

Differential Filamentation of *Candida albicans* and *Candida dubliniensis* Is Governed by Nutrient Regulation of *UME6* Expression^{∇†}

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Candida dubliniensis is closely related to *Candida albicans*; however, it is responsible for fewer infections in humans and is less virulent in animal models of infection. *C. dubliniensis* forms fewer hyphae *in vivo*, and this may contribute to its reduced virulence. In this study we show that, unlike *C. albicans*, *C. dubliniensis* fails to form hyphae in yeast extract-peptone-dextrose (YPD) medium supplemented with 10% (vol/vol) fetal calf serum (YPDS medium). However, *C. dubliniensis* filaments in water plus 10% (vol/vol) fetal calf serum (WS), and this filamentation is inhibited by the addition of peptone and glucose. Repression of filamentation in YPDS medium could be partly overcome by preculture in synthetic Lee's medium. Unlike *C. albicans*, inoculation of *C. dubliniensis* in YPDS medium did not result in increased *UME6* transcription. However, >100-fold induction of *UME6* was observed when *C. dubliniensis* was inoculated in nutrient-poor WS medium. The addition of increasing concentrations of peptone to WS medium had a dose-dependent effect on reducing *UME6* expression. Transcript profiling of *C. dubliniensis* hyphae in WS medium identified a starvation response involving expression of genes in the glyoxylate cycle and fatty acid oxidation. In addition, a core, shared transcriptional response with *C. albicans* could be identified, including expression of virulence-associated genes including *SAP456*, *SAP7*, *HWP1*, and *SOD5*. Preculture in nutrient-limiting medium enhanced adherence of *C. dubliniensis*, epithelial invasion, and survival following coculture with murine macrophages. In conclusion, *C. albicans*, unlike *C. dubliniensis*, appears to form hyphae in liquid medium regardless of nutrient availability, which may account for its increased capacity to cause disease in humans.

Candida dubliniensis is the closest known relative of *Candida albicans*, the predominant fungal pathogen of humans (27, 28). Epidemiological evidence has shown that *C. albicans* is more prevalent in the human population as a commensal of the oral cavity and is responsible for more infections (both oral and systemic) than *C. dubliniensis* (10, 13, 15). *C. albicans* is responsible for approximately 60% of cases of candidemia, whereas *C. dubliniensis* accounts for fewer than 2% of cases (13). Evidence from animal infection models also suggests that *C. dubliniensis* is less virulent than *C. albicans* (26, 29). Following oral-intragastric inoculation, *C. dubliniensis* strains are more rapidly cleared from the gastrointestinal tract than *C. albicans* strains and are less able to establish disseminated infection (26). Following tail vein inoculation in the systemic mouse model of infection, only a small number of *C. dubliniensis* isolates have been shown to establish disseminated infections, and most studies conclude that *C. dubliniensis* isolates are generally less virulent than *C. albicans* isolates (1, 29).

Virulence studies have associated the reduced capacity of *C. dubliniensis* to establish infection with a reduced ability to undergo the transition from yeasts to hyphae (1, 26). In the oral-intragastric infection model, *C. dubliniensis* cells in the

stomach and kidney were found to be in the yeast form only, while using the same models *C. albicans* cells were found to be in both the yeast and hyphal forms (26). Asmundsdottir et al. (1) also noted that *C. dubliniensis* produced significantly fewer hyphae than *C. albicans* following dissemination to the liver and kidney in mice. *In vitro*, *C. dubliniensis* forms true hyphae less efficiently than *C. albicans* in response to serum, pH shifts in Lee's medium, and CO₂ and in certain defined media such as RPMI 1640 medium (16, 26). Poor hypha production has also been observed in *C. dubliniensis in vitro* during coculture with murine macrophages and during infection of reconstituted human oral epithelial tissues (16, 24). This results in an inability of *C. dubliniensis* to evade macrophage killing and limited invasion of epithelial surfaces.

Although *C. dubliniensis* produces true hyphae less efficiently than *C. albicans*, *C. dubliniensis* can produce abundant pseudohyphae and chlamydo spores on certain solid media (27). Recently, Staib and Morschhauser (25) demonstrated that the propensity for *C. dubliniensis* to form large numbers of chlamydo spores on these media was due to species-specific downregulation of the *NRG1* repressor. Further studies have shown that downregulation of the *NRG1* transcript is also required for efficient production of true hyphae in *C. albicans* in response to serum (22). We have shown that under conditions where *C. dubliniensis* fails to filament, for example, following phagocytosis by murine macrophages, this species does not downregulate *NRG1*, whereas *C. albicans* responds to these conditions by shutting down *NRG1* transcription (16). Deletion of the *NRG1* gene in *C. dubliniensis* can partly offset the failure of this species to filament *in vitro* and leads to more

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efficient production of hyphae in response to serum and CO₂ and during coculture with murine macrophages (16).

In this study, we have examined in detail the environmental signals required for filamentation in *C. dubliniensis*. We have shown that nutrient-rich conditions inhibit efficient hypha formation by suppressing *UME6* expression in *C. dubliniensis*. This study also includes the first description of a *C. dubliniensis*-specific microarray that we used to generate a transcript profile for *C. dubliniensis* true hyphae. The effects of inducing hypha formation in *C. dubliniensis* under these conditions on the ability to infect reconstituted oral epithelial tissues and to evade macrophage killing were also examined.

MATERIALS AND METHODS

Candida strains and culture conditions. All *Candida* strains were routinely cultured on yeast extract-peptone-dextrose (YPD) agar at 37°C. For liquid culture, cells were grown with shaking (at 200 rpm) in YPD broth at 30°C or 37°C, as indicated in the figure legends (9). Genotypes of strains used in this study are listed in Table S1 in the supplemental material. Liquid culture was also carried out at 30°C in the liquid medium of Lee et al. (14) supplemented with 400 mM arginine, 0.001% (wt/vol) biotin, and trace metals (0.2 mM ZnSO₄, 0.25 mM CuSO₄, 1 mM FeCl₃, 1 mM MgCl₂, and 1 mM CaCl₂). Where indicated in Fig. 5b and 7f, Lee's medium was buffered to pH 5.0 or pH 7.2 with 0.1 M potassium phosphate buffer. Supplementation of Lee's and other media with peptone was carried out with bacteriological peptone (Oxoid). Peptone supplementation up to 2% (wt/vol) did not significantly alter the pH of Lee's medium or serum. Hyphal induction was carried out in liquid YPD plus 10% (vol/vol) fetal calf serum (YFDS) or in sterile Milli-Q H₂O supplemented with 10% (vol/vol) fetal calf serum (WS) at 37°C. The proportion of germ tubes or hyphae in each culture was assessed at intervals by microscopic examination of an aliquot of culture with a Nikon Eclipse 600 microscope (Nikon U.K., Surrey, United Kingdom).

Genetic manipulation of *C. dubliniensis*. Ectopic expression of *C. albicans* *UME6* (*CaUME6*) in *C. dubliniensis* was achieved using plasmid pCaUme6-3, containing *UME6* under the control of a doxycycline-inducible promoter (32). The expression cassette was released from pCaUme6-3 by *ApaI* and *PmlI* digestion and was used to transform strains Wü284 and CDM10 by electroporation, as described previously (16). Plasmid pNRG1 was generated from plasmids pNIM1 and pTET42 (18). *NRG1* was removed from pTET42 as a *SalI*/*BglII* fragment and ligated to *SalI*/*BglII*-digested pNIM1 to generate pNRG1. The expression cassette was released from pNRG1 by *SacI* and *KpnI* digestion and was used to transform Wü284 and CDM10 by electroporation, as described previously (16). Integration of pNIM1 derivatives at the *ADHI* locus was confirmed by PCR.

In order to create strains harboring a *P_{ECE1}-GFP* fusion, we used the integrating vector pCDRI (16). A derivative of this plasmid was created by inserting enhanced green fluorescent protein (yEGFP) fused to the actin terminator on a *HindIII*/*MluI* fragment to create pGM175. An *ECE1* promoter fragment from bases -1 to -921 was amplified from *C. albicans* SC5314 with primers ECEAF (GTACGGGCCCAAGAGTCTCATTTCAGATAACG) and ECEXR (GCATCTCGAGTTTAAACGAATGGAAATAGTTG) and cloned upstream of yEGFP following digestion of both fragments with *ApaI* and *XhoI*. The plasmid was linearized within the *CDRI* region and used to transform *C. albicans* SC5314, *C. dubliniensis* Wü284, and the *nrg1Δ* derivative CDM10 as described previously (16). Ectopic integration in the *CDRI* gene was confirmed by Southern hybridization.

Transcriptional profiling with oligonucleotide microarrays. A set of 5,999 open reading frames (ORFs) from the CD36 genome was used to design a *C. dubliniensis* expression microarray. Two unique 60-mer oligonucleotides specific for each ORF were designed using the Agilent eArray probe design tool. Each 60-mer was printed in quadruplicate on glass slides by Agilent technologies. To examine the hyphal transcript profile of *C. dubliniensis* strain Wü284, the strain was grown for 18 h in Lee's medium (pH 4.5) at 30°C with shaking, washed in sterile H₂O, and inoculated in 200 ml of H₂O plus 10% (vol/vol) fetal calf serum to a density of 2 × 10⁶ cells/ml. Samples (50 ml) were removed for RNA preparation at 1, 3, and 5 h postinoculation. To examine the effects of cell density changes, nutrient depletion, a shift to 37°C, and a shift to alkaline pH, identical 18-h Lee's medium cultures were washed and inoculated at 2 × 10⁶ cells/ml in (i) fresh Lee's medium (pH 4.5) at 30°C, (ii) 10% (vol/vol) Lee's medium (pH 4.5) at 30°C, (iii) Lee's medium (pH 4.5) at 37°C, and (iv) Lee's medium (pH 7.2) at 30°C. RNA was extracted from these cultures following 3 h of incubation under

each condition. To identify *NRG1*-regulated genes in *C. dubliniensis*, RNA was extracted from Wü284 and its *nrg1Δ* derivative, CDM10, following growth to an optical density at 600 nm (OD₆₀₀) of 1.0 in YPD broth at 30°C. For RNA preparation, cell pellets were snap-frozen in liquid N₂ and disrupted using Mikro-Dismembrator S system (Sartorius Stedim Biotech, Göttingen, Germany). RNA was prepared using TRI-Reagent (Sigma Chemical Co.) according to the manufacturer's instructions. Poly(A) mRNA was then isolated using a Sigma Genelute mRNA isolation kit. A 200-ng aliquot of mRNA was labeled with Cy5 or Cy3 using an Agilent Two-Color Low RNA Input Linear Amplification Kit PLUS, according to the manufacturer's instructions. Hybridization and washing of the arrays were carried out using an Agilent Gene Expression Hybridization Kit and Gene Expression Wash Pack according to the manufacturer's instructions. For each condition, four biological replicate experiments were performed, including two dye swap experiments. Slides were scanned using a GenePix personal 4100A scanner (Axon), and data were extracted using GenePix Pro, version 6.1 (Axon). Spots were flagged absent if the signal was less than background +1 standard deviation in both fluorescent channels. Raw data were exported to GeneSpring GX11, and signals for each replicate spot were background corrected and normalized using Loess normalization. Log₂ fluorescence ratios were generated for each replicate spot and averaged. Oligonucleotides were excluded from analysis if >50% of replicates in each condition were flagged absent. Genes differentially expressed across all conditions were identified by analysis of variance (ANOVA) with a Student Newman Keuls (SNK) posthoc test in GeneSpring GX11. A total of 7,107 oligonucleotide probes were significantly differentially expressed, with a corrected *P* value (Benjamini-Hochberg false discovery rate [FDR]) of ≤0.05. Hierarchical clustering was used to compare gene expression under each condition using the default settings in GeneSpring GX11. Some individual samples (sera from 1 h, 3 h, and 5 h) were also analyzed using a one-sample *t* test in order to identify genes exhibiting significant differential expression (2-fold or greater) from preculture cells. All *P* values were adjusted using the Benjamini-Hochberg multiple correction test to limit false differential gene expression, and oligonucleotides with *P* values of ≤0.015 were selected for analysis.

