Candida dubliniensis: phylogeny and putative virulence factors

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Candida dubliniensis is a recently identified species which is implicated in oral candidosis in HIV-infected and AIDS patients. The species shares many phenotypic characteristics with, and is phylogenetically closely related to, Candida albicans. In this study the phylogenetic relationship between these two species was investigated and a comparison of putative virulence factors was performed. Four isolates of C. dubliniensis from different clinical sources were chosen for comparison with two reference C. albicans strains. First, the distinct phylogenetic position of C. dubliniensis was further established by the comparison of the sequence of its small rRNA subunit with representative Candida species. The C. dubliniensis isolates formed true unconstricted hyphae under most induction conditions tested but failed to produce true hyphae when induced using N-acetylglucosamine. Oral C. dubliniensis isolates were more adherent to human buccal epithelial cells than the reference C. albicans isolates when grown in glucose and equally adherent when grown in galactose. The C. dubliniensis isolates were sensitive to fluconazole, itraconazole, ketoconazole and amphotericin B. Homologues of seven tested C. albicans secretory aspartyl proteinase (SAP) genes were detected in C. dubliniensis by Southern analysis. In vivo virulence assays using a systemic mouse model suggest that C. dubliniensis is marginally less virulent than C. albicans. These data further confirm the distinct phenotypic and genotypic nature of C. dubliniensis and suggest that this species may be particularly adapted to colonization of the oral cavity.

INTRODUCTION

Numerous reports have described a significant increase in the incidence and diagnosis of opportunistic and systemic candidosis during the last decade (Hazen, 1995; Sullivan et al., 1996; Coleman et al., 1997a). The reasons for this increase are many, but one of the most important contributory factors has been the increase in numbers of immunocompromised individuals, particularly those infected with HIV. Candida albicans is the most pathogenic species of the genus Candida and is the species most frequently associated with candidosis (Odds et al., 1990). However, recently the proportion of infections diagnosed as being caused by this species has decreased relative to the increasing incidence of infections apparently caused by other, less pathogenic species such as C. tropicalis, C. glabrata and C. krusei (Powderly, 1992; Hazen, 1995; Pfaller, 1996; Sullivan et al., 1996; Coleman et al., 1997b). The reasons for this epidemiological shift are not clear; however, the decreased susceptibility of these species relative to C. albicans to commonly used antifungal agents such as fluconazole has been implicated as being at least partially responsible (Powderly, 1992; Chavanet et al., 1994).

**Keywords:** aspartic proteinase, Candida dubliniensis, HIV and AIDS, rRNA gene sequences, virulence

**Abbreviations:** BEC, buccal epithelial cell; SAP, secretory aspartyl proteinase.

The EMBL accession number for the sequence reported in this paper is X99389.
In addition to the reports of the increased detection of non-*C. albicans* species in cases of human infection, there have been several reports describing the emergence of what were originally described as ‘atypical *C. albicans*’. Sullivan et al. (1993) and Boerlin et al. (1997) have subjected further detailed analysis (Sullivan et al., 1995, 1997). These isolates can be distinguished from conventional *C. albicans* isolates by a number of phenotypic and genetic characteristics. They produce germ tubes and chlamydospores, although the latter are produced abundantly and in unusual formations. They have distinct carbohydrate assimilation profiles, grow poorly or not at all at 42°C and yield dark green colonies on primary isolation from the broth to make the solid medium for 48 h at 37°C. The identity of *C. dubliniensis* isolates was confirmed by API LAB ID32C (bioMérieux) carbohydrate assimilation assays, analysis of growth at 42°C and by DNA fingerprinting and karyotype analysis, according to the methods described by Sullivan et al. (1995).

**METHODS**

**Yeast strains.** Representative *C. dubliniensis* and control *C. albicans* isolates used in this study are shown in Table 1. The yeasts were grown routinely on Potato Dextrose Agar (PDA, Oxoid) at pH 5.5 and Yeast Peptone Dextrose (YPD) agar and broth [YPD (g l−1): yeast extract (Oxoid), 10; peptone (Oxoid), 20; glucose, 20; pH 5.5; 20 g agar (Oxoid) was added to the broth to make the solid medium] for 48 h at 37°C. The identity of *C. dubliniensis* isolates was confirmed by API LAB ID32C (bioMérieux) carbohydrate assimilation assays, analysis of growth at 42°C and by DNA fingerprinting and karyotype analysis, according to the methods described by Sullivan et al. (1995).

