Lower filamentation rates of *Candida dubliniensis* contribute to its lower virulence in comparison with *Candida albicans*

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Abstract

Candida albicans and *Candida dubliniensis* are very closely related yeast species. In this study, we have conducted a thorough comparison of the ability of the two species to produce hyphae and their virulence in two infection models. Under all induction conditions tested *C. albicans* consistently produced hyphae more efficiently than *C. dubliniensis*. In the oral reconstituted human epithelial model, *C. dubliniensis* isolates grew exclusively in the yeast form, while the *C. albicans* strains produced abundant hyphae that invaded and caused significant damage to the epithelial tissue. In the oral-intragastric infant mouse infection model, *C. dubliniensis* strains were more rapidly cleared from the gastrointestinal tract than *C. albicans*. Immunosupression of *Candida*-infected mice caused dissemination to internal organs by both species, but *C. albicans* was found to be far more effective at dissemination than *C. dubliniensis*. These data suggest that a major reason for the comparatively low virulence of *C. dubliniensis* is its lower capacity to produce hyphae.

Keywords: *Candida albicans, Candida dubliniensis*, hyphae, morphogenesis, infection models, virulence

1. Introduction.

Candida albicans is routinely reported as the most common cause of superficial and systemic candidiasis, indicating that it is the most pathogenic *Candida* species (Edmond et al, 1999; Eggimann and Pittet, 2001; Garber, 2001; Asmundsdottir et al., 2002; Ellis, 2002, Kibbler et al, 2003). *Candida dubliniensis,* which was first identified as a separate species in 1995, is the most closely related species to *C. albicans* (Sullivan et al, 1995). Due to this close phylogenetic relatedness the two species are phenotypically very similar and exhibit a similar range of putative virulence factors (Gilfillan et al, 1998; Hannula et al., 2000; Vilela et al., 2002). In particular, *C. dubliniensis* has the capacity to produce hyphae, pseudohyphae and chlamydospores, and thus like *C. albicans*, this species is polymorphic in nature (Gilfillan et al., 1998). Interestingly, despite the fact that the two species are so genotypically and phenotypically similar, one clear difference between them is their capacity to cause disease. *C. dubliniensis* is only rarely identified as a cause of systemic infection (Kibbler et al., 2003; Sullivan et al., 2004), suggesting that *C. dubliniensis* may be less virulent than *C. albicans.*

In a previously published comparison of the phylogeny and virulence factors expressed by *C. albicans* and *C. dubliniensis*, Gilfillan et al (1998) investigated the ability of the two species to produce hyphae, a trait widely regarded as one of the most important virulence factors of *C. albicans* (Gilfillan et al., 1998). This study, which was limited to two strains of each species and to a limited range of experimental conditions conducive to the induction of hyphae by *C. albicans,* revealed that while *C. dubliniensis* has the capacity to produce true hyphae, it appears that the hyphae are produced at a slower rate (Gilfillan et al., 1998). Since this original study a far greater range of *C.*

dubliniensis isolates from various clinical sources have been identified. Population analysis using DNA fingerprinting has also revealed that *C. dubliniensis* is comprised of four distinct genotypes (Gee et al., 2002). Due to the importance of hyphae in candidal virulence, in the current study we have compared the dynamics and the levels of hypha-induction in a comprehensive range of *C. dubliniensis* isolates (representative of the four known genotypes and from a wide range of anatomic sites) with the production of hyphae by *C. albicans* under a broad range of in vitro hypha-induction conditions and in two infection models.

The most commonly used infection model to investigate candidal virulence is the murine model for systemic infection, in which the inoculum is administered directly into the mouse's blood stream by injection into the tail vein (Ghannoum et al, 1995; Lo et al., 1997; Navarro-Garcia et al., 2001; Brand et al, 2004; MacCallum and Odds, 2005). Two studies have already investigated the comparative virulence of *C. albicans* and *C. dubliniensis* using this model (Gilfillan et al, 1998; Vilela et al., 2002). The data obtained in both of these studies indicate that *C. albicans* is significantly more pathogenic than *C. dubliniensis*, reflecting the higher incidence of *C. albicans* in human systemic disease. However, this infection model bypasses the normal barrier to infection provided by human gastrointestinal epithelial tissue. The gastrointestinal tract in up to 80% of humans is colonized by *Candida* species and these organisms may act as a reservoir for systemic infection, especially in patients with neutropenia. Microorganisms colonizing the gut can traverse the gut wall and enter the blood stream, leading to dissemination of the yeasts throughout the body (Odds, 1988; Bougnoux et al., 2006). For this reason we have compared the gastrointestinal colonization and dissemination of the two species in the

oral-intragastric infant mouse infection model (Cole et al., 1990; 1993;1996). The advantages associated with this model include the ability to precisely control inoculum size and the maintenance of the natural host barriers to infection, such as the gut wall and gastric and intestinal secretions. In this study we have compared the ability of *C. albicans* and *C. dubliniensis* in single and in mixed species inocula to colonize the mouse gastrointestinal tract and to disseminate to internal organs following immunocompromization. In order to enhance our ability to accurately enumerate the proportions of each species present at specific time points in mixed inoculum experiments representative strains of each species were tagged with a molecular cassette containing genes encoding mycophenolic acid resistance (MPA^R) and green fluorescent protein (GFP).