The *C. albicans* hypha-induced gene set used in this study included the hypha-regulated genes identified by Nantel et al. (17) and by Kadosh and Johnson (12). Additional *C. albicans* hypha-regulated genes were identified in the data set of Kadosh and Johnson (12) following analysis of the data set with GeneSpring GX11. These additional genes were included if they exhibited significant (>2-fold) regulation (*t* test, *P* ≤ 0.01) in the 2-h and 3-h data sets (12).

Real-time PCR analysis of gene expression. Cultures for RNA preparation for quantitative reverse transcription-PCR (QRT-PCR) were set up in identical fashion to those used for microarray analysis. RNA for QRT-PCR was isolated using an RNeasy Mini-kit (Qiagen). Cells were disrupted using a FastPrep bead beater (Bio101). RNA samples were rendered DNA free by incubation with Turbo-DNA free reagent (Ambion, Austin, TX). cDNA synthesis was carried out as described by Moran et al. (16). Primers used in this study are listed in Table S2 in the supplemental material and were designed using Primer Express software, version 1.5 (Applied Biosystems, Foster City, CA). These primers yielded single, specific amplicons from genomic DNA and cDNA templates. Primer pairs for *UME6* and *NRG1* were selected that yielded similar amplification efficiencies as the *TEF1* primer pair against a serial dilution of template DNA. Real-time detection of amplicons was carried out using a Power SYBR green PCR Master Mix (Applied Biosystems, Foster City, CA) and an ABI 7500 sequence detector, with separate reactions performed for each gene. Gene expression levels were normalized against the expression levels of the constitutively expressed *TEF1* gene in the same cDNA sample.

Epithelial adhesion and invasion studies. Adherence of *Candida* strains to monolayers of the oral epithelial cell line TR146 was determined using the assay of Rotrosen et al. (20). Monolayers of TR146 cells were cultured in six-well tissue culture dishes in complete medium (CM), which consisted of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). A suspension of 2 × 10² yeast cells per ml was prepared in CM, and 1 ml was added to triplicate wells and incubated at 37°C in 5% (vol/vol) CO₂ for 30, 60, or 90 min. The same suspension was also plated on YPD agar to enumerate CFU in the starting inoculum. Following incubation, nonadherent cells were removed from the monolayer by washing with 10 ml of phosphate-buffered saline (PBS). The monolayer was then overlaid with 2 ml of YPD agar and incubated at 37°C overnight. The number of colonies present on the monolayers relative to the starting inoculum was determined, and results are expressed as percentage adherence. Statistical analysis of the data was performed using ANOVA in Prism, version 4.0 (GraphPad Software).

Invasion of reconstituted human oral epithelial (RHE) tissue of TR146 cells was determined using RHE tissues purchased from Skinethic Laboratories (Nice,

France) and used as described previously (23, 26). The release of lactate dehydrogenase (LDH) from epithelial cells into the cell culture medium was measured to quantify the extent of epithelial cell damage using a CytoTox 96 non-radioactive cytotoxicity assay (Promega Corp., Madison, WI) as described by Moran et al. (16).

Macrophage cell culture and infection with *Candida*. Infection of the murine macrophage-like cell line RAW264.7 with *Candida* isolates was carried out as described by Moran et al. (16). Evaluation of yeast cell proliferation in coculture with macrophages was assessed after 18 h of incubation using an XTT [2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide] dye reduction assay (Sigma-Aldrich), also described by Moran et al. (16).

Microarray data accession number. Results from all 32 microarrays have been submitted to the Gene Expression Omnibus (GEO) archive under accession number GSE20537.

RESULTS

Effect of nutrient concentration on hypha formation in *C. dubliniensis*. Previous studies have examined the transcript profile of *C. albicans* hyphae when they are induced in YPD medium supplemented with 10% (vol/vol) fetal calf serum (YPDS) at 37°C (12, 17). In this study, we wished to compare the transcript profiles of *C. dubliniensis* Wü284 hyphae induced in YPDS medium. However, preliminary experiments demonstrated that *C. dubliniensis* did not produce sufficient numbers of true hyphae under these conditions over a period of 5 h (Fig. 1a). This differential filamentation phenotype was confirmed with an additional 11 *C. dubliniensis* isolates and five *C. albicans* isolates (Fig. 1d). On average, 21% (range, 9 to 43%) of yeast cells in *C. dubliniensis* YPDS cultures produced germ tubes or filaments following 2 h of incubation (Fig. 1d). In contrast, 80% (range, 61 to 95%) of cells in *C. albicans* cultures produced germ tubes or filaments under the same conditions (Fig. 1d). Previous studies by Stokes et al. (26) demonstrated that water supplemented with 10% fetal calf serum (WS) was a more potent inducer of *C. dubliniensis* hyphae. Under these conditions, *C. dubliniensis* Wü284 was approximately 90% hyphal after 3 h of incubation (Fig. 1b). Eleven additional *C. dubliniensis* isolates exhibited significantly increased rates of filamentation in WS medium compared to filamentation in YPDS medium, whereas the rate of filamentation in six *C. albicans* isolates was similar in both media (Fig. 1d). Induction of hypha-specific gene expression was examined by observing induction of yEGFP expression from the *CaECE1* promoter in both species. *C. albicans* produced fluorescent hyphae in WS and YPDS media, whereas cells of *C. dubliniensis* produced fluorescence only in WS medium (Fig. 1c).

These data suggest that efficient filamentation in *C. dubliniensis* requires nutrient depletion. We investigated whether the addition of nutrients present in YPD medium such as glucose or peptone to *C. dubliniensis* incubated in WS medium could inhibit filamentation. The addition of 2% (wt/vol) glucose to WS cultures had no significant effect on the rate of filamentation of *C. dubliniensis* Wü284 (Fig. 1e). However, a reduction in filamentation was observed upon the addition of 2% (wt/vol) peptone, and a greater effect was observed when WS medium was supplemented with both glucose and peptone (Fig. 1e). As the addition of a nitrogen source (peptone) determined whether filamentation could proceed in *C. dubliniensis*, we carried out further experiments to characterize whether different nitrogen sources could mediate this effect. Addition of whole protein (bovine serum albumin [BSA]) to *C. dubliniensis*

WS cultures elicited similar effects as peptone but did not affect filamentation by *C. albicans*. Ammonium sulfate (100 mM) had no effect on filamentation of *C. albicans* but resulted in an ~50% reduction in filamentation by *C. dubliniensis*. Addition of specific amino acids (up to 10 mM methionine, tryptophan, histidine, or proline) to WS medium did not affect filamentation of either species.

Preculture in Lee's medium at pH 4.5 enhances filamentation in *C. dubliniensis*. We investigated whether preculture in Lee's medium, a peptone-free synthetic medium, could affect subsequent filamentation of *C. dubliniensis* in YPDS medium. Cells precultured in Lee's medium (pH 4.5) at 30°C showed a greater capacity to form true hyphae than cells precultured in YPD medium (pH 5.6), also at 30°C (Fig. 2a). Following preculture in Lee's medium, approximately 56% of cells were observed to produce germ tubes (Fig. 2a). However, budding growth resumed after several hours of incubation, indicating that Lee's medium preculture alone could not maintain hyphal elongation under these conditions (Fig. 2a). We examined whether the pH shift, the temperature shift, or the nutrient composition of Lee's medium was responsible for this phenotype. Preculture in Lee's medium at 37°C or in Lee's medium buffered to pH 7.2 could inhibit filamentation in strain Wü284, indicating that a pH and temperature shift was required (Fig. 2b). However, we also showed that addition of 1% peptone to Lee's medium could inhibit subsequent filamentation by Wü284 in YPDS medium, indicating that the medium composition also played a role (Fig. 2b). In *C. albicans* SC5314, the addition of peptone (1%) to the Lee's preculture medium could not inhibit filamentation in YPDS medium (Fig. 2b), whereas preculture of *C. albicans* at 37°C in Lee's medium increased the numbers of pseudohyphae relative to true hyphae (Fig. 2b).

Lee's medium preculture enhanced filamentation in 10 of 12 additional *C. dubliniensis* isolates examined, exhibiting an average rate of filamentation of 48% following 2 h of incubation in YPDS medium (Fig. 1d). Analysis of six independent *C. albicans* isolates showed that Lee's medium preculture also enhanced filamentation in YPDS medium by approximately 10% in these isolates relative to cells precultured in YPD medium (Fig. 1d).

