Differential virulence of *Candida albicans* and *C. dubliniensis* 

A role for Tor1 kinase?

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*Candida albicans* and *Candida dubliniensis* are two very closely related species of pathogenic yeast. *C. albicans* is the most prevalent species in the human gastrointestinal tract and is responsible for far more opportunistic infections in comparison with *C. dubliniensis*. This disparity is likely to be due to the reduced ability of *C. dubliniensis* to undergo the yeast to hypha transition, a change in morphology that plays an important role in *C. albicans* virulence.

We have recently shown that hypha formation by *C. dubliniensis* is specifically repressed by nutrients at alkaline pH. In this article, we present new data showing that this can be partly reversed by treatment with rapamycin, an inhibitor of the nutrient sensing kinase Tor1 (Target Of Rapamycin). We also provide a speculative model to describe why *C. albicans* filaments more efficiently in nutrient rich environments, citing recently described data on Mds3, a pH responsive regulator of Tor1 kinase activity.

Several yeast species of the genus *Candida* have evolved to colonize the human gastrointestinal tract. *C. albicans*, the most commonly recovered species, is highly adapted for growth on mucosal surfaces and even minor disturbances in the host’s immunity can lead to superficial infection. Recent comparative genomic studies have identified a range of gene families in *C. albicans* with putative roles in adhesion and nutrient acquisition that may contribute to its greater success in vivo relative to other yeast species. In addition to these factors, the ability of *C. albicans* to undergo a reversible switch from a budding yeast morphology to filamentous hyphal cells is widely regarded as an important contributory factor to its success as a commensal and opportunistic pathogen. This morphological transition (termed morphogenesis) is crucial for virulence and hyphal forms have been shown to be highly adherent and express specific degradative enzymes and adhesins that may contribute to tissue invasion. Both yeast and hyphal cells can be recovered from mucosal surfaces; however, cells restricted to either phase of growth display reduced virulence, demonstrating the requirement for morphogenesis at different stages of infection. The only other member of the genus Candida that forms true hyphae (as opposed to pseudohyphae) is the closely related *C. dubliniensis*. Despite the close relatedness of *C. albicans* and *C. dubliniensis*, epidemiological and virulence data show that there is a dramatic difference in the ability of these organisms to cause disease.

Analysis of the incidence of *C. dubliniensis* in bloodstream infection shows that this organism is responsible for fewer than 2% of systemic *Candida* infections compared with over 60% by *C. albicans*. Data from animal models of infection confirm that *C. dubliniensis* isolates are less virulent than *C. albicans*. In the oral-intragastric murine infection model, *C. dubliniensis* colonized mice less efficiently than *C. albicans* and were less able to establish systemic infection. In addition, mice infected systemically by tail vein inoculation with *C. dubliniensis* have greater survival times relative to *C. albicans* infected mice. Data from both models indicate that *C. dubliniensis* forms fewer

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**Key words**: *Candida albicans*, *Candida dubliniensis*, fungal morphogenesis, TOR, rapamycin

**Abbreviations**: abbreviations

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mRNA families of unknown function such as the also has expansions in gene C. dubliniensis tion, possibly as a result of niche special - tive genomic studies have shown that Although genetically similar, compara- for its reduced capacity to cause disease. proteins. The contribution of these such as form and have putative roles in virulence are expressed exclusively in the hyphal of the species-specific genes of C. dublinien- since its divergence from C. albicans.11 In addition, C. albicans has acquired over 200 genes since its divergence from C. dubliniensis.11 In addition, C. albicans is indicative of reductive evolution, possibly as a result of niche specialization by C. dubliniensis.11 In addition, C. albicans has acquired over 200 genes since its divergence from C. dubliniensis.11 These additional genes are largely the result of gene duplication events. Several of the species-specific genes of C. albicans are expressed exclusively in the hyphal form and have putative roles in virulence such as ALS3, HYR1, SAP5 and SAP6. C. albicans also has expansions in gene families of unknown function such as the telomeric TLO genes and the IFA genes encoding a family of leucine-rich repeat proteins.11 The contribution of these genetic differences to virulence and filamentation are currently under investigation in our laboratory.

