it) were generally less stress tolerant and less fit when confronted with macrophages, an observation consistent with their low clinical impact.

The robustness in our assays of three species—*C. dubliniensis*, *C. lusitaniae*, and *C. parapsilosis*—deviated from expectations from clinical incidence and animal models. *C. dubliniensis*, although closely related to *C. albicans* is a substantially weaker pathogen: only 24 cases of invasive infections from *C. dubliniensis* were identified among over 4,000 isolates from candidiasis patients (3). The disparity between the general fitness of this species and its low incidence may come from the absence of several par-
particularly important virulence factors found in C. albicans, including the Als3 adhesion/iron acquisition protein, the Sap4-6 proteases, and the invasin Ifi4 (48). Thus, a plausible mechanism for the difference in virulence and clinical incidence between these species is a combination of loss of specific genes (in C. dublinitensis) with expansion of gene families that mediate host interactions (in C. albicans).

C. lusitaniae clustered with the more virulent species in some measures and with the less virulent ones in others. In general, it performed well in assays more reflective of in vitro, laboratory conditions, including general growth rates and morphogenesis under nitrogen starvation, but was more sensitive to stresses and macrophage contact. The biofilm assay is illustrative: C. lusitaniae adhered well when grown in the minimal yeast media YNB but not when grown in the tissue culture medium RPMI. Further work will be needed to understand the genetic mechanisms by which this species has adapted to environmental but not host niches.

In contrast, C. parapsilosis performed poorly in nearly every assay, a finding consistent with published reports indicating that it is much less virulent than C. albicans in animal models (8, 9, 49), despite its clinical incidence. Although intraspecies strain variation can be significant (see, for instance, references 50, 51, 52, and 53), several additional C. parapsilosis species fared no better than did CDC317, the sequenced reference isolate used in most of our studies. Another recent study that looked at a larger set of C. parapsilosis clinical isolates also concluded that, while there were phenotypic differences in vitro, the effects on host interactions were modest (54). The discrepancy between the lab and the clinic might be explained if C. parapsilosis were more common as a commensal or more easily transmitted from person to person, such that patients were exposed to it more frequently. Indeed, there is some evidence for this: C. parapsilosis is more commonly isolated from skin than C. albicans, with one study identifying it as the most commonly isolated yeast species on hands (55–57; see also reference 58). A few studies have linked cases in neonatal intensive care units to transmission from health care workers (59–61), in-


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170:1371/journal.ppat.1003995. http://dx.doi.org/10.1038/nature02579.


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170:1371/journal.ppat.1003995. http://dx.doi.org/10.1038/nature02579.


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