In order to further investigate the comparative ability of *C. albicans* and *C. dubliniensis* isolates to colonize and infect invade human epithelial tissues we investigated the effects of each species on oral epithelial tissues using the ex vivo Reconstituted Human Epithelial model (RHE). This model entails the use of commercially available tissue samples obtained by culturing the TR146 human keratinocyte cell line (derived from a buccal mucosal squamous cell carcinoma) on an inert supporting membrane (Rupniak et al., 1985). These tissues, which superficially resemble human oral epithelium, have proved useful in investigating the virulence of *C. albicans*, due to their standardization and high potential throughput and the potential for direct microscopic observation of the interaction between the candidal and host cells (Korting et al., 1998; Schaller et al., 1998a,b; Korting et al., 1999; Korting et al., 2003, Schaller et al., 2004; Zhao et al., 2004; Jayatilake et al., 2005, Zhao et al., 2005).

2. Materials and Methods

2.1. Candida clinical isolates, strains and derivatives

All *Candida* clinical isolates, strains and derivatives were routinely cultured on Potato Dextrose Agar (PDA) medium (Oxoid, Basingstoke, Hampshire, UK) pH 5.6, at 37°C for 18 h (Table 1). For routine liquid culture, isolates were grown in Yeast-Extract-Peptone-Dextrose (YPD) broth (10 g yeast extract (Oxoid), 20 g peptone (Difco, Becton Dickinson, Franklin Lakes, NJ, USA), 20 g glucose per liter, pH 5.5) at 37°C in a Gallenkamp (Model G25) orbital incubator (New Brunswick Scientific Company Incorporated, Edison, New Jersey, USA) at 200 rpm.

2.2. Chemicals, enzymes and radioisotopes

All chemicals used were of analytical-grade or molecular biology-grade and were purchased from the Sigma-Aldrich Chemical Co. (Tallaght, Dublin, Ireland) or from Roche Diagnostics Ltd. (Lewes, East Sussex, UK). Restriction enzymes were purchased from the Promega Corporation (Madison, Wisconsin, USA) and from New England Biolabs (Beverley, Massachusetts, USA) and used according to the manufacturer's instructions. [α-^{32}P]dATP (6,000 Ci/mmol; 222 TBq/mmol) was purchased from Amersham International Plc. (Little Chalfont, Buckinghamshire, UK).

2.3. Induction of germ tubes and hyphae

Production of hyphae by *Candida* isolates was induced by inoculation of cells grown for 18 h at 37°C in YPD into media that promote the yeast-hypha transition. The media used

included Medium-199, with and without 10% (v/v) newborn calf serum (v/v), RPMI-1640 medium, with and without 10% (v/v) newborn calf serum, YNB medium, with and without 10% (v/v) newborn calf serum, water with 10% (v/v) newborn calf serum, Lee's medium with pH/temperature shift (Buffo et al., 1984) and N-acetylglucosamine-yeast-nitrogen base-proline medium (NYP) medium (Schaude et al., 1990). Cells were inoculated to a cell density of 2 x 10^6 cfu/ml in 20 ml of each medium and incubated at 37°C. In the case of NYP medium the cells were incubated in the presence of 5% (v/v) CO_2. The percentage of cells that produced germ tubes or hyphae was determined by counting one hundred cells every hour from t=0 h to t=6 h and then again at t=24 h by microscopic examination using a Nikon Eclipse 600 microscope (Nikon Europe B.V., Badhoevedorp, Holland). All experiments were performed in triplicate.

2.4. Cloning of HWP1

PCR amplimers of *CdHWP1* and *CaHWP1* were cloned into pBluescript II KS (-) by standard procedures and were sequenced and subsequently used as probes for Northern blot analysis.

2.5. DNA isolation and Southern hybridization analysis

Total genomic DNA of *C. dubliniensis* and *C. albicans* isolates and derivatives was prepared from cells grown for 18 h in YPD broth culture, as described by Gallagher et al., 1992. Southern hybridization was carried out at high stringency using DNA probes labeled with [α-^{32}P]dATP by random primer labeling using the Prime-A-Gene kit (Promega) (Gallagher et al., 1992).

2.6. RNA extraction and Northern hybridization analysis

Candida cells were grown overnight in 50 ml YPD medium and washed three times in ultra pure water and then inoculated at a concentration of 2 x 10^6 cells/ml into 200 ml of hyphal inducing media and harvested at t = 0,1,2,3,4,5 and 6 h. Cells were harvested by vacuum filtration with Supor®-450 membrane filters with a pore size of 0.45 μm (Pall Gelman laboratory, Ann Arbor, MI, USA). The cells were scraped from the filter using a scalpel and placed in a 1.5 ml screw capped tube with 1 ml of TRIreagent and 0.1 g of acid washed glass beads. Extractions were carried out by the glass bead disruption method described by Hube et al., 1994. RNA electrophoresis and Northern hybridization with [α-^{32}P]dATP labelled probes was carried out as described by Moran et al., 1998.