**Chemicals, enzymes and radioisotopes.** Analar-grade or molecular biology-grade chemicals were purchased from BDH, Boehringer Mannheim, Fisons and Sigma. Restriction endonucleases and Klenow fragment DNA polymerase were purchased from Boehringer Mannheim or Promega and were used as directed by the manufacturers. [α-32P]dCTP (3000 Ci mmol−1; 111 TBq mmol−1) used in random priming of DNA probes was purchased from Amersham.

**MIC antifungal assays.** MIC assays were conducted for fluconazole, itraconazole, ketoconazole and amphotericin B using the broth dilution method (Pfaller et al., 1990).

**Hypthal formation.** Hypthal formation assays were performed on cells grown to stationary phase in the defined medium of Lee et al. (1975) supplemented with 400 mM arginine, 0.001% (w/v) biotin and trace metals (0.25 mM CuSO₄, 1 mM FeCl₃, 1 mM MgCl₂, 1 mM CaCl₂). Cells were grown at either 25 or 37°C in orbital incubators (New Brunswick Scientific) set at 180 r.p.m. The pH/temperature shift induction of hyphae (Buffo et al., 1984) was performed by inoculating 25 ml supplemented Lee’s medium, pH 6.5, 37°C with 0.5 ml of cells grown to stationary phase in supplemented Lee’s medium, pH 4.5, 25°C (Buffo et al., 1984). N-Acetyl-d-glucosamine (GlcNAc) induction of hypthal formation (Mattia et al., 1982) was performed by resuspending cells that had been grown to stationary phase in supplemented Lee’s medium, pH 4.5, 37°C, washed and starved in distilled water for 24 h, into 25 ml basal salt medium [0.5% (%/w) (NH₄)₂SO₄, 0.02% (%/w) MgSO₄, 0.5% (%/w) NaCl, 0.001% biotin] with GlcNAc added to 4 mM, to a cell density of 2 x 10⁶ cells ml⁻¹ at 37°C. Serum induction of hypthal formation (Gow & Gooday, 1982) was performed by resuspending cells that had been grown to stationary phase in supplemented Lee’s medium, pH 4.5, 37°C, and washed in genetically closely related and share several phenotypic characteristics, further studies are required to elucidate the phenotypic and genetic relationships between these two species. The aims of the present study were twofold. First, to further investigate the phylogenetic position of *C. dubliniensis* in relation to other *Candida* species by comparing the sequences of the small rRNA subunit (small rRNA) genes from representative species. Comparative sequence analysis of small rRNA genes has been used most extensively in phylogenetic studies with a wide variety of microbial species (Olsen & Woese, 1993). Second, to compare *C. dubliniensis* and *C. albicans* isolates with respect to potential virulence factors, such as adhesion, hyphal production and the possession of genes encoding secretory aspartyl protease (SAP) homologues.

**Boucher et al., 1996.**

Isolates of *C. dubliniensis* have mainly been recovered from the oral cavities of HIV-infected individuals (Coleman et al., 1997b). However, a small number of isolates have been recovered from the oral cavities and the vaginas of normal healthy individuals (Sullivan et al., 1995; Moran et al., 1997). Although most isolates to date have been recovered in Ireland, they have also been recovered from patients throughout Europe, North and South America and Australia (Sullivan et al., 1997). Recently, it has been demonstrated that although most isolates of *C. dubliniensis* are sensitive to the commonly used antifungal agent fluconazole, a significant proportion of isolates are resistant to the drug (Moran et al., 1997). Furthermore, some isolates of *C. dubliniensis* have been shown to become resistant on exposure to subinhibitory doses of fluconazole, suggesting that this may be the reason why this species has emerged in recent years (Moran et al., 1997).