The reduced capacity of C. dublini - ensis to form hyphae in vivo has been highlighted in several studies of virulence using murine models and these data have been corroborated by in vitro studies of filamentation in alkaline liquid media (e.g. Lee’s medium, RPMI, serum supplemented broths) where C. dubliniensis generally forms far fewer true hyphae than C. albicans.3,12,13 Genomic comparisons have revealed that all of the major transcriptional regulators and signaling pathways implicated in filamentation at alkaline pH in C. albicans are highly conserved in C. dubliniensis, including the cAMP-protein kinase A pathway and the pH responsive RIM101 pathway (Fig. 1). The first studies to investigate these pathways for a molecular basis for differential morphogenesis focused on differences in the expression of Nrg1, a DNA binding protein that acts in a complex with the transcriptional repressor Tup1 to repress the expres- sion of genes regulating morphogenesis (Fig. 1). Morphogenesis in both species is associated with a reduction in NRG1 transcription. C. dubliniensis forms abun- dant chlamydospores on several nutrient poor media such as Staub medium, in con- trast to C. albicans, which grows solely as yeast cells.14 Staub et al. showed that this enhanced chlamydospore formation by C. dubliniensis on Staub medium was due to

hyphae in vivo, which may partly account for its reduced capacity to cause disease. Although genetically similar, comparative genomic studies have shown that C. dubliniensis has undergone gene loss and extensive pseudogenization relative to C. albicans, indicative of reductive evolution, possibly as a result of niche specialization by C. dubliniensis.11 In addition, C. albicans has acquired over 200 genes since its divergence from C. dubliniensis.11 These additional genes are largely the result of gene duplication events. Several of the species-specific genes of C. albicans are expressed exclusively in the hyphal form and have putative roles in virulence such as ALS3, HYR1, SAP5 and SAP6. C. albicans also has expansions in gene families of unknown function such as the telomeric TLO genes and the IFA genes encoding a family of leucine-rich repeat proteins.11 The contribution of these genetic differences to virulence and filamentation are currently under investigation in our laboratory.