2.7. Construction and chromosomal integration of a GFP/MPA^R expression cassette into the RP10 locus in C. albicans and C. dubliniensis

Firstly, a 2.9 kb *Xba*I fragment from the MPA-flipper cassette (Wirsching et al., 2000; Staib et al., 2001), containing the entire mycophenolic acid resistance gene (*IMH3*) and its promoter was used to replace the *URA3* gene in the integrating plasmid CIp10 (Murad et al., 2000) to generate CdIp1. This plasmid encodes the *C. albicans RP10* gene and allows direct integration into the RP10 locus in both *C. albicans* and *C. dubliniensis*. In order to construct a GFP expression cassette for each species, the yeast optimized *GFP* gene was PCR amplified from plasmid pYGFP3 (Cormack et al., 1997) with the primer pair GFPF/GFPR (Table 2) and cloned between the *Sal*I and *Hin*dIII recognition

sequences of CdIp1. In order to obtain constitutive expression of the *GFP* gene, the *CaACT1* promoter region (positions -1019 to -7) was amplified from *C. albicans* SC5314 by PCR with primers ACTPF/ACTPR (Table 2) and inserted upstream of the *GFP* gene between the *Xho*I/*Sal*I recognition sites. The *CaACT1* terminator region (positions +1789 to + 2182 with respect to the *CaACT1* start codon) was amplified from SC5314 by PCR with primers ACTTF/ACTTR (Table 2), and cloned in the *Eco*RI site to create plasmid pCC1.

Transformation of *C. albicans* and *C. dubliniensis* with pCC1 linearized with *Stu*I (within the *RP10* region) was performed by electroporation (Kohler et al., 1997). The *CaRP10* locus was found to be sufficiently homologous to the *C. dubliniensis* homolog to allow integration of the cassette into the *C. dubliniensis* genome as well as that of *C. albicans*. Integrative transformation into the *RP10* locus in each species was confirmed by PCR and Southern hybridization. Amplification reactions were carried out using standard conditions and *Taq* DNA polymerase (Promega). The primers used were MPA1 (which anneals within the cassette) and TEM1 (which anneals downstream of the *RP10* gene in the *C. albicans* and *C. dubliniensis* genomes (Table 2)). Integration of the cassette into the *RP10* locus was identified by the presence of a 1.5 kb PCR product (data not shown). Correct integration of the cassette was further verified by Southern hybridization analysis of *Hin*dIII-digested genomic DNA using the *RP10* gene as a probe (data not shown). *C. albicans* and *C. dubliniensis* derivatives tagged with this cassette are denoted by the abbreviation (M/G) after the strain name.

To confirm that the molecular tagging of strains with the MPA^R/GFP cassette (M/G) had no effect on growth, the growth rate of parent strains and their tagged

derivatives (e.g. SC5314 vs SC5314 (M/G) and CD36 vs CD36 M/G) was compared. No significant difference was observed in the growth of any of the untagged vs tagged pairs investigated (data not shown), indicating that insertion of the cassette into the genome of either species had no detectable effect on growth rate.

Fluorescence of GFP was visualized using a Nikon Eclipse 600 microscope fitted with a super high power mercury lamp (Nikon) and the GFP-specific filter set (Endow GFP Bandpass Emission (FGP®-BP) filter combination).

2.8. Reconstituted Human Epithelium (RHE)

Reconstituted human oral epithelial tissues were supplied by Skinethic Laboratory (Nice, France) and used as described previously (Schaller et al., 1998a,b, 1999). Triplicate infection experiments were performed for each strain and each time point. Reconstituted epithelium samples (0.5 cm^2) were inoculated with 2 x 10^6 yeast cells in 50 µl of PBS and controls were inoculated with 50 µl of PBS. Inoculated cultures were incubated at 37°C with 5% (v/v) CO_2 at 100% humidity for 12, 24, 36, 48, 60 and 72 h.

The release of lactate dehydrogenase (LDH) from epithelial cells into the surrounding medium was measured to quantify the extent of epithelial cell damage. The CytoTox 96® non-radioactive cytotoxicity asssay (Promega) was used to measure the amount of LDH in each sample. The reaction was assayed at 480 nm using a Genios plate reader (Tecan UK Ltd., Reading, UK). One unit of LDH activity is equivalent to 1 mmol of formazan formed min/l.

Prior to sectioning and staining for light microscopy, RHE tissues were fixed in 4% (v/v) paraformaldehyde in PBS (pH 7.4), dehydrated in ethanol and embedded in

paraffin wax. Sections were stained with Periodic Acid Schiff (PAS) reagent for visualization of fungal elements (Luna, 1968). Tissues were examined using a Nikon Eclipse 600 microscope.

2.9. The oral intragastric infant mouse infection model

Crl:CFW(SW)BR mice obtained from Charles River Laboratories (Wilmington, MA, USA), were used to establish a breeding colony and the offspring of these animals were used in all experiments. Infant mice (6-9 days old) derived from these animals were isolated from their mothers 3-4 h before inoculation and held at room temperature to enable clearance of the mothers milk from the infant stomach. Animal husbandry and research were conducted in accordance with approved institutional protocols.