Because *C. dubliniensis* and *C. albicans* are phylo-
Table 1. C. albicans and C. dubliniensis strains used

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Origin</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. albicans</td>
<td>3153</td>
<td>Systemic isolate</td>
<td>Odds (1974)</td>
</tr>
<tr>
<td>C. albicans</td>
<td>SC5314</td>
<td>Systemic isolate</td>
<td>Gillum et al. (1984)</td>
</tr>
<tr>
<td>C. dubliniensis</td>
<td>CD36</td>
<td>Irish oral isolate from HIV+ patient</td>
<td>Sullivan et al. (1995)</td>
</tr>
<tr>
<td>C. dubliniensis</td>
<td>CD41</td>
<td>Irish oral isolate from HIV+ healthy subject</td>
<td>Sullivan et al. (1995)</td>
</tr>
<tr>
<td>C. dubliniensis</td>
<td>CD57</td>
<td>Irish vaginal isolate from HIV+ patient</td>
<td>Moran et al. (1997)</td>
</tr>
<tr>
<td>C. dubliniensis</td>
<td>CD43</td>
<td>Irish oral isolate from HIV- intravenous drug user</td>
<td>Sullivan et al. (1997)</td>
</tr>
</tbody>
</table>

Adherence assays. Adherence assays were conducted on yeast cells grown for exactly 24 h in medium containing 0.67% (w/v) yeast nitrogen base without amino acids (Difco) and either 50 mM glucose or 500 mM galactose (Douglas et al., 1981). Yeast cells (1 x 10^6 ml^-1) and human buccal epithelial cells (BECs; 1 x 10^6 ml^-1) were incubated together for 45 min at 37 °C, then recovered on polycarbonate filters (pore size 12 μm; Costar) and Gram-stained. Adherence assay results are presented as the mean number of yeasts adhering to 100 BECs, with samples being taken in triplicates of 100 BECs and on 3 consecutive days.

Isolation of plasmid and genomic DNA. Genomic DNA was isolated from spheroplasts of Candida isolates by the method described by Rose et al. (1990). Plasmid DNA was isolated from Escherichia coli DH5α by the alkali lysis method described by Kraft et al. (1988).

Cloning and sequencing of the small rRNA gene from C. dubliniensis strain CD36. The gene encoding the C. dubliniensis small rRNA was amplified using primers specific for the conserved sequences at the 3' and 5' ends of eukaryotic small rRNA genes (primers A and B, Medlin et al., 1988). The amplification reaction was performed in a Perkin-Elmer 9600 thermal DNA cyclers in a 100 μl volume containing 10 mM Tris/ HCl, pH 8.4, 50 mM KCl, 2 mM MgCl₂, 200 mM each of dATP, dTTP, dGTP and dCTP, 20 mM each of primer A and B and 2.5 μl U Taq polymerase. The cycling conditions used were 95 °C for 2 min, 37 °C for 2 min and 72 °C for 6 min for 30 cycles. Following amplification, a 90 μl aliquot of the amplified PCR mix was added to 210 μl ddH₂O and purified on a Microcon 100 column (Amicon) as recommended by the manufacturer. The purified amplimer was cloned into T-tailed pGEM3Zf and subsequently sequenced using the PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems). The sequencing primers used were 395-343R, 536-519R, 705-689R and 1242-1255R (Barns et al., 1991) and the M13 forward and reverse primers. All sequencing was performed on a 373A STRETCH automated sequencer (Applied Biosystems) and the results analysed using the computer program SEQUED. Comparison of small rRNA gene nucleotide sequences from various species was performed using multiple sequence alignments generated using the program CLUSTAL W. The sequences compared included those from C. dubliniensis (this study; accession no. X99399), C. albicans (Hendriks et al., 1989; X53497), C. tropicalis (Hendriks et al., 1991; M55527), C. glabrata (Wong & Clark-Walker, 1990; X51831), C. lusitaniae (Hendriks et al., 1991; M55526), C. krusei (Hendriks et al., 1991; M55528) and Saccharomyces cerevisiae (Rubtsov et al., 1980; M27607). These data were then used to construct a genetic distance matrix incorporating corrections for multiple base changes according to the method of Jukes & Cantor (1969). A phylogenetic tree was produced from this matrix using the neighbour-joining method of Saitou & Nei (1987) and bootstrap values for the tree were generated according to Felsenstein (1985).

PFGE. Yeast chromosomes were prepared in agarose plugs as described by Vazquez et al. (1991) and separated in 1:4% (w/v) agarose gels using the CHEF-Mapper PFGE system (Bio-Rad) under conditions described previously (Sullivan et al., 1995).