Regulation of *UME6* and *NRG1* transcription. Previous studies have shown that in *C. albicans*, filamentation in YPDS medium is associated with downregulation of *NRG1* transcript levels and increased expression of *UME6* (16). Examination of *NRG1* transcript levels in *C. dubliniensis* in YPDS medium demonstrated that *NRG1* transcript levels increased following 1 h of incubation in YPDS medium at 37°C (Fig. 3a). However, inoculation of cells precultured in Lee's medium resulted in a transient drop in *NRG1* transcript levels by approximately 50% following 1 h (Fig. 3a). Inoculation of *C. dubliniensis* in WS medium yielded a 70% decrease in *NRG1* transcript levels by 3 h (Fig. 3a), similar to the decreases observed during filamentation of *C. albicans* in YPDS medium (data not shown). Analysis of *UME6* transcript levels in *C. dubliniensis* in YPDS medium revealed no significant change (Fig. 3b). However, when cells were precultured in Lee's medium (pH 4.5), we observed an ~30-fold increase in *UME6* expression in YPDS medium (Fig. 3b). In addition, we observed >100-fold induction of

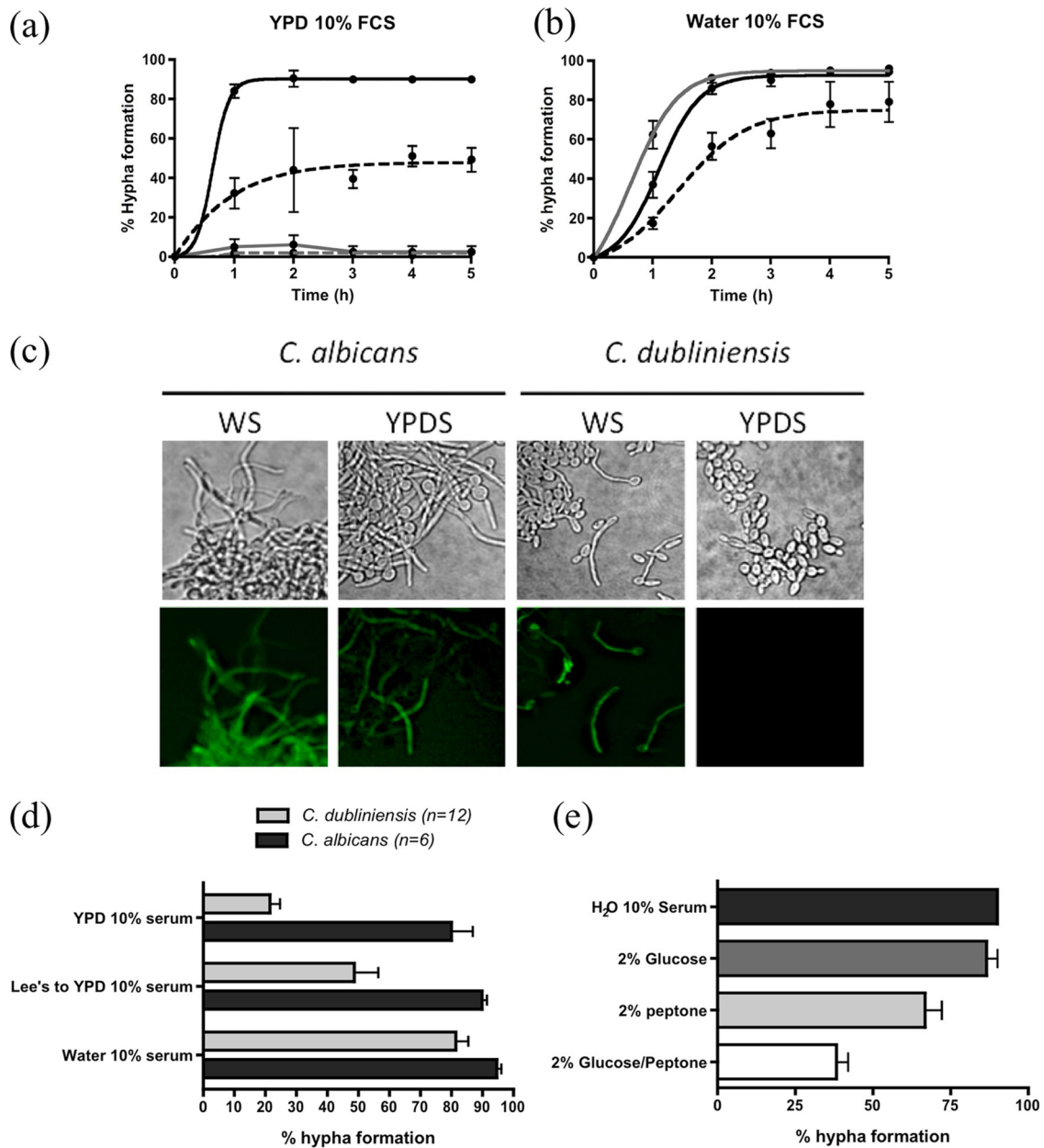


FIG. 1. (a) Hypha formation in YPD medium plus 10% FCS (YPDS) by *C. dubliniensis* Wü284 (gray lines) and *C. albicans* SC5314 (black lines) following preculture in YPD medium at 30°C (solid lines) or 37°C (dashed lines). (b) Enhanced filamentation of *C. dubliniensis* Wü284 in water plus 10% fetal calf serum (WS) following preculture in YPD medium at 30°C (solid black line), YPD medium at 37°C (dashed black line), or in Lee's medium at pH 4.5 and 30°C (gray line). Error bars correspond to standard deviations in at least three replicate experiments. A sigmoidal curve was fitted to the data for visualization using Prism, version 4.0 (GraphPad Software, Inc.). (c) Examination of induction of GFP expression from the hypha-specific *ECE1* promoter in *C. albicans* and *C. dubliniensis* in YPDS and WS media. (d) Average percent hypha formation in *C. dubliniensis* (12 isolates) and *C. albicans* (6 isolates) at 37°C in YPD medium plus 10% serum (YPDS), in YPDS following preculture in Lee's medium at pH 4.5, and in water plus 10% serum (WS). Error bars correspond to the standard error of the mean. (e) Filamentation of *C. dubliniensis* Wü284 in WS medium supplemented with 2% peptone, 2% glucose, or both peptone and glucose.

UME6 in *C. dubliniensis* following inoculation in WS medium (Fig. 3b).

Addition of peptone to WS cultures showed that peptone could decrease *UME6* expression in *C. dubliniensis* in a concentration-dependent manner, with 2% (wt/vol) peptone reducing *UME6* expression by approximately 80%. Glucose (2% wt/vol) alone did not significantly decrease *UME6* expression

although the combination of glucose and peptone had an additive effect on *UME6* expression.

Overexpression of *UME6* enhances filamentation in *C. dubliniensis*. We further investigated the roles of *NRG1* and *UME6* in hypha formation in the *C. dubliniensis* *nrg1*Δ mutant CDM10. Previously, we have shown that the *nrg1*Δ strain, unlike the wild type, forms hyphae in response to CO₂ and fila-

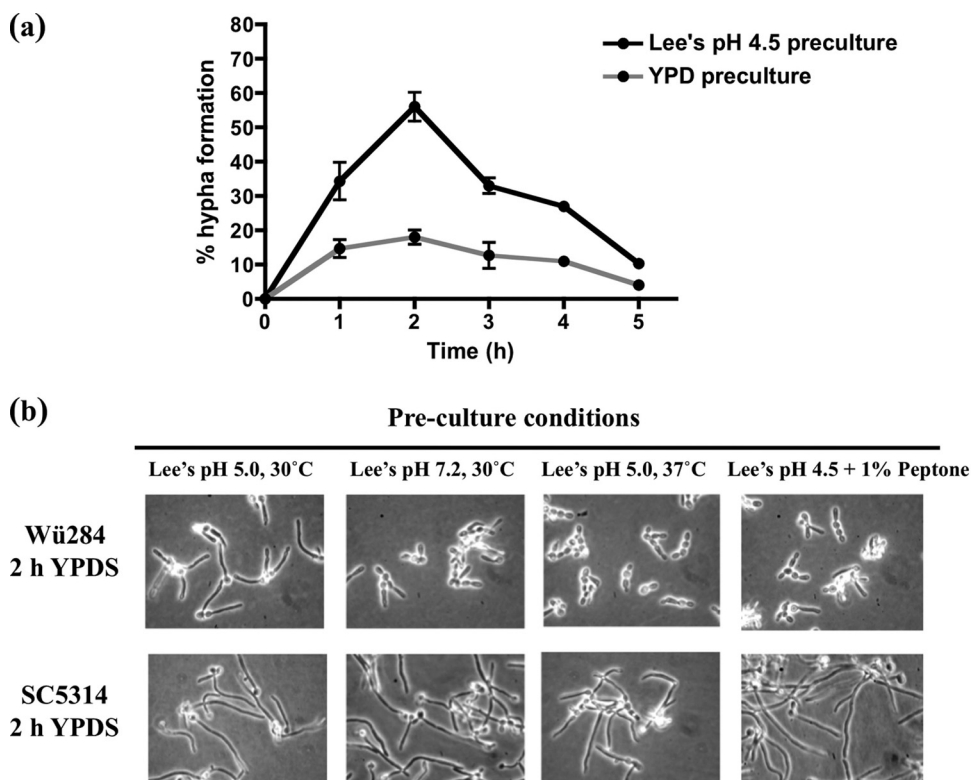


FIG. 2. (a) Filamentation rate of strain Wü284 in YPDS medium at 37°C following preculture in YPD broth at 30°C or following preculture in Lee's medium at pH 4.5 and 30°C. Error bars correspond to standard deviations in three replicate experiments. (b) Photomicrographs showing typical morphologies of *C. dubliniensis* Wü284 and *C. albicans* SC5314 following 2 h of incubation in YPD medium plus 10% (vol/vol) FCS following preculture in Lee's medium. Cells were precultured for 24 h in modified Lee's medium, buffered to pH 5.0 or 7.2 with 0.1 M potassium phosphate buffer or supplemented with 1% (wt/vol) peptone.

ments more rapidly in response to serum in water (16). In this study, a derivative of CDM10 harboring a P_{ECE1} -*GFP* promoter fusion (M10EGFP) formed elongated filaments in YPDS medium; however, these filaments possessed the characteristic constrictions of pseudohyphae (Fig. 4a). Strain M10EGFP was weakly fluorescent in YPD and YPDS media (Fig. 4a), whereas in WS medium the same strain emitted strong fluorescence and formed masses of true hyphae (Fig. 4a). We tested whether overexpression of *UME6* from a doxycycline-inducible promoter could enhance true hypha production by CDM10 in YPDS medium. Addition of 20 μ g/ml doxycycline promoted conversion of pseudohyphae to true hyphae in this strain (Fig. 4b). Similarly, introduction of the same construct in the parent isolate Wü284 could promote the formation of true hyphae in YPDS medium (Fig. 4b).

We also tested whether constitutive *NRG1* expression from the doxycycline-inducible promoter could prevent filamentation. Constitutive expression of *NRG1* in Wü284 and CDM10 could block pseudohypha formation in YPDS medium. However, expression of *NRG1* from this promoter was not sufficient to block true hypha formation in WS medium (data not shown).