Inocula containing 2.0 x 10^8 yeast were prepared in nonpyrogenic saline (Travenol; Travenol laboratories Inc., Deerfield, ILL. USA) and delivered by the oral-intragastric route with a 24-gauge feeding needle attached to a 1.0 ml syringe (Popper and sons, Rhode Island, USA). In co-infection experiments 1.0 x 10^8 cfu of each species was used in the inocula. After day 10 post infection mice infected by oral intra-gastric inoculation were examined for the presence of *Candida* in their faecal pellets, which were homogenized in 1.0 ml of chilled, sterile saline and plated on SDA containing 50 μg/ml chloramphenicol (Sigma-Aldrich). Mice used in the colonization experiments were not immunocompromised in any way and were simply inoculated and then sacrificed every two days over a period of 10 days.

To investigate dissemination of the yeasts, on day 11, animals with *Candida*-positive fecal pellets were immunocompromised with an intraperitoneal injection of

cyclophosphamide (Mead Johnson, BMS Co.; New Jersey, USA) 0.2 mg/ml plus 1.25 mg/ml cortisone acetate (Westward Pharmaceutical Corporation, New Jersey USA), followed by a second administration of cyclophosphamide (0.1 mg/ml) and cortisone acetate (1.25 mg/ml) on day 14 post-challenge. Immunocompromised animals were sacrificed at 20-24 days post challenge by asphyxiation with CO_2. Mice were sacrificed earlier if they were unresponsive. Mice were immediately dissected to remove the entire gastrointestinal tract and along with the liver and kidneys were prepared for homogenization and plating, or for histological studies.

The stomach, intestine, liver and kidney were examined for the presence of abscesses and homogenized, the enumeration of cfu of *Candida* was determined by dilution plating on YNB agar (Sigma-Aldrich) containing chloramphenicol (50 μg/ml). When differentiation was necessary between tagged and untagged strains the level of each species was determined by replica plating on YNB agar containing chloramphenicol (50 μg/ml) and MPA (0.01 mg/ml).

Selected organs were chemically fixed for 12 h at 4°C in 3% (v/v) glutaraledyhyde and 2% (v/v) paraformaldeyhyde, each prepared separately in cacodylate buffer (0.1 M, pH 7.4) and mixed just before use. The tissues were rinsed in buffer, post fixed in 2% osmium tetroxide (2 h) prepared in the same buffer, dehydrated and embedded in Spurr's low viscosity resin as described previously (Seshan and Cole, 1994). Thick sections (approximately 1 μm) were stained with azure 11-methylene blue for light microscopy as reported in Cole et al., 1989. Smears of organs were also examined in the absence of any fixative. Organs for histological examination were examined with a Zeiss Photomicroscope II. Fluorescence of GFP was detected using a FITC filter.

2.10. Statistical analysis.

The data from the animal models were compared using the nonparametric Mann-Whitney U test. All other statistical analysis was carried out using the two sample *t*-test assuming unequal variances. *P* values of <0.05 were considered significant. Statistical comparisons were performed using SPSS version 9.0 software package for Windows.

3. Results

3.1. Induction of hyphae

The results of a previous study based on two strains of *C. dubliniensis* and a limited range of induction conditions, had suggested that *C. albicans* and *C. dubliniensis* may differ in their capacity to produce hyphae (Gilfillan et al., 1998). To investigate this more comprehensively, in the present study we included a greater number of *C. dubliniensis* isolates (i.e. 11) representative of all four *C. dubliniensis* genotypes recovered from a wide range of anatomic sites from patients from around the world. In addition we compared the induction of hyphae by each strain under a wider range of conditions that are known to induce the production of hyphae in *C. albicans*. In all of the nine experimental conditions examined hyphal production was less efficient in *C. dubliniensis* than in *C. albicans* and for some strains of *C. dubliniensis* no significant hyphal production (<10%) was detected at all (see Table 3). Representative graphs showing the dynamics of hyphal induction by each species when incubated under four induction conditions are shown in Fig. 1. By far the most effective means of inducing hyphae in *C. dubliniensis* was incubation in water and 10% (v/v) serum. Under these conditions 8/11 (73%) *C. dubliniensis* strains tested produced significant amounts of hyphae (i.e. 50% or more of cells were in the hyphal phase). In all of the media tested, the efficiency of induction of hyphae by *C. dubliniensis* varied from strain to strain. Under many of the conditions tested some strains failed to produce hyphae at all, while one strain, P7718, showed relatively high levels of induction under all conditions, although the levels produced were always less than those produced by *C. albicans*. Northern

hybridization analysis using the hypha-specific *HWP1* gene as a probe confirmed that the hyphae produced by *C. dubliniensis* expressed a gene which has previously been associated with true hyphal formation in *C. albicans*, in addition the level of expression of this gene in *C. dubliniensis* correlated precisely with the levels of hyphae observed at each timepoint (data not shown).