Plasmids and SAP probe DNA. DNA probes for use in hybridization experiments were generated from C. albicans SAP genes 1, 2, 3, 5 and 7. The SAP1 probe was a 1.35 kb NcoI–XhoI fragment of the SAP1 ORF from plasmid pAS1 (Hube et al., 1994); the SAP2 probe was a 0.65 kb BamHI–HindIII fragment of the SAP2 ORF from plasmid pCA2 (Monod et al., 1994); the SAP3 probe was a 1 kb EcoRI fragment of the SAP3 ORF from plasmid pCA3 (Monod et al., 1994); the SAP5 probe (referred to later as SAP4-6 due to the cross-hybridization of these three highly homologous genes) was a 0.6 kb BglII fragment of the SAP5 ORF from plasmid pCA5 (Monod et al., 1994) and the SAP7 probe was a 1 kb EcoRI–HindII fragment of the SAP7 ORF from plasmid pCA7 (Monod et al., 1994). After plasmid isolation, restriction digestion and fragment separation by agarose gel electrophoresis, the desired probe fragments were purified from the agarose by the Prep-A-Gene kit (Bio-Rad) in preparation for random priming.

Southern transfer and hybridization. Restriction digestion, agarose (1%) gel electrophoresis in 1x TAE buffer (40 mM Tris/acetate, 1 mM EDTA) and Southern transfer using a vacuum manifold (Pharmacia) were completed as described by Sambrook et al. (1989). Nylon membrane filters were then rinsed with 5x SSC (1x SSC is 0.15 M sodium chloride, 0.015 M sodium citrate) and prehybridized at 42 °C for at least 5 h in 40% (v/v) formamide, 5x SSC, 5x Denhardt’s reagent (1x Denhardt’s is 0.2 g 1^-1 each of Ficoll, polyvinilpyrrolidone and BSA), 0.5% (w/v) SDS, 0.01 M EDTA, 0.025 M NaPO₄ and 100 mg denatured sheared herring sperm DNA ml^-1. Denatured radiolabelled probes were added and incubated with fresh prehybridization solution for 12–18 h. Membranes were then washed twice for 15 min with each of 2x SSCP (1x SSCP is 0.15 M NaCl, 15 mM sodium citrate, 5 mM NaH₂PO₄, 7 mM Na₂HPO₄), 0.1% SDS at 42 °C (very low stringency), 0.5x SSCP, 0.1% SDS at 55 °C (low stringency) and 0.2x SSCP, 0.1% SDS at 60 °C (high stringency). PFGE gels were Southern blotted as described by Lee et al. (1991) and hybridized to the probes under the high stringency conditions described above.

In vivo virulence tests. Candida cells grown overnight on Sabouraud’s dextrose agar were washed off in saline and inoculated at two inoculum densities into the tail vein of CR...
CD1 female mice (mean weight 22 g) and the animals were monitored for 8 d.

RESULTS

Comparison of the nucleotide sequences of the small rRNA genes of *C. dubliniensis* and other *Candida* species

Comparison of the nucleotide sequence of the V3 region of the large ribosomal subunit genes from a variety of species was previously used as evidence for the designation of *C. dubliniensis* as a taxonomically distinct species (Sullivan et al., 1995). Previously, comparisons of the nucleotide sequences of the entire small rRNA genes have been used most extensively for phylogenetic analyses with a wide range of microbial species. To confirm this phylogenetic position of *C. dubliniensis* we cloned the entire small rRNA gene from the type strain of *C. dubliniensis* (CD36), determined its complete nucleotide sequence and compared it to the corresponding sequences previously obtained from other representative yeast species.

PCR amplification of the *C. dubliniensis* small rRNA gene with primers previously used in the amplification of small rRNA sequences from other *Candida* species (Medlin et al., 1988) yielded a single product of approximately 1800 bp. This product was then cloned into T-tailed pGEM3Zf and sequenced using standard protocols. The amplified sequence was found to be 1791 bp. When this sequence was compared with the corresponding sequence in *C. albicans* (accession no. X53497) it was found to differ at 25 bp (corresponding to 1.4% sequence divergence). An evolutionary distance matrix (Table 2) incorporating corrections for multiple base changes was generated by the method of Jukes & Cantor (1969) using the small rRNA genes of *C. dubliniensis* (X99399), *C. albicans* (X53497), *C. tropicalis* (M55527), *C. glabrata* (X51831), *C. lusitaniae* (M55528), *S. cerevisiae* (M27607). It is interesting to note that the *C. tropicalis* and *C. albicans* sequences differed by approximately 1.4%; this difference is similar to the sequence difference found between *C. albicans* and *C. dubliniensis* (Table 2). An evolutionary tree was generated from these data using the neighbour-joining method of Saitou & Nei (1987) (Fig. 1). It is evident from this tree, and from the bootstrap values also obtained, that *C. dubliniensis* is phylogenetically distinct from the other *Candida* species, including *C. albicans*.