Transcript profiling of *C. dubliniensis* in serum. This study has shown that under nutrient depleted conditions, *C. dubliniensis* can form hyphae as effectively as *C. albicans*. In order to determine whether *C. dubliniensis* hyphae can express the same range of virulence-associated factors as *C. albicans* hyphae, we

carried out whole-genome transcript profiling of *C. dubliniensis* during growth in WS medium. Samples were analyzed at 1 h, 3 h, and 5 h postinoculation in WS medium. Within 1 h, we observed a 2.5-fold or greater change in transcription in 1,095 genes relative to preculture cells (*t* test, $P < 0.015$) (see Table S3 in the supplemental material). This corresponds to 18% of the genome. Analysis of the upregulated genes ($n = 526$) for significant shared Gene Ontology (GO) terms identified large groups of genes associated with transport (102 genes), organelle organization (73), the cell cycle (44), and translation (43) (Fig. 5a). Many of these genes were associated with processes known to be involved in hyphal development, such as the assembly of actin cables (*TPM2*, *ARF3*, *MEA1*, *ARP9*, and *YEL1*), Spitzenkörper assembly (*MLC1*), and GTPases with roles in actin organization (*RSR1*, *RAC1*, *RDII*, and *RHO3*) (see Fig. S1 in the supplemental material). These data also highlighted some processes not previously associated with hypha formation, such as downregulation of vacuolar metabolism, including vacuolar protein catabolism (8 of 10 annotated genes) (see Fig. S1), suggesting a shutdown in autophagic processes. However, increased expression of genes with roles in vacuolar biogenesis and inheritance was also observed (*VAM3*, *YPT7*, *YPT72*, and *YKT6*) (see Fig. S1). Reorganization of membrane lipid structure was indicated by a significant decrease in sphingolipid metabolism (9 of 25 annotated genes) (see Fig. S1). Reorganization of the cell surface was indicated by an increase in expression of genes associated with glyco-

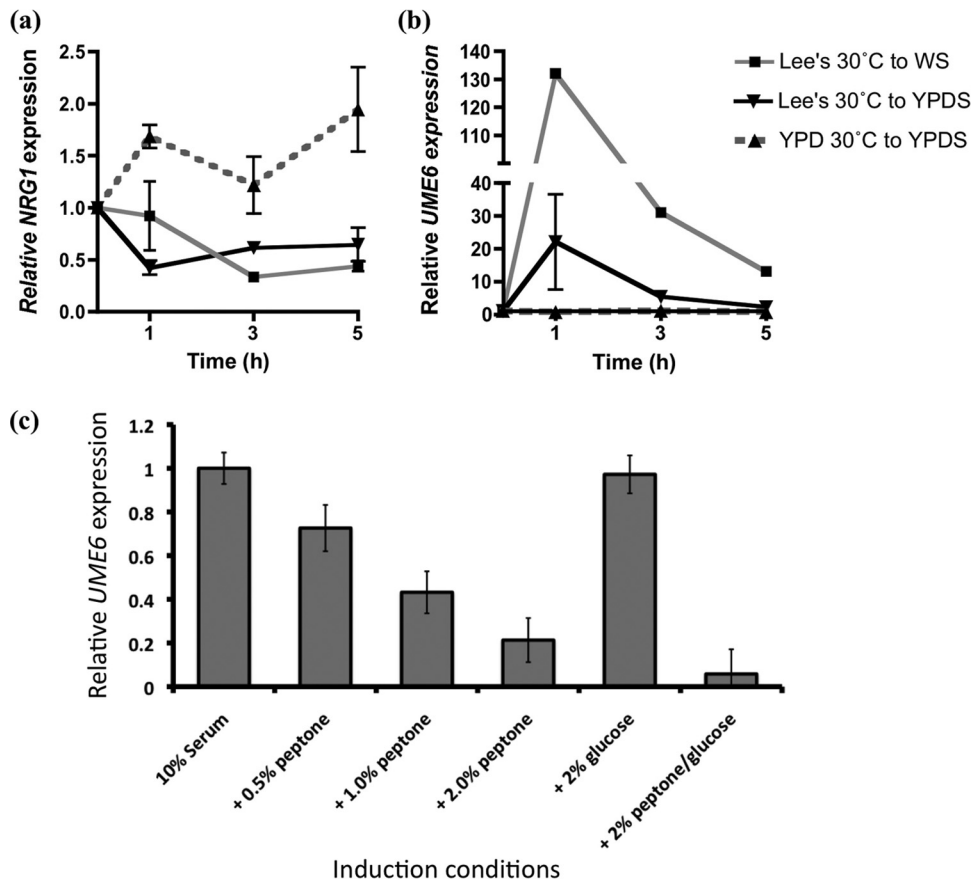


FIG. 3. Real-time PCR analysis showing relative levels of *NRG1* transcript (a) and relative levels of *UME6* transcript (b) in *C. dubliniensis* incubated in serum-containing medium. Expression levels were normalized to *TEF1* expression levels in each sample. *C. dubliniensis* was cultured in WS medium following preculture in Lee's medium at 30°C, in YPDS medium following preculture in Lee's medium at 30°C, and in YPDS medium following preculture in YPD medium at 30°C. Error bars represent standard deviations of results from three replicate RNA preparations. In the case of *UME6* expression in WS medium, representative data from one replicate are shown; additional experiments all showed >100-fold induction at 1 h. (c) Relative expression of *UME6* in *C. dubliniensis* in WS medium supplemented with additional nutrients. Cells were precultured in YPD medium at 30°C and inoculated in WS medium alone or supplemented with the indicated concentrations of peptone or glucose. *UME6* expression levels were normalized to *TEF1* expression levels in the same sample.

syphosphatidylinositol (GPI) anchor biosynthesis (*DPM1*, *MCD4*, and *orf19.538*) and glycosylation (*PMI1*, *PMT2*, *PMT5*, *ALG5*, *ALG6*, *ALG7*, *GFA1*, *DPM1*, *orf19.2298*, and *orf19.7426*).

Within 1 h, significant upregulation of *RAS1*, an upstream regulatory element of the cyclic AMP-protein kinase A (cAMP-PKA) pathway was detected (Fig. 5b). Regulation of several transcriptional regulators of filamentous growth was also observed, including that of *EFH1*, *TEC1*, and *UME6* (Fig. 5b). Induction of the pH regulator *RIM101* was also observed. Downregulation of *EFG1* and the transcriptional repressor *NRG1* was also observed by 1 h (Fig. 5b). We also observed increased expression of Cd36_54430, the putative orthologue of *CaSFL2*, a novel regulator of hypha production that we have previously shown to be uniquely expressed by *C. albicans* during infection of oral epithelial tissues *in vitro* (Fig. 5b) (24).

By 3 h, approximately 90% of cells in WS medium produced true hyphae. At this time point, 345 genes exhibited a >2.5-fold induction and 348 exhibited a >2.5-fold decrease in expression, relative to the preculture cells (*t* test, $P \leq 0.015$) (see Table S4 in the supplemental material). A significant propor-

tion of the genes upregulated at 3 h were orthologous to *C. albicans* genes annotated with the GO term pathogenesis ($n = 20$; $P \leq 0.044$), including the secreted proteinase *SAP7* and *C. dubliniensis* *SAP4-SAP5-SAP6* (*CdSAP4,5,6*), the single *C. dubliniensis* orthologue of *C. albicans* *SAP4*, *SAP5*, and *SAP6* (Fig. 5c). We also observed induction of the predicted GPI-anchored proteins *SOD5*, *HWPI1*, and *ALS1* and downregulation of the orthologues of *ALS4*, *ALS9*, and *RBT5* (Fig. 5c).

Environmental regulation of gene expression in *C. dubliniensis*. In order to understand how different environmental stimuli shaped the transcriptional response to growth in 10% serum, we also analyzed the transcript profile of *C. dubliniensis* following a change in cell density, a shift to 37°C, nutrient depletion (10% [vol/vol] Lee's medium) or a shift to alkaline pH (pH 7.2). None of these conditions alone could induce morphogenesis in *C. dubliniensis*. Although both *UME6* and *NRG1* exhibited regulation under the conditions examined, the changes did not reach the levels seen in WS cultures, indicating perhaps that multiple environmental signals are required to alter their expression levels sufficiently to allow filamentation to proceed (see Fig. S2 in the supplemental material). We

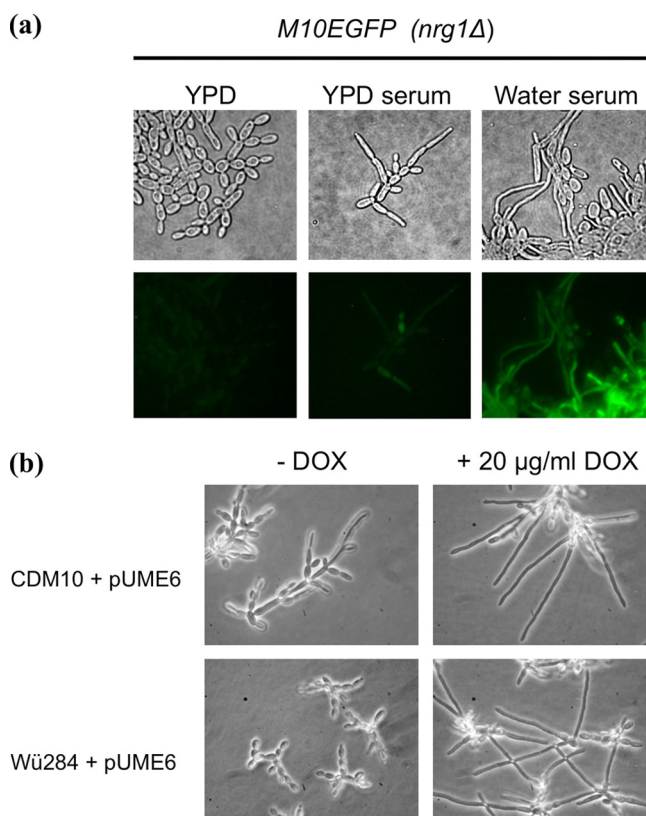


FIG. 4. (a) Photomicrographs showing morphology of a derivative of the *C. dubliniensis nrg1Δ* mutant harboring a P_{ECE1} -GFP construct (CDM10). Top panel shows morphology in YPD, YPDS, and WS media. Lower panel shows levels of fluorescence expressed from the P_{ECE1} -GFP fusion under each condition. (b) Morphology of CDM10 and Wü284 derivative strains harboring plasmid pCaUME6, containing the *UME6* gene under the control of a doxycycline-inducible promoter. Morphology is shown following 3 h of incubation in YPDS medium with (+) or without (-) 20 μ g/ml doxycycline (DOX).

carried out ANOVA to identify differentially regulated transcripts ($P \leq 0.05$) and visualized the results using hierarchical clustering. From this analysis we could identify two large clusters of genes regulated by changes in cell density (Fig. 6a). Cluster I genes ($n = 163$) were induced in all experiments involving a change in cell density and were significantly enriched for genes encoding ribosomal subunits or proteins involved in ribosome biogenesis (Fig. 6b). Cluster IV ($n = 167$) included genes downregulated by cell density changes and was significantly enriched for genes involved in glycolysis and trehalose biosynthesis (Fig. 6b). Three clusters of serum-specific genes could also be identified (clusters II, III, and V) (Fig. 6a), and these were largely involved in metabolism of alternative carbon sources (*ECII*, *ICL1*, and *PXP2*) (Fig. 6c) and nutrient transport (e.g., *HGT1* and *JEN1*) (Fig. 6c). These data show that growth in WS medium resulted in a switch from carbohydrate catabolism to fatty acid oxidation and the glyoxylate cycle for energy production (see Fig. S3 in the supplemental material). Smaller clusters of genes were identified that were induced by alkaline pH or relief of *NRG1* repression (Fig. 6d and e). The regulation of genes in response to nutrient-depleted Lee's medium and the temperature shift was more complex

(Fig. 6f and g). Some transcripts exhibited clear temperature induction (*MET14* and *CEK2*) (Fig. 6f) or nutrient depletion induction (*GAP2* and *MNN4*) (Fig. 6g). Other transcripts responded to several conditions (e.g., *HGT12* was *NRG1* repressed and nutrient regulated, whereas *CFL11* was induced by both temperature and pH).