3.2. Reconstituted Human Epithelial infection model

Reconstituted human epithelial (RHE) cells have been used widely as a model of oropharyngeal candidiasis (Korting et al., 1998; Schaller et al., 1998a,b; Korting et al., 1999; Schaller et al., 1999; Korting et al., 2003; Zhao et al., 2004; Jayatilake et al., 2005; Zhao et al., 2005). In order to compare the ability of *C. albicans* and *C. dubliniensis* to colonize and infect these tissues, RHE samples were inoculated with *C. albicans* strains SC5314 and 132A and *C. dubliniensis* strains CD36, CD519 and P7718. *Candida dubliniensis* strains P7718 and CD519 were chosen because they belong to genotypes 3 and 4 and are distantly related to the genotype 1 reference strain CD36.

In RHE samples infected with *C. albicans*, extensive growth (of both yeasts and hyphae) was evident by 12 h post-inoculation. Between 12 and 24 h post-infection, the RHE showed signs of tissue damage, characterized by vacuolization and detachment of the keratinocytes in all cell layers. Invasion of deeper parts of the epithelium by *C. albicans* was observed after 24 h, whereby hyphal cells had penetrated through the epithelium and made contact with the inert supporting membrane. These results are in agreement with the findings reported by Schaller et al., 1998a. At 48 h post-inoculation, for RHE colonized by *C. albicans* SC5314, very few epithelial cells were left attached to

the membrane (Fig. 2, panel A). Similar results were also found for *C. albicans* 132A, although more yeast cells were observed than with SC5314 (data not shown). In contrast, inoculation of RHE tissues with *C. dubliniensis* CD36 resulted in major differences in candidal morphology and tissue damage. At all time points examined *C. dubliniensis* was found to grow exclusively as yeasts (data for 48 h are shown in Fig. 2, panel B), although a very small number of hyphae or pseudohyphae were present in a small minority of sections examined. By 12 h, RHE that had been inoculated with *C. dubliniensis* showed no oedema and very few yeast cells attached directly to the epithelium. After 24 h slight tissue damage was evident as some epithelial cells in the top layer appeared to be detached, however the lack of hyphal invasion by *C. dubliniensis* meant that the integrity of the tissue appeared to be largely in tact. However, by 36 h obvious holes were visible within the epithelial layers. At 48 a layer of epithelial cells was still visible over the supporting membrane, in contrast to *C. albicans*-infected tissue at these time points which showed little or no epithelial cells still visible attached to the membrane (Fig. 2). Another difference between the two species was the comparatively poor adhesion of the *C. dubliniensis* cells to the RHE. At each timepoint the *C. dubliniensis* yeast cells were easily dislodged from the tissue surface by shaking or during the fixation/staining process. Similar results were also found for the two other strains of *C. dubliniensis* tested (CD519 and P7718; data not shown).

An estimation of the degree of damage caused by each of the *Candida* strains was also assessed by measuring the levels of the enzyme lactate dehydrogenase (LDH) released by the RHE into the culture medium at 12, 24, 36 and 48 (Fig. 3). Up to and including the 36 h timepoint the level of LDH released from the tissue infected with

tissue invasive *C. albicans* hyphae was significantly higher than that observed with the apparently non-invasive *C. dubliniensis* yeast cells ($P < 0.05$). However, by 48 h the differences between the two species were not significant($P > 0.05$).

3.3. Colonization of the stomach and intestine in the oral-intragastric infant mouse infection model

Candida species are commonly found as commensals in the gastrointestinal tracts of normal healthy individuals and it has been proposed that these organisms may serve as a reservoir for systemic infection when the immune system and/or gut wall are compromised (Cole et al., 1989). To compare the ability of *C. albicans* and *C. dubliniensis* to colonize and infect gastrointestinal tissue we compared the growth of each species using wild type strains and strains tagged with the (M/G) cassette in the infant mouse oral-intragastric infection model (Cole et al., 1989;1990;1993;1995).

When mice were inoculated with either *C. albicans* or *C. dubliniensis* alone there was no significant difference between the levels of *C. albicans* and *C. dubliniensis* recovered from the stomach at days 2 to 6. However at day 8 and day 10 there was no *C. dubliniensis* detectable, whereas the numbers of *C. albicans* remained high (Table 4). Data from colony counts obtained from the intestine show that the levels of *C. dubliniensis* present at days 2-8 were lower than *C. albicans* and decreased during the time course of the experiment until practically no counts were observed at day 10 (Table 4). Faecal pellet counts were not obtained from any of these mice as pellets were not produced by the mice prior to day 10 as they were still being fed by their mothers.

Since *C. albicans* and *C. dubliniensis* are often found in mixed culture in the oral cavity (Sullivan and Coleman, 1998) we investigated the dynamics of co-infection by performing experiments using mixed inocula (containing 1 x 10^8 cfu of each species) in competition experiments in which one or other species was tagged with the GFP/MPA^R expression cassette. As shown in Table 5, when inoculated together the levels of *C. albicans* SC5314 and *C. dubliniensis* CD36(M/G) in the stomach were similar at day 2, but from day 4 onwards *C. albicans* was consistently present in higher numbers than *C. dubliniensis,* with no *C. dubliniensis* detectable at days 8 and 10. Comparable numbers of *C. albicans* and *C. dubliniensis* were found in the intestine at days 2 to 6, however, by day 8 the proportion of *C. dubliniensis* had fallen dramatically, while by day 10 only *C. albicans* was detectable. Similar data were obtained when mice were infected with CD36 and SC5314(M/G) (data not shown).