Susceptibility to antifungals

The four *C. dubliniensis* isolates and *C. albicans* 3153 were assayed for their susceptibility to the antifungal drugs fluconazole, ketoconazole, itraconazole and amphotericin B using the broth dilution method (Pfaller et al., 1990). All strains were susceptible to the antifungals tested, with only minor variations in susceptibility between strains (Table 3).

Hyphal formation

The relative ability of reference *C. albicans* and *C. dubliniensis* isolates to produce true non-constricted hyphae under a variety of induction conditions was assayed. Hyphal growth was induced using serum (Gow & Gooday, 1982), *N*-acetyl-d-glucosamine (GlcNAc; Mattia et al., 1982) and pH/temperature shift (Buffo et al., 1984). All results are presented as the formation of

<table>
<thead>
<tr>
<th></th>
<th><em>C. du.</em></th>
<th><em>C. al.</em></th>
<th><em>C. tr.</em></th>
<th><em>C. gl.</em></th>
<th><em>S. ce.</em></th>
<th><em>C. lu.</em></th>
<th><em>C. kr.</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. dubliniensis</em></td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>1.4</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><em>C. tropicalis</em></td>
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<td>1.4</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. glabrata</em></td>
<td>5.4</td>
<td>4.8</td>
<td>4.5</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td>5.5</td>
<td>4.6</td>
<td>4.4</td>
<td>2.2</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. lusitaniae</em></td>
<td>7.6</td>
<td>7.3</td>
<td>7.0</td>
<td>7.8</td>
<td>8.2</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>C. krusei</em></td>
<td>8.9</td>
<td>8.4</td>
<td>7.8</td>
<td>8.0</td>
<td>8.5</td>
<td>10.1</td>
<td>-</td>
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Table 3. MIC of selected antifungals

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (μg ml⁻¹)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Fluconazole</td>
</tr>
<tr>
<td>C. albicans 3153</td>
<td>0.39</td>
</tr>
<tr>
<td>C. dubliniensis CD36</td>
<td>0.39</td>
</tr>
<tr>
<td>C. dubliniensis CD41</td>
<td>0.39</td>
</tr>
<tr>
<td>C. dubliniensis CD57</td>
<td>0.78</td>
</tr>
<tr>
<td>C. dubliniensis CD43</td>
<td>0.19</td>
</tr>
</tbody>
</table>

**Fig. 2.** Hyphal formation by C. albicans strains 3153 and SC5314 and C. dubliniensis strains CD36 and CD57 under different induction conditions. (a) Newborn-calf serum (10%), (b) pH/temperature shift and (c) GlcNAc (4 mM). Similar results to those obtained with C. dubliniensis isolates CD36 were also obtained with C. dubliniensis isolates CD41 and CD43. A value of 100% represents germ tube outgrowth from all cells. Error bars indicate SD (n = 3).

Hyphal induction of C. dubliniensis isolates CD36, CD41 and CD43 produced results similar to that shown for CD36 (data not shown). The four C. dubliniensis isolates tested produced hyphae less rapidly than control C. albicans isolates under the *in vitro* induction conditions described above. This effect was seen under all conditions tested, with the exception of hyphal induction of CD57 in serum medium, but was most pronounced when hyphae were induced in medium containing GlcNAc, where all C. dubliniensis isolates failed to produce hyphae. In GlcNAc induction medium, however, CD57 displayed a low level of hyphal induction. In this case hyphae were formed during the 24 h period of starvation in water – part of the published protocol to generate inocula for germ tube induction experiments. The inability of C. dubliniensis to form hyphae in GlcNAc was not due to an incapacity to utilize GlcNAc as a carbon source, as N-acetylglucosamine assimilation has been demonstrated for this species (Sullivan et al., 1995).

**Adherence to human buccal epithelial cells**

The