Comparison of the *C. albicans* and *C. dubliniensis* hypha-regulated gene sets. We compared the list of *C. dubliniensis* hypha-expressed genes (see Table S3 in the supplemental material) with a list of genes regulated during hypha formation in serum by *C. albicans* (see Material and Methods). We identified a core set of 65 hypha-induced genes in both species (Table 1). Sixty-seven genes were found to be downregulated by both species (Table 2). This analysis could identify common sets of cell surface, stress response, and regulatory genes induced or repressed in hyphae of both species. The specific transcriptional response of *C. dubliniensis* to WS medium was largely associated with the nutrient-poor conditions used and included genes of the glyoxylate cycle and fatty acid beta-oxidation (Fig. 6c; see also Fig S3). Increased expression of several species-specific hypothetical genes in *C. dubliniensis* could also be detected (Cd36_41370, Cd36_63200, and Cd36_65070) as well as downregulation of a putative glutamate decarboxylase (Cd36_10760) and a predicted ORF (Cd36_34790).

The specific response of *C. albicans* included several predicted GPI-anchored proteins, including *RBT4*, *PGA54*, and *PGA55* (see Table S5 in the supplemental material). Nine of the *C. albicans*-specific genes had no direct orthologue in *C. dubliniensis* (i.e., genes without BLAST matches in *C. dubliniensis* or where the top BLAST hit in *C. dubliniensis* was not reciprocal). These included *EEDI*, *SAP4*, *SAP5*, *ALS3*, and *HYR1* and several members of the *C. albicans* telomeric *TLO* gene family (Table 3). The transcriptional regulator *BCR1* was also induced in *C. albicans*, which may contribute to the concomitant upregulation of the *BCR1*-regulated genes *HYR1*, *ALS3*, *GCN1*, and orf19.6079.

Can stimulation of hypha formation in *C. dubliniensis* result in tissue damage? We wished to determine, using simple infection models, whether induction of hyphae can increase the invasive potential of *C. dubliniensis*. Previous studies have demonstrated that *C. dubliniensis*, in contrast to *C. albicans*, does not invade reconstituted oral epithelium tissue when it is precultured in YPD medium at 37°C (16, 24, 26). These findings were confirmed here when *C. dubliniensis* Wü284 was inoculated on the surface of RHE cultures following preculture in YPD medium at 37°C. Cells grown under these conditions remained exclusively in the yeast phase and attached poorly to the surface of the tissue. Penetration of the tissue by filaments did not occur (Fig. 7a). In contrast, when *C. dubliniensis* cells precultured in Lee's medium, pH 4.5, at 30°C were inoculated on the tissue, we observed a mixture of morphologies (yeasts, pseudohyphae, and some true hyphae), and the cells adhered more closely to the surface of the epithelial tissue (Fig. 7b). In addition, localized invasion was observed by hyphae and pseudohyphae at 24 h postinfection (Fig. 7b and c). When a quantitative assessment of epithelial damage was made by measuring the release of lactate dehydrogenase from epithelial cells, we observed a significant increase in damage caused by cultures incubated at 30°C in Lee's pH 4.5 medium

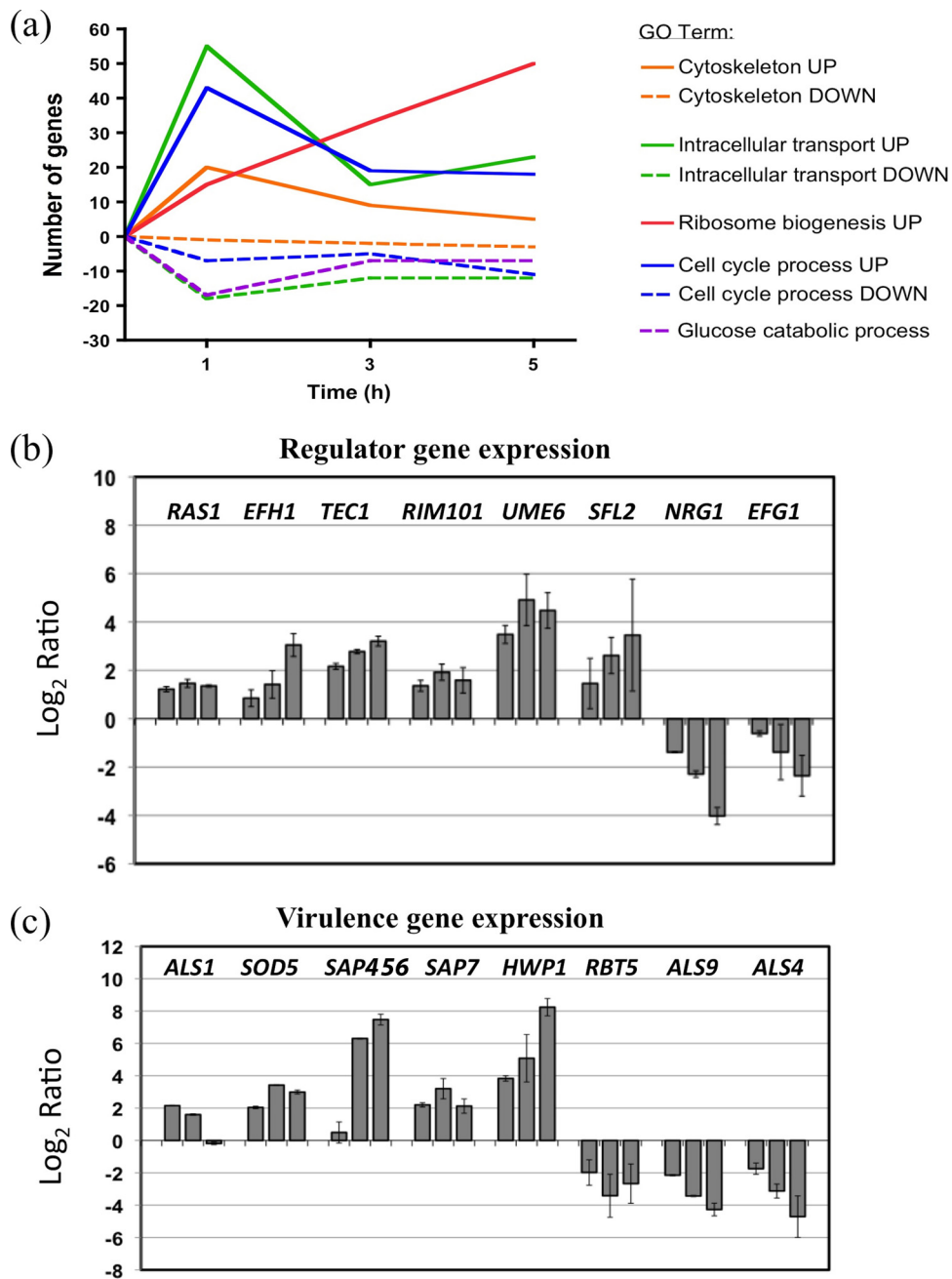


FIG. 5. (a) Graphical representation of the changes in expression in selected Gene Ontology (GO) groups during filamentation in *C. dubliniensis*. The total number of genes up- or downregulated 2.5-fold in each group is shown at each time point. (b) Microarray expression of selected regulators of filamentous growth during hypha formation in *C. dubliniensis* Wü284 in WS medium. Columns for each gene show expression levels relative to preculture cells at (left to right) 1, 3, and 5 h postinoculation. (c) Microarray expression of selected virulence-associated genes during hypha formation in *C. dubliniensis* Wü284 in WS medium. Columns for each gene show expression levels relative to preculture cells at (left to right) 1, 3, and 5 h postinoculation. Error bars in panels b and c represent standard deviations from the mean generated in Genespring GX11 from two distinct oligonucleotide probes per gene in four biological replicate experiments.

compared to YPD-grown cultures (Fig. 7d). Increased cell damage was also recorded in RHE infections with *C. dubliniensis* strain CD36 following preculture in Lee's medium (7.0 ± 0.6 LDH U/liter) relative to YPD medium (5.2 ± 0.2 LDH U/liter).

These data suggest that the difference in tissue damage and invasion elicited by *C. dubliniensis* cells grown in YPD medium

and Lee's medium may be due to differences in adherence. We carried out a more detailed investigation of the adhesion of *C. dubliniensis* to TR146 cell monolayers over 90 min. Within 30 min of inoculation, 10 to 20% of yeast cells had adhered to the monolayer (Fig. 7e). Adherence of *C. albicans* SC5314 increased by 60 min, and this was independent of preculture conditions and corresponded with germ tube formation by *C.*