3.4. Dissemination of C. albicans and C. dubliniensis from the gastrointestinal tract

Dissemination of *C. albicans* from the stomach to other organs such as the liver, kidney and brain has been examined previously (Cole et al., 1989;1990), but corresponding data on *C. dublinienis* are lacking. In order to examine the dissemination of *C. dubliniensis*, alone and in co-inoculation with *C. albicans* (using tagged strains), infant mice were inoculated with *Candida* as in the colonization experiments described above and levels of *Candida* in fecal pellets were monitored to confirm the presence of *Candida* in the GIT at the time of immunocompromization. Following immunocompromization at days 11 and 14, mice were sacrificed at day 20 and the cfu of each species enumerated in the stomach and liver. All 5 mice infected with *C. albicans*

SC5314 (100%), showed colonization/infection of the stomach, whereas only 2/5 (40%) *C. dubliniensis* CD36-infected mice showed colonization/infection. Dissemination to the liver was evident in 5/5 (100%) mice infected with *C. albicans* and 2/5 (40%) mice infected with *C. dubliniensis*. When the stomach and liver were infected with *C. dubliniensis* colony counts were comparable with those of *C. albicans*-infected tissues. Similar data were obtained when mice were infected with the GFP/MPA^{R}-tagged derivatives of SC5314 and CD36 (data not shown)..

When *C. albicans* SC5314 and *C. dubliniensis* CD36 (M/G) were co-inoculated into 10 mice, at the time of sacrifice *C. albicans* was detected in the stomach of all 10 mice while *C. dubliniensis* was only detected in the stomach of 2/10 (20%) mice. The mean levels of *C. albicans* and *C. dubliniensis* in the stomach were 1.7 x 10^6 and 2.4 x 10^2 cfu/ml, respectively ($P<0.001$). *Candida albicans* was also found in the liver of 7/10 (70%) of the mice infected with a mean value of 1.9 x 10^3 cfu/ml, however, *C. dubliniensis* was only found in the liver of a single (10%) mouse and with a mean value of 1.5 x 10 cfu/ml. The difference between the levels of *Candida* found in the livers of the two species was found to be significant ($P = 0.042$).

Histological analysis of stomach tissues obtained 20 days post-inoculation revealed that penetration of the stomach by *C. albicans* strain SC5314 had occurred with both yeast and hyphal elements evident in the stomach submucosa and in the gastric lumen (data not shown). Histological analysis of stomachs removed from mice infected with *C. dubliniensis* and prepared for light microscopy showed no detectable colonization. However, smears of stomachs from mice that were known to be infected with *C. dubliniensis* derivative CD36 (M/G) and *C. albicans* derivative SC5314 (M/G)

examined using FITC filters to detect GFP expression showed the presence of *C. dubliniensis* cells in the yeast phase only, while *C. albicans* cells were predominantly in the hyphal phase (Fig. 4). When additional infected organs were examined by light microscopy the livers obtained from mice infected with both species contained both hyphal and yeast cells (Fig. 5, panels A and B). However, sections of a kidney abscess in a mouse infected with *C. dubliniensis* CD36 demonstrated the presence of mainly yeast cells (Fig. 5, panel C) while a combination of yeast and hyphal cells were found in the kidney of a mouse which had been infected with *C. albicans* strain SC5314 (Fig. 5, panel D).

4. Discussion

Despite the close phylogenetic relationship and the sharing of many phenotypic traits of *C. albicans* and *C. dubliniensis*, there is a major dichotomy in the virulence of the two species as evidenced by epidemiological and limited infection model data. In this study, using comparisons with a wide range of *C. dubliniensis* isolates, we have shown that *C. albicans* forms hyphae more rapidly in vitro than *C. dubliniensis*. In addition, *C. albicans* causes greater damage to reconstituted human oral epithelial tissues, and shows more extensive colonization and more efficient dissemination to internal organs in the oral-intragastric infant mouse model. In both experimental models of infection production of hyphae by *C. dubliniensis* was rarely, if ever, observed, while *C. albicans* produced high levels of hyphae under all conditions examined. These data suggest that the increased capacity of *C. albicans* to colonize and penetrate gastrointestinal epithelium is strongly associated with its ability to efficiently produce hyphae, and that this very likely contributes to the higher prevalence of *C. albicans* in comparison with *C. dubliniensis* in the gastrointestinal tract and in the far higher incidence of *C. albicans* in cases of systemic candidiasis.