The reduced capacity of C. dublini - ensis to form hyphae in vivo has been highlighted in several studies of virulence using murine models and these data have been corroborated by in vitro studies of filamentation in alkaline liquid media (e.g. Lee’s medium, RPMI, serum supplemented broths) where C. dubliniensis generally forms far fewer true hyphae than C. albicans.3,12,13 Genomic comparisons have revealed that all of the major transcriptional regulators and signaling pathways implicated in filamentation at alkaline pH in C. albicans are highly conserved in C. dubliniensis, including the cAMP-protein kinase A pathway and the pH responsive RIM101 pathway (Fig. 1). The first studies to investigate these pathways for a molecular basis for differential morphogenesis focused on differences in the expression of Nrg1, a DNA binding protein that acts in a complex with the transcriptional repressor Tup1 to repress the expres- sion of genes regulating morphogenesis (Fig. 1). Morphogenesis in both species is associated with a reduction in NRG1 transcription. C. dubliniensis forms abun- dant chlamydospores on several nutrient poor media such as Staub medium, in con- trast to C. albicans, which grows solely as yeast cells.14 Staub et al. showed that this enhanced chlamydospore formation by C. dubliniensis on Staub medium was due to species-specific downregulation of NRG1 transcription.14 Subsequent to this study, we hypothesized that differential NRG1 transcription might also suppress hypha formation by C. dubliniensis, as NRG1 has been shown to repress UME6, a transcription factor required for hyphal extension (Fig. 1).15 However, although deletion of NRG1 in C. dubliniensis could restore fila- ment production under some conditions, the mutant grew mainly as pseudohyphae in liquid media and did not differ in virulence from the wild-type, suggesting that other Nrg1-independent factors are also involved in repressing hypha formation in C. dubliniensis.15 More recently, O’Connor et al.13 carried out a more thorough investiga- tion of the environmental cues involved in differential filament production in C. albicans and C. dubliniensis. This study showed that efficient filamentation could be induced in C. dubliniensis in both wild-type and nrg1Δ cells by incubation in liquid media that were nutrient poor and alkaline (pH 7.0-7.5). For example, whereas C. albicans formed abundant hyphae in standard yeast extract peptone dextrose (YPD) broth supplemented with 10% bovine serum (YPDPS), C. dubliniensis formed only pseudohyphae. However, in water supplemented with 10% bovine serum (WS), C. dubliniensis produced true hyphae at levels equivalent to C. albi- cans in YPDS. The addition of 2% glucose to WS medium had little effect on filamentation in C. dubliniensis, whereas the addition of peptone up to 2% greatly inhibited filamentation (Fig. 1). A combi- nation of glucose and peptone also had a greater effect than peptone alone. These data suggested that nutrient starvation, in particular amino acid limitation, may be required for filamentation in C. dubliniensis.15 Several mechanisms of pep- tide and amino acid sensing have been described in C. albicans. In liquid media, the presence of amino acids such as proline (10 mM) have been shown to stimulate hypha formation via the plasma mem- brane SPS amino acid sensing complex. In contrast, amino acid starvation on nutri- ent poor solid media such as synthetic low ammonium dextrose (SLAD) medium can also stimulate hypha formation in C. albicans. Filamentation under nitrogen starvation conditions requires the activity
of two permeases, the amino acid permease GAP118 and the ammonium permease MEP2,7 which may act as sensors of nitrogen in the environment. Furthermore, in response to amino acid starvation, C. albicans activates the general amino acid control (GCN) response, which is activated by the transcription factor Gcn4.18 In C. albicans, Gcn4 has also been shown to have an additional role in stimulating filamentation via interaction with the cAMP-PKA pathway, leading to activation of the transcription factor Efg1 (Fig. 1). These nitrogen starvation responses are critical for filamentation of C. albicans on nitrogen poor solid media such as SLAD as the addition of ammonium or amino acids acts to repress filamentation. However, the addition of serum to nitrogen-rich media can reverse this inhibition, indicating that the alkaline pH response can override nutrient repression. In contrast, in C. dubliniensis the addition of serum to amino acid rich medium such as YPD is not sufficient to stimulate true hypha formation,13 indicating that perhaps the alkaline pH response alone cannot induce filamentation in nutrient rich environments. This phenotype is unexpected as transcript profiling of C. dubliniensis grown in alkaline medium (e.g. Lee’s medium pH 7.5) shows that this organism possesses a robust alkaline pH response with all of the hallmarks of an active RIM101-mediated transcriptional response.13 However, incubation of C. dubliniensis in WS, a nutrient-poor alkaline medium that promoted filamentation, activated a transcriptional response highly similar to that described during hypha formation in C. albicans, including expression of genes encoding septins, GTPases, DNA replication factors and cell surface proteins. Critically, nutrient depletion in C. dubliniensis resulted in significantly increased transcription of UME6,13 a transcription factor required for hyphal elongation in C. albicans that acts by inducing HGC1, encoding a component of a regulatory kinase complex required for filament extension (Fig. 1).19,20 Addition of peptone to nutrient poor WS medium could sequentially decrease filament formation in C. dubliniensis and this was associated with a sequential decrease in UME6 expression (Fig. 1). Conversely, forced UME6 expression in C. dubliniensis, either from a doxycycline-inducible promoter or by preculturing in peptone-free media could enhance filamentation in nutrient rich environments and increase subsequent adhesion and invasion of epithelial cells.13 These data suggest a clear role for nutrients, specifically complex mixtures of amino acids, in suppressing filamentation at alkaline pH in C. dubliniensis.