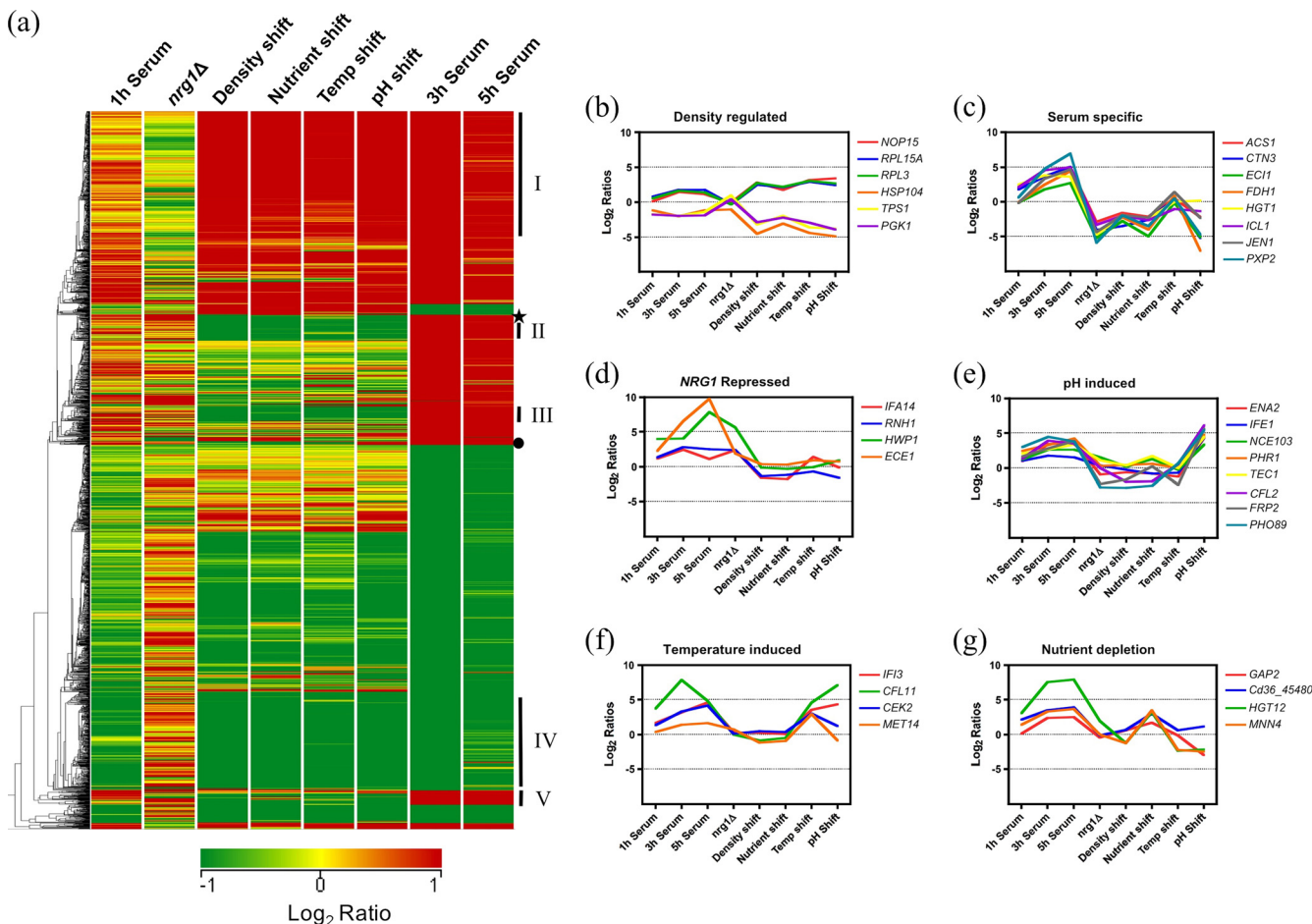


FIG. 6. (a) Hierarchical cluster analysis showing expression patterns of differentially regulated genes in *C. dubliniensis* (ANOVA, $P \leq 0.05$) induced 2-fold or greater in WS medium at 3 h. Clustering was carried out in Genespring GX11 using default hierarchical clustering parameters. Colors refer to \log_2 ratio values as depicted in the bar legend. Conditions include a change in cell density (density shift), a switch to 10% (vol/vol) Lee's medium (nutrient shift), a switch to growth at 37°C (temperature shift), a shift to pH 7.5 (pH shift), or expression in an *nrg1*Δ background. Solid bars to the right (labeled I, II, III, IV, and V) indicate major clusters of coregulated genes (see text). The star shows the location of the major group of *NRG1*-regulated genes, and the circle shows the position of the main pH-regulated group. (b to g). Graphs showing expression plots of representative genes identified from clusters in panel a.

albicans (Fig. 7e). In contrast, only *C. dubliniensis* cells precultured in Lee's medium at 30°C exhibited an increase in adherence over time (Fig. 7e). The difference in adherence at 90 min was highly significant ($P < 0.01$, by two-way ANOVA). An additional *C. dubliniensis* strain, CD36, was also shown to exhibit increased adhesion to TR146 monolayers following preculture in Lee's medium (see Fig. S4 in the supplemental material). In additional experiments, we altered the preculture conditions in order to determine the role of the temperature shift, the pH shift, and the nutrient composition of Lee's medium in this phenotype (Fig. 7f). Preculture at 37°C or at pH 7.2 reduced adhesion by 48% and 35%, respectively (Fig. 7f). The addition of 1% (wt/vol) peptone to the preculture medium also significantly reduced adhesion by 43% ($P < 0.05$, ANOVA). Preculture at 37°C with peptone did not have any significant additive effect on adhesion ($P > 0.05$).

In addition, we have previously observed that *C. dubliniensis* is engulfed and killed more efficiently than *C. albicans* by RAW264.7 murine macrophages (16). This phenotype was associated with the inability of *C. dubliniensis* to filament and

destroy the macrophage. However, preculture of *C. dubliniensis* in Lee's medium at pH 4.5 at 30°C led to an increase in the rate of filamentation following phagocytosis by murine macrophages compared to cells grown in YPD medium at 37°C (Fig. 8). Assessment of candidal growth in coculture with the macrophage cells demonstrated that cells grown Lee's medium at pH 4.5 could proliferate to a significantly greater level than cells grown in YPD medium (Fig. 8a). No difference in proliferation was noted with *C. albicans* cultures pregrown in YPD medium at 37°C or Lee's medium grown at 30°C (data not shown).

DISCUSSION

In our attempts to generate a hyphal transcript profile for *C. dubliniensis*, we initially encountered problems in inducing ~100% hyphal growth in liquid medium with this species. This led us to carry out a thorough investigation of the environmental conditions that favor the yeast-to-hypha transition in *C. dubliniensis* in liquid medium. Nutrient depletion was found to

TABLE 1. Selected genes of *C. albicans* and *C. dubliniensis* commonly upregulated during hypha formation in response to serum^a

Category	GeneDB no.	CGD no. ^b	Fold change ^c		Name	Description ^d
			<i>C. albicans</i>	<i>C. dubliniensis</i>		
Cell surface/secreted	Cd36_43360	orf19.1321	71	48	<i>HWP1</i>	Hyphal wall protein
	Cd36_52240	orf19.4255	5.9	4.8	<i>ECM331</i>	GPI-anchored protein
	Cd36_64370	orf19.5760	4.3	5.0	<i>IHD1</i>	GPI-anchored protein
	Cd36_63420	orf19.5542	75	28*	<i>SAP456</i>	Secreted aspartyl proteinase
	Cd36_43260	orf19.3374	87	52	<i>ECE1</i>	Secreted cell elongation protein
	Cd36_44230	orf19.3829	7.7	8.8	<i>PHR1</i>	GPI-anchored protein
Stress response	Cd36_60850	orf19.85	9.8	9.1	<i>GPX1</i>	Glutathione peroxidase
	Cd36_15620	orf19.2060	11.2	15.9	<i>SOD5</i>	Copper-zinc superoxide dismutase
	Cd36_33470	orf19.3710	8.6	3.4*	<i>YHB5</i>	Protein related to flavohemoglobins
DNA replication	Cd36_23200	orf19.201	3.5	8.0	<i>CDC47</i>	DNA helicase
	Cd36_20640	orf19.5487	5.7	10.8	<i>CDC46</i>	Part of ARS replication complex
	Cd36_21620	orf19.1901	3.3	4.4	<i>MCM3</i>	Part of ARS replication complex
	Cd36_63950	orf19.5597	2.5	4.7	<i>POL5</i>	DNA polymerase V, 5-prime end
	Cd36_41670	orf19.4616	5.2	9.5	<i>POL30</i>	Accessory for DNA polymerase delta
Cytoskeleton	Cd36_03010	orf19.3013	5	4.7	<i>CDC12</i>	Septin
	Cd36_29930	orf19.548	2.5	2.9	<i>CDC10</i>	Septin
	Cd36_11250	orf19.5265	9.5	9.7	<i>KIP4</i>	Kinesin heavy chain homolog
GTPase	Cd36_81390	orf19.1702	14.8	2.8	<i>ARF3</i>	GTP-binding ADP-ribosylation factor
	Cd36_18700	orf19.815	2.7	2.8*	<i>DCK1</i>	DOCK180 protein
	Cd36_71380	orf19.6573	5.3	3.8	<i>BEM2</i>	Bud emergence protein
	Cd36_24270	orf19.1760	2.6	2.2	<i>RAS1</i>	Small monomeric GTPase
	Cd36_84970	orf19.5968	3.4	2.8*	<i>RDI1</i>	Rho GDP dissociation inhibitor
	Cd36_73140	orf19.6705	7.2	11	<i>YEL1</i>	Conserved hypothetical protein
Secretion	Cd36_86230	orf19.7409	3.3	2.1	<i>ERV25</i>	Component of ER- derived vesicles
	Cd36_40670	orf19.4181	4.2	3.3	<i>SPC2</i>	Subunit of signal peptidase complex
	Cd36_72140	orf19.6476	3.0	3.8	<i>AVL9</i>	Conserved Golgi protein
	Cd36_51450	orf19.586	3.9	2.1	<i>ERV46</i>	Component of ER-derived vesicles
Glycosylation	Cd36_07530	orf19.5073	4.3	2.3	<i>DPM1</i>	Dolichol-P-mannose synthesis
	Cd36_60365	orf19.1203.1	9.0	3.3	<i>DPM2</i>	Regulator of dolichol-P-mannose
	Cd36_32420	orf19.1843	2.3	2.3	<i>ALG6</i>	Glucosyltransferase
	Cd36_02340	orf19.2937	8.4	5.5	<i>PMM1</i>	Phosphomannomutase
	Cd36_23720	orf19.1390	3.9	3.7	<i>PMI1</i>	Mannose-6-phosphate isomerase
Transcription factors	Cd36_81290	orf19.1715	5.5	4.9	<i>IRO1</i>	Transcription factor
	Cd36_01290	orf19.3328	3.9	2.0	<i>HOT1</i>	Osmostress transcription factor
	Cd36_05880	orf19.1822	21	4.3	<i>UME6</i>	Regulator of filamentation
Kinases/phosphatases	Cd36_08920	orf19.4809	4.3	4.2	<i>ERG12</i>	Mevalonate kinase
	Cd36_40980	orf19.4698	2.6	4.7	<i>PTC8</i>	Serine/threonine phosphatase
	Cd36_42970	orf19.2678	3.3	2.8	<i>BUB1</i>	Protein kinase in mitosis checkpoint

^a Ribosomal proteins were excluded.

^b CGD, *Candida* Genome Database.

^c Expression relative to yeast cells. *C. albicans* values are taken from the data of Kadosh and Johnson (12) except those marked with an asterisk, which are taken from Nantel et al. (17).