Previous observations based on a small number of *C. albicans* and *C. dubliniensis* isolates and a limited set of hypha-induction conditions suggested that hyphal production is less efficient in *C. dubliniensis* compared to *C. albicans* (Gilfillan et al., 1998). In the present study, we have used a larger number of *C. dubliniensis* and *C. albicans* isolates to investigate production of hyphae under a wide range of conditions commonly used to induce hyphae in *C. albicans*. The only medium that consistently induced hyphae in *C. dubliniensis* was water with 10% (v/v) serum in which 73% (8/11) of strains tested were

≥ 50% in the hyphal phase of growth following 6 h incubation. However, in this and in the other liquid induction media examined the rate of induction of hyphae was significantly lower in *C. dubliniensis* (Fig. 1). Confirmation of these data was obtained by investigating the expression of the *HWP1* gene, a gene that is known to be expressed only in hyphae in *C. albicans* and for which a divergent homolog has recently been identified in *C. dubliniensis* (Moran et al., 2004). The levels of *HWP1* expression in each species was directly associated with the proportion of germinated cells, indicating that expression of this gene is tightly associated with hyphal formation in *C. dubliniensis*, despite the major sequence divergence between *CaHWP1* and *CdHWP1*. In addition, the expression of *HWP1* by *C. dubliniensis* hyphae confirms that when *C. dubliniensis* does manage to produce hyphae they can express genes specifically associated with true hypha formation in *C. albicans*. In contrast to our data Vilela et al. have reported that when cultured in RPMI 1640 containing 50% fetal bovine serum the rate of germ tube formation was higher in *C. dubliniensis* than in *C. albicans* (Vilela et al., 2002). It is not clear what the reasons are for this divergence in results are, but both studies used different strains and the induction conditions used in the two studies were different (e.g. 50% versus 10% serum).

Our data indicate that *C. dubliniensis* is far less capable than *C. albicans* of producing hyphae under a wide range of hyphal inducing conditions. This suggests that there are likely to be differences in the signaling and regulatory pathways governing the induction of hyphae in the two species when grown on specific media (e.g. in the presence of CO_2 and different sources of nutrients). It has already been shown by Staib and Morschhäuser that differential regulation of the Nrg1 repressor is responsible for the

different growth morphology of each species when grown on Staib agar, a medium on which *C. albicans* grows exclusively as yeasts while *C. dubliniensis* growth is characterized by the production of pseudohyphae and chlamydospores (Staib and Morrschäuser, 2005). In addition, the repertoire of hypha-specific genes encoded in the genomes of both species is significantly different, with the absence of genes such as *SAP5, SAP6* and *HYR1* and the divergence of genes such as *HWP1* in the *C. dubliniensis* genome (Moran et al., 2004). Given the reduced efficiency of hyphal induction and the apparent loss of important hypha-specific genes in *C. dubliniensis* it is possible that this species may grow preferentially in the yeast phase in its natural environment.

When we compared the growth of *C. albicans* and *C. dubliniensis* in the RHE infection model, the latter grew exclusively in the yeast form, forming a non-invasive layer along the surface of the epithelial tissue that appeared to be poorly adherent as it was easily dislodged from the RHE surface. This was in strong contrast to *C. albicans*, the cells of which were attached to the epithelium and produced abundant hyphae early during the infection and which penetrated and invaded deep into the tissue. These findings clearly indicate that there are major differences in the regulation of hyphal formation and tissue adherence between the two species. The lack of efficient colonization by *C. dubliniensis* reflects the observations made by Dietrich *et al.* investigating *C. albicans Δefg1/Δefg1* mutants. In this mutant *C. albicans* strain the inability to form hyphae correlated with the inability to adhere to or penetrate a reconstituted epithelium model of infection (Dietrich et al., 2002).

Lactate dehydrogenase (LDH) activity (a marker of mammalian cell injury) in the RHE culture medium, was examined every 12 h as a crude method of determining the

effects of each species on the integrity of the RHE tissue. It was found that within 12-36 h, *C. albicans* caused significantly greater RHE tissue damage than *C. dubliniensis*, correlating with the high levels of filamentation tissue invasion and greater tissue damage evident microscopically with this species. During the same time period limited LDH activity was detected in the culture medium of *C. dubliniensis*–infected tissue, however, from 36 h on increased tissue damage was evident and LDH levels increased dramatically. Since no invasion of the tissue by *C. dubliniensis* was evident it is likely that the tissue damage observed at 48 h was due to the production of secreted factors, such as secreted aspartyl proteinases or lipases, produced by *C. dubliniensis* yeast cells., suggesting that even in the absence of hyphae, when cell burdens are high, this species can cause significant tissue damage.

Previous studies have compared the virulence of *C. albicans* and *C. dubliniensis* using the murine systemic infection model and have shown that animals infected with *C. dubliniensis* survive longer than those infected with *C. albicans* (Gilfillan et al., 1998; Vilella et al., 2002). In this study we used the oral intragastric infant mouse infection model to investigate if the low incidence of *C. dubliniensis* systemic infection may due to a reduced capacity of this species to colonize and disseminate from the gastrointestinal tract. Infant mice were inoculated with the two species alone and in co-culture and the cfu/ml determined in both the stomach and the intestine. When mice were infected with either *C. albicans* or *C. dubliniensis* the levels of each species were relatively similar up until and including day 6. However, at days 8 and 10 no *C. dubliniensis* were detectable while the levels of *C. albicans* remained relatively high. Similarly, when the two species were used in mixed inocula (when either species was tagged with the molecular (M/G)

cassette), levels of *C. albicans* remained high throughout the experiment, while the *C. dubliniensis* had disappeared almost entirely from the stomach and intestine by day 8, suggesting that *C. dubliniensis* is cleared from the mouse gastrointestinal tract far more quickly than *C. albicans*. These data and the apparent low adhesion of *C. dubliniensis* to oral RHE suggests that the gastrointestinal tract may not be the ideal environmental niche for this species and that additional anatomic sites should be investigated for the its presence.

The ability of *C. albicans* to disseminate from the gastrointestinal tract to other organs such as the liver, kidney and brain has been examined in *C. albicans* in previous studies using the oral intragastric infant mouse infection model (Cole et al., 1989; 1990). We therefore decided to compare the ability of *C. dubliniensis* and *C. albicans* to disseminate from the gastrointestinal tract to other organs following colonization of the infant mouse and subsequent treatment with immunosuppressant drugs to render the mice immunocompromised. The results from these experiments suggest that *C. dubliniensis* whether inoculated alone or in conjunction with *C. albicans* has a significantly lower capacity to colonize and establish itself in the gastrointestinal tract than *C. albicans* and that it is far less efficient at dissemination to other body organs. Upon histological analysis it was found that in the stomach, liver and kidney, *C. albicans* was present in both the yeast and hyphal phase. In contrast, in the small number of animals in which *C. dubliniensis* was detected in tissue this species was only present in the yeast phase in the stomach and kidney, although hyphal cells were observed in the liver. These data are in agreement with those of Vilela *et al.* who observed in a systemic mouse infection model that the fungal cells in *C. dubliniensis*–infected kidneys were predominantly in the yeast

phase, while in the same model *C. albicans* strains produced high levels of hyphae and pseudohyphae (Vilela et al., 2002).

While the reasons for the lower virulence of *C. dubliniensis* are likely to be multifactorial, our data suggest that the low prevalence of *C. dubliniensis* in systemic candidiasis is due at least partly to the lower capacity of *C. dubliniensis* to adhere to and invade oral and gastrointestinal epithelia and that this is associated with its limited capacity to produce hyphae in vivo. Low levels of germination are likely to result in a reduced ability to establish a secure level of colonization and subsequent infection in the GIT. Adhesion of yeast cells to epithelia may be insufficient to ward off the effects of fluids such as saliva and gastric acid washing the cells away. In contrast, the ability of *C. albicans* to produce hyphae results in these cells becoming embedded in the tissue and therefore less prone to being dislodged and eliminated. Similarly, the ability to form hyphae very likely facilitates the transmigration of *C. albicans* across the intestinal wall, especially if the wall is compromised by the use of immunosuppressant drugs. Interestingly even in co-infection experiments invasion of the gut wall by *C. albicans* does not appear to promote increased infection by *C. dubliniensis*. However, even if *C. dubliniensis* cells do find themselves in the blood stream their reduced capacity to produce hyphae may also result in faster clearance by professional phagocytes. This is supported by a study showing that the non-filamentous *C. albicans Δefg1/Δcph1* double mutant is avirulent in a mouse model and is killed more effectively by macrophages (Lo et al., 1997). The molecular basis of the differences in the regulation of dimorphism in *C. albicans* and *C. dubliniensis* have yet to be elucidated, however, it is hoped that further investigation of this will enhance our understanding of candidal virulence.

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Figure legends

Fig. 1. Graphs showing the mean percentage of hyphal cells produced by 4 strains of *C. albicans* (□, continuous line) and 11 strains of *C. dubliniensis* (◊, dotted line) during a 6 h incubation period in hypha-inducing media; (A) Medium-199, (B) NYP medium incubated with 5% (v/v) CO_2, (C) Water with 10% (v/v) newborn calf serum and (D) Lee's medium with a pH and temperature shift. The strains examined are listed in Table 1.

Fig. 2. Light micrographs of RHE infected with *C. albicans* SC5314 (panel A) and *C. dubliniensis* CD36 (panel B) following 48 h incubation. Histological sections were stained with Periodic-Acid Schiff reagent. Scale bars are shown.

Fig. 3. Graph showing levels of LDH (U/l) released from RHE tissue infected with *C. albicans* strains 132A and SC5314 and *C. dubliniensis* strains CD36, CD519 and P7718 over 48 h.

Fig. 4. Fluorescent micrographs of cells from stomach smears of a mouse infected with (A) *C. dubliniensis* derivative CD36 (M/G) which shows the presence of only yeast cells and (B) *C. albicans* derivative SC5314 (M/G) which shows only hyphal cells.

Fig. 5. Light micrographs of PAS stained *Candida* abscesses in body organs of infected, immunocompromised mice 20 days post challenge. Thin sections through abscesses in the liver (A) for *C. dubliniensis* strains CD36 and (B) for *C. albicans* strain SC5314. Thin sections through abscesses in the kidney for (C) *C. dubliniensis* strain CD36 and (D) *C. albicans* strain SC5314. The scale bar of 50 μm is representative of light micrographs A, C and D and the scale bar of 30 μm is representative of light micrograph B.