Since publication of these data, we hypothesized that a nutrient-responsive mechanism must repress expression of UME6 in C. dubliniensis and that perhaps in C. albicans, this repression can be removed by growth at alkaline pH. Recently, Bastidas et al. showed that the nutrient regulated protein kinase Tor1 plays a novel role in regulating the expression of several cell wall genes and the transcriptional repressor Nrg1.21 Tor1 is a multifunctional kinase conserved throughout the eukaryotic kingdom that coordinates cell growth and morphogenesis in response to nutrient-derived signals.22 In nutrient rich environments, Tor1 activates transcription of genes involved in ribosome biogenesis and glycolysis and represses starvation responses, such as NCR (nitrogen catabolite repressed) genes and GCN responses (Fig. 2).22 Bastidas et al. showed that rapamycin, a specific inhibitor of Tor1, could reduce NRG1 expression in Spider medium and induce extensive aggregation of C. albicans in an adhesin-dependent fashion.21 As Tor1 kinase is activated by nutrients, particularly amino acids, we hypothesized that nutrient activated Tor1 could be involved in repression of filamentation in C. dubliniensis. In order to investigate this, we determined whether inhibition of Tor1 by rapamycin could enhance filamentation in C. dubliniensis. Addition of 20 nM rapamycin to YPDS at 37°C significantly enhanced filamentation in C. dubliniensis Wü284 at 3–5 h post-incubation compared to solvent (DMSO) treated cultures (2-way ANOVA, p < 0.001; Fig. 3A). The effect of rapamycin was greatest at 3 h post inoculation, with the production of filaments by 40% (± 6.7 standard deviation) of cells compared to 9.7% (± 1.9) of those incubated with DMSO alone (Fig. 3A). Rapamycin had no significant effect on C. albicans isolates in YPDS; however these isolates produced abundant filaments in the absence of rapamycin (data not shown). In total, 12 C. dubliniensis isolates examined exhibited an increase in filamentation in response to rapamycin (data not shown). The addition of rapamycin (20 nM) also reversed the inhibitory effects of peptone.
and glucose on filamentation in C. dubliniensis incubated in 10% serum (data not shown). These data indicate that Tor1 may play a role as a sensor of nutrient rich conditions that acts to inhibit filamentation in C. dubliniensis. We investigated whether rapamycin could mediate similar effects on NRG1 or UME6 transcription in C. dubliniensis, thereby promoting filamentation in YPDS. Exposure of C. dubliniensis to rapamycin in YPDS prevented induction of NRG1 transcription normally observed in YPDS and in addition, we observed a -2-fold increase in UME6 expression (Fig. 3D). The effect of reducing NRG1 transcription and boosting UME6 expression may provide a mechanism whereby some cells in the population can undergo the yeast to hypha switch.