^d ARS, autonomously replicating sequence; ER, endoplasmic reticulum.

be the most important requirement for filamentation of *C. dubliniensis* in liquid medium. Highly efficient filamentation was observed in *C. dubliniensis* when a nutrient-poor inducing medium (water plus 10% [vol/vol] FCS) was used, and this could be suppressed by the addition of peptone and glucose. Although nutrient limitation has been shown to induce hypha formation in *C. albicans* in liquid and solid media, this species still filaments efficiently in nutrient-rich YPD medium in the presence of a shift to alkaline pH at 37°C (5). In *C. dubliniensis*, a shift from YPD medium to nutrient-rich YPDS medium (pH ~7.5) could not induce significant morphological changes. However, filamentation of *C. dubliniensis* was partly induced in

YPDS medium when this species was precultured in synthetic Lee's medium. This Lee's medium induction could also be suppressed by the addition of 1% peptone to the preculture medium. These data indicate that nutrient-sensing mechanisms, specifically those that sense complex mixtures of peptides, may somehow suppress pH- and temperature-induced filamentation in *C. dubliniensis*. We have shown that the mechanism of inhibition involves suppression of *UME6* induction. Addition of peptone to WS medium could inhibit filamentation in *C. dubliniensis* and suppressed *UME6* induction in a concentration-dependent manner. We also observed induction of *NRG1* transcription in *C. dubliniensis* following inoculation

TABLE 2. Genes of *C. albicans* and *C. dubliniensis* commonly downregulated during hypha formation in response to serum

Category	GeneDB no.	CGD no. ^a	Fold change ^b		Common name	Description
			<i>C. albicans</i>	<i>C. dubliniensis</i>		
Cell surface	Cd36_64800	orf19.1097	14.3	7.1	<i>CdALS21</i>	Agglutinin-like sequence protein
	Cd36_65010	orf19.1097	14.3	10.0	<i>CdALS22</i>	Agglutinin-like sequence protein
	Cd36_64610	orf19.4555	7.1	6.7	<i>ALS4</i>	Agglutinin-like sequence protein
	Cd36_26450	orf19.2531	5.0	2.5	<i>CSP37</i>	Cell surface protein
	Cd36_51670	orf19.575	3.8	3.7	<i>HYR5</i>	Similar to <i>HYR1</i>
	Cd36_29770	orf19.532	3.7	3.8	<i>RBR2</i>	Hypothetical protein
	Cd36_43810	orf19.5305	12.5	5.0	<i>RHD3</i>	Conserved protein repressed in hyphal development
	Cd36_22720	orf19.3618	80.0	10.0	<i>YWPI</i>	Putative cell wall protein
	Cd36_23050	orf19.220	25.0	7.1	<i>PIR1</i>	Cell wall structural constituent with tandem repeats
Transport	Cd36_20820	orf19.23	4.0	10.0	<i>RTA3</i>	Putative transporter or flippase upregulated during the acquisition of azole resistance
	Cd36_28130	orf19.2425	5.3	2.4	<i>HGT18</i>	Putative glucose transporter
	Cd36_29200	orf19.473	1.7*	2.6	<i>TPO4</i>	Spermidine transporter
	Cd36_27990	orf19.2849	33.3	2.0	<i>AQY1</i>	Aquaporin
	Cd36_27190	orf19.3749	2.0	4.5	<i>IFC3</i>	Peptide transporter
	Cd36_41090	orf19.4679	5.0	4.5	<i>AGP2</i>	Amino acid permease
	Cd36_71860	orf19.6514	3.0	2.5	<i>CUP9</i>	Copper homeostasis
	Cd36_35530	orf19.7666	2.2	16.7	<i>SEO3</i>	Permease
	Cd36_83640	orf19.6956	5.9	5.3	<i>DAL9</i>	Allantoate permease
Mitochondrial	Cd36_17750	orf19.5805	5.9	12.5	<i>DLD3</i>	Mitochondrial D-lactate ferricytochrome c oxidoreductase
	Cd36_41790	orf19.4602	7.1	4.2	<i>MDH1</i>	Mitochondrial malate dehydrogenase
	Cd36_01500	orf19.3353	9.1	6.3	<i>CIA30</i>	Possible complex I intermediate associated protein
	Cd36_60630	orf19.3656	2.0	2.0	<i>COX15</i>	Cytochrome oxidase assembly factor
Transcription factor	Cd36_84590	orf19.5924	5.6	2.5	<i>ZCF31</i>	Conserved hypothetical protein
	Cd36_12210	orf19.4941	1.7*	2.4	<i>TYE7</i>	Basic helix-loop-helix transcription factor
	Cd36_73890	orf19.7150	2.0	5.3	<i>NRG1</i>	Transcriptional repressor
	Cd36_06830	orf19.4438	33.3	14.3	<i>RME1</i>	Zinc-finger transcription factor
	Cd36_52720	orf19.4318	1.7	3.0	<i>MIG1</i>	Transcriptional regulator
Stress response	Cd36_80290	orf19.5437	1.7*	2.3	<i>RHR2</i>	DL-Glycerol-3-phosphatase
	Cd36_01850	orf19.4526	100.0	33.3	<i>HSP30</i>	Plasma membrane heat shock protein
	Cd36_01930	orf19.3664	9.1	2.6	<i>HSP31</i>	Membrane heat shock protein
	Cd36_10070	orf19.2344	33.3	4.0	<i>ASR1</i>	Similar to heat shock proteins
Glutamate metabolism	Cd36_01650	orf19.4543	10.0	3.0	<i>UGA22</i>	Succinate-semialdehyde dehydrogenase
	Cd36_10950	orf19.1153	5.3	2.9	<i>GAD1</i>	Glutamate decarboxylase
	Cd36_45660	orf19.4716	2.0*	7.1	<i>GDH3</i>	NADP-glutamate dehydrogenase

^a CGD, *Candida* Genome Database.

^b Expression relative to yeast cells. *C. albicans* values are taken from the data of Kadosh and Johnson (12) except values marked by an asterisk, which are taken from Nantel et al. (17).

in YPDS medium, and this may also play a significant role in preventing filamentation in this medium as Saville et al. (22) have shown that induced *NRG1* transcription can prevent filamentation in YPDS medium by *C. albicans*. In previous studies we have hypothesized that the lack of filamentation observed in *C. dubliniensis* in certain media may be due to lack of *NRG1* downregulation (16). However, in the present study, examina-

tion of the *nrg1Δ* mutant in YPDS medium showed that although removal of *Nrg1* repression could enhance filamentation in this medium, the mutant still exhibited pseudohyphal characteristics and exhibited only moderate fluorescence from a *P_{ECE1}-GFP* fusion, suggesting an additional mechanism of nutrient repression (Fig. 3a). Induction of *UME6* expression from a doxycycline-inducible promoter promoted true hypha

TABLE 3. *C. albicans*-specific genes expressed during hyphal development

ORF no.	Common name	Description
orf19.5716	<i>SAP4</i>	Secreted aspartyl proteinase
orf19.5585	<i>SAP5</i>	Secreted aspartyl proteinase
orf19.4975	<i>HYR1</i>	Predicted GPI anchored cell wall protein
orf19.1816	<i>ALS3</i>	ALS family; role in epithelial adhesion, endothelial invasiveness
orf19.7561	<i>EED1</i>	Protein required for filamentous growth and for escape from epithelial cells
orf19.7544	<i>TLO1</i>	Member of a family of telomere-proximal genes
orf19.4054	<i>TLO12</i>	Member of a family of telomere-proximal genes
orf19.7127	<i>TLO16</i>	Member of a family of telomere-proximal genes
orf19.3074	<i>TLO10</i>	Member of a family of telomere-proximal genes

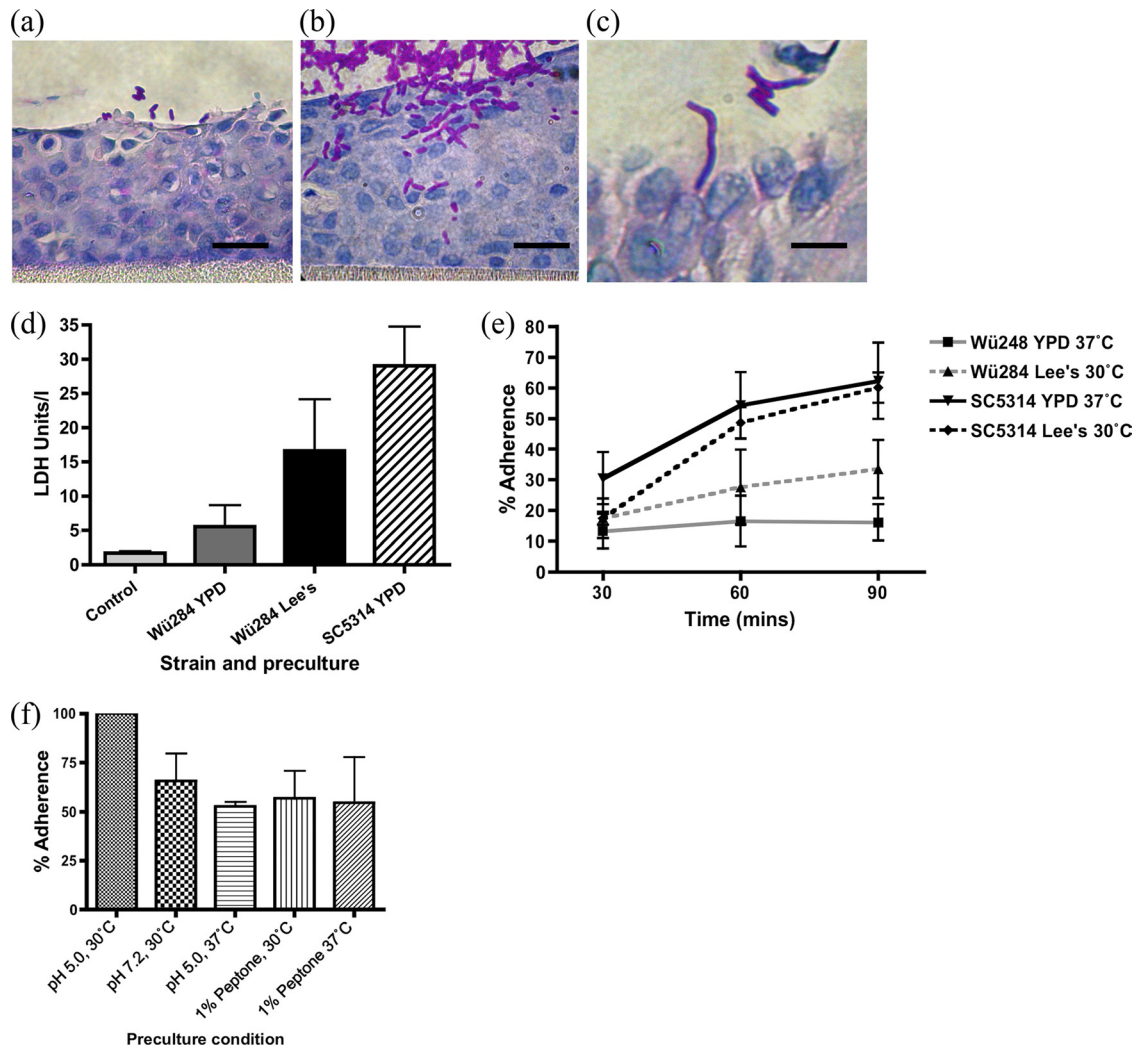


FIG. 7. Interaction of *C. dubliniensis* Wü284 with reconstituted human oral epithelium (RHE) following 24 h of incubation. (a) Photomicrograph of *C. dubliniensis* yeast cells at the surface of RHE following preculture in YPD medium at 37°C. Scale bar, 25 μ m. (b) Localized invasion of the surface of the RHE by *C. dubliniensis* following preculture in Lee's medium at pH 4.5 and 30°C. Scale bar, 25 μ m. (c) High-magnification photomicrograph of a hyphal *C. dubliniensis* cell penetrating the surface of the RHE, following preculture in Lee's medium at pH 4.5 and 30°C. Scale bar, 10 μ m. (d) Damage to the RHE tissues estimated by measurement of lactate dehydrogenase (LDH) release in control (uninfected) tissues, tissues infected with cells pregrown in YPD medium, and tissues infected with cells pregrown in Lee's medium at pH 4.5 after 24 h of incubation. (e) Adherence of *C. dubliniensis* Wü284 and *C. albicans* SC5314 to TR146 monolayers over time. Adherence was determined in cells precultured in YPD medium at 37°C and in Lee's medium at 30°C and is expressed as the percentage of adherent CFU relative to the inoculum. Error bars represent standard deviation from the mean of three replicate experiments. (f) Examination of adherence of *C. dubliniensis* Wü284 precultured in various modifications of Lee's medium, including medium buffered to pH 5.0 and pH 7.2, incubated at 37°C, or supplemented with peptone. Error bars represent standard deviations from the mean of three replicate experiments.

formation in the *nrg1* Δ mutant in YPDS medium (Fig. 3b). In addition, overexpression of *UME6* in the wild-type strain could also induce filamentation in YPDS medium, indicating that differential expression of *UME6* may be the key reason for reduced filamentation of *C. dubliniensis* in these media. In *C. albicans*, it has been shown that *UME6* may also play a role in suppressing *NRG1* transcription during filamentation, and the differential expression of *NRG1* observed in *C. dubliniensis* may also be *UME6* dependent (2, 8). Unexpectedly, constitutive expression of *NRG1* from the doxycycline-inducible promoter could not prevent hypha formation in WS medium, suggesting that high-level *UME6*

expression may also affect *NRG1* function posttranscriptionally.

To further examine the response of *C. dubliniensis* in a nutrient-poor medium, we examined the transcript profile of Wü284 grown in 10% (vol/vol) Lee's medium. Nutrient depletion induced expression of genes involved in amino acid, carbohydrate, and iron uptake (*GAP2*, *HGT12*, and *FET3*). In *C. albicans*, expression of the hexose transporter *HGT12* is induced by glucose limitation, and the glucose sensor Hgt4 mediates this induction (7). In addition, *HGT4* is required for filamentation under some conditions (spider medium) in *C. albicans* (7). However, low glucose stimulation is not essential

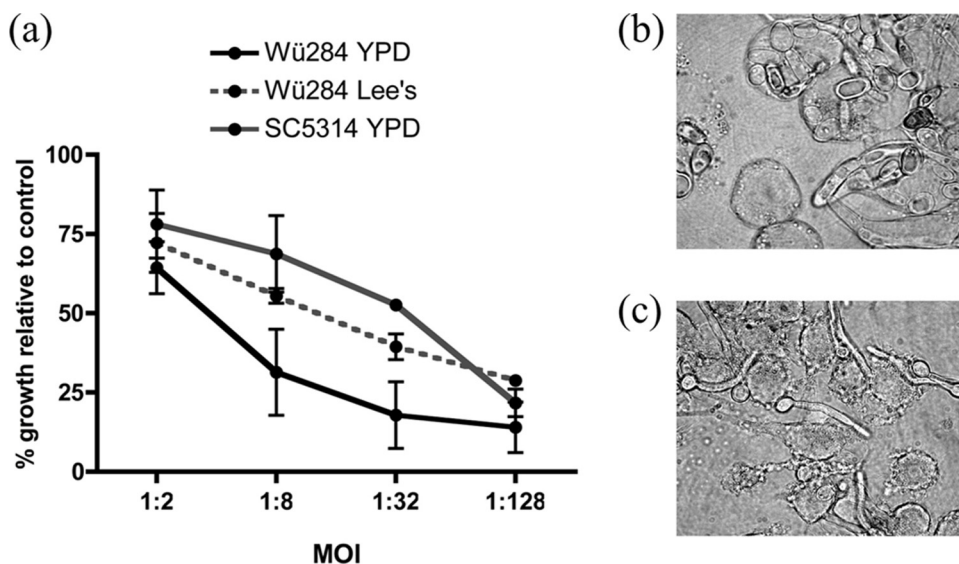


FIG. 8. Survival of *C. dubliniensis* Wü284 following coculture with murine RAW264.7 macrophages. (a) Proliferation of viable *Candida* cells was assayed using an XTT dye reduction assay following 18 h of coculture at several multiplicities of infection (MOI; *Candida* CFU to macrophages). Wü284 cells precultured in Lee's medium at pH 4.5 exhibited significantly greater proliferation at MOIs of 1:8 and 1:32. Morphology of *C. dubliniensis* Wü284 cells grown in YPD medium (b) and in Lee's medium at pH 4.5 (c) following 5 h of incubation with murine RAW264.7 macrophages. Error bars represent standard deviations from the means of three replicate experiments.

for filamentation in *C. albicans* as *HGT4* mutants form filaments normally in glucose-rich YPDS medium (7). It is also unlikely that *HGT4* signaling is required for filamentation of *C. dubliniensis* in WS medium as addition of 2% glucose to WS medium did not significantly inhibit filamentation in *C. dubliniensis*. Repression of filamentation was more apparent when *C. dubliniensis* was exposed to a complex mixture of peptides or whole protein (BSA), indicating that nitrogen-sensing mechanisms may be important. Growth in WS medium was associated with induction of several general amino acid permeases (*GAP2*, *GAP4*, *CAN2*, and *CAN3*), suggesting that this medium is amino acid limiting. However, addition of amino acids to WS medium did not affect filamentation in *C. dubliniensis*, indicating that cell surface amino acid sensors such as *SSY1* may not be involved in repression of filamentous growth (6). However, high concentrations of ammonium sulfate (100 mM) could reduce filamentation. Ammonium sulfate has been shown to inhibit filamentation of *C. albicans* on solid medium via the Mep2 sensor; however, as shown here and previously by Biswas and Morschhauser (4), this effect is not seen in *C. albicans* in the presence of serum. However, ammonium sulfate-mediated repression of filamentation may be serum independent in *C. dubliniensis*. Interestingly, the most repressive effects on filamentation were observed when WS medium was supplemented with a mixture of both peptone and glucose, suggesting that either a combination of sensing mechanisms or possibly a more general nutrient-sensing mechanism is involved. It has recently been shown in *C. albicans* that an orthologue of the general nutrient sensor Tor1 can modulate *NRG1* expression in spider medium (3). In addition, it has also been shown that a *C. albicans* *MDS3* mutant can form hyphae only in the presence of the Tor1 inhibitor rapamycin (30). We are currently assessing whether *C. dubliniensis* Tor1 could play novel role in nutrient sensing and filamentation.

The transcript profiling data presented here also indicate important roles for pH, temperature, and cell density changes in activating the transcription of hypha-specific genes in *C. dubliniensis*. The transcript profiling data presented here show a key role for the pH response in activating the filamentous growth regulators *SFL2*, *UME6*, *TEC1*, and *RIM101* (5, 8). *UME6* was also found to be *NRG1* repressed, whereas *TEC1* also exhibited induction due to cell density changes. Temperature changes also induced *EFH1* and *CPH1*. These data show that induction of filamentation under the conditions examined in *C. dubliniensis* involves multiple environmental signals.

The microarray data presented here highlighted some novel processes regulated during filamentation in *C. dubliniensis* and identified a strong core transcriptional response shared with *C. albicans*. The data show rapid induction of genes involved in regulating polarized growth, including genes involved in actin polymerization, vesicle transport, and septin formation. The data also provide evidence for processes not previously described during the morphological switch. This includes evidence for changes in lipid composition, with a shutdown in transcription of genes involved in sphingolipid synthesis and an increase in fatty acid biosynthesis gene expression. Changes in vacuole function are also indicated with an increase in expression of genes involved in vacuolar biogenesis and inheritance and decreases in expression of vacuolar proteases, suggesting that the vacuole plays a structural rather than metabolic role in hyphae. Comparison of this transcript profile with previously published studies of gene expression in *C. albicans* allowed us to identify a core transcriptional response to filamentation in both species which consists of 132 genes regulated 2-fold or greater (12, 17). This strongly conserved core response supports the hypothesis that a specific program of transcriptional changes may be essential for filamentation to proceed in both species, in addition to posttranscriptional events. Induction of

several secreted and cell wall-associated proteins was specific to *C. albicans* under the conditions examined, and these included *RBT4*, *PGA54*, and *PGA55*. Several species-specific genes were also induced in *C. albicans*, including *HYR1*, *ALS3*, and *EED1*. *C. albicans* expresses three *SAP* genes, *SAP4*, *SAP5*, and *SAP6* during filamentation, whereas *C. dubliniensis* possesses only one orthologue of these genes, termed *CdSAP456*, which is also induced during hyphal growth (11, 21). However, secreted aspartyl proteinase (*SAP*) activity in *C. dubliniensis* may be supplemented by *SAP7* expression, which exhibited an 8-fold increase in expression. *C. albicans* also expresses the putative invasin *ALS3* (19). However, we did not identify any compensatory expression of *ALS* genes in *C. dubliniensis* although orthologues of *C. albicans* *ALS2*, *ALS4*, and *ALS9* all exhibited decreased expression during hyphal growth.

Overall, transcript profiling revealed that *C. dubliniensis* hyphae express a number of genes associated with virulence, suggesting that induction of filamentation in *C. dubliniensis* could promote tissue invasion. Recently, Spiering et al. concluded that the reduced virulence of *C. dubliniensis* in the RHE model was a result of a failure to initiate filamentation and the specific transcriptional program associated with this (24). In the present study we have shown that induction of *UME6* expression in *C. dubliniensis* by preculturing in Lee's medium at 30°C could enhance filamentation in the RHE model. This resulted in greater attachment of *C. dubliniensis* cells to the tissue surface and localized invasion of the epithelium. We have never previously identified RHE invasion in a wild-type strain of *C. dubliniensis* (16, 24, 26). Examination of adhesion of *C. dubliniensis* to TR146 monolayers demonstrated that this adherent phenotype could be partly inhibited by the addition of peptone to the preculture medium, as well as by removing the pH or temperature shift. However, the level of damage to the RHE tissues was still significantly lower than that routinely observed when tissues are infected with *C. albicans*. There may be several reasons for this. First, the transition following Lee's preculture is largely short-lived, and by 24 h most cells have reverted to budding growth. Second, although *C. dubliniensis* can be induced to form hyphae, the absence of several *C. albicans*-specific hypha-associated genes (*ALS3*, *SAP5*, *HYR1*, and *EED1*) may also attenuate the virulence of this species (19, 21, 31). Studies are under way to determine if these genetic differences are crucial to the greater pathogenicity of *C. albicans*.

Finally, this study suggests that the ability of *C. albicans* to form filaments at alkaline pH, irrespective of nutrient availability, may enable it to colonize and infect a wider range of niches relative to *C. dubliniensis*. *C. dubliniensis* may have lost or perhaps failed to acquire this morphological flexibility since the divergence of the two species. The genome sequence of *C. dubliniensis* suggests that, due to gene loss and pseudogenization, *C. dubliniensis* may be undergoing niche specialization. It may be possible that reduced filamentation is part of this specialization process and that it may even be of benefit to *C. dubliniensis* in certain niches, particularly where tissue damage, inflammation, and attraction of the host's defenses is unfavorable.

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