These preliminary data suggest that Tor1 kinase is at least partly responsible for nutrient repression of filamentation in C. dubliniensis. As the Tor1 kinase pathway is highly conserved in fungi, it is difficult to speculate why Tor1 activation does not also repress filamentation in C. albicans. It appears that C. albicans forms filaments in media containing serum, irrespective of nutrient availability. The addition of serum to growth medium renders the pH of that medium alkaline, suggesting that perhaps C. albicans can repress Tor1 functions at alkaline pH. The mechanism for this could involve a recently described pH response pathway in C. albicans that acts in parallel to the traditional Rim101 pH response pathway pathway.23 Davis et al. first identified MDS3 in C. albicans as a novel pH response regulator with roles in morphogenesis, biofilm formation and chlamydospore formation.24 The S. cerevisiae ortholog of MDS3 has roles in induction of sporulation at alkaline pH.25 Subsequently, Zacchi et al. showed that a C. albicans mds3Δ mutant, like C. dubliniensis, displays greatly reduced filamentation in alkaline liquid media (M199).23 In addition, deletion of Mds3 results in a Tor ‘hyperactive’ transcriptional response, with decreased expression of starvation response genes such as GAP2 and the NCR response. Treatment of the mds3Δ mutant with the Tor1 inhibitor rapamycin could initiate starvation responses and restore filamentation in -45% of the mds3Δ cells, similar to the level of restoration induced by rapamycin in C. dubliniensis. These data show that active Tor1 can inhibit filamentation and that Mds3 is required to reverse this inhibition at alkaline pH. Zacchi et al. have also shown that Mds3 modulates Tor1 activities via interaction with a phosphatase, Sit4, positioned downstream of Tor1 in the TOR pathway (Fig. 2).23 In C. albicans Sit4 has been shown to be required for filament extension and stress responses in addition to starvation responses. Under nutrient rich conditions, Tor1 represses starvation responses by inactivating Sit4 (Fig. 2). Zacchi et al. have shown that mds3Δ and sit4Δ mutants are phenotypically similar and exhibit similar transcriptional changes indicative of Tor1 hyperactivity. In addition, Mds3 was shown to physically interact with Sit4, thereby providing the first evidence of a link between the pH response and Tor1 activity in C. albicans.23 As described, deletion of MDS3 prevented filamentation in alkaline M199 and prevented activation of Sit4 mediated starvation responses. Therefore, in alkaline hypha-inducing media, Mds3 may activate Sit4 to promote filamentation and this activation may occur irrespective of nutrient availability and Tor1 repression (Fig. 2). Zacchi et al. speculate that due to the presence of several Kelch repeats that are involved in protein-protein interactions, Mds3 may act as a scaffold to facilitate interactions between TOR pathway members.23 It is not clear how Sit4 may activate filamentous growth; however it could involve interaction with the GCN response regulator Gcn2. In S. cerevisiae, amino acid starvation results in activation of Sit4, which activates the GCN regulator Gcn2, leading to enhanced translation of GCN4.26 As described previously, C. albicans GCN4 is also involved in activating filamentous growth. In C. albicans, Gcn2 has also been shown to increase GCN4 transcript levels, providing
a possible mechanism for Sit4 regulation of filamentous growth (Fig. 2).27

Due to the inability of C. dubliniensis to filament at alkaline pH in nutrient rich conditions and the ability to rescue this phenotype with rapamycin (similar to mds3Δ) it is our hypothesis that signaling of alkaline pH signals via the Mds3/Sit4 complex is somehow defective. Alternatively, Tor1 may simply have greater kinase activity or perhaps a lower activation threshold in C. dubliniensis relative to C. albicans, which acts to repress downstream activities like filamentation. Interestingly, defective Sit4 in C. albicans is also associated with increased sensitivity to NaCl and high temperatures, both of which are characteristic traits of C. dubliniensis. Inspection of the orthologous Sit4 and MDS3 genes in C. dubliniensis indicates high levels of homology in the encoded proteins. Experiments are planned to determine whether C. dubliniensis Sit4 and Mds3 are functionally equivalent to their C. albicans orthologs. However, this phenotype may be due to loss or mutation of another protein in the Mds3-Sit4 complex. Several genes encoding putative filamentous growth regulators (FGRs) are predicted to be pseudogenes in C. dubliniensis and may account for this phenotypic disparity.11

It is difficult to speculate at this stage why these species have evolved such different responses to nutrients and pH. Certainly, the ability of C. albicans to filament at alkaline pH, irrespective of nutrients may allow this organism to colonize a wider range of niches relative to C. dubliniensis. The reduced virulence of C. dubliniensis suggests that nutrient concentrations in its in vivo niche may be sufficient to supress filamentous growth in this organism, which may account for the attenuated virulence of C. dubliniensis in the murine infection model. Although this morphogenetic defect may result in a reduced capacity to cause disease, loss of filamentous growth under some conditions could be part of a specialization process that may be advantageous in specific niches.

For example, filamentation and cellular invasion are likely to trigger inflammatory responses and attract immune cells, and perhaps C. dubliniensis has evolved to be predominantly non-filamentous in vivo to avoid triggering these responses. Although this may have restricted the range of niches available to C. dubliniensis, it may have allowed successful colonization of new niche(s) that are under close surveillance by the immune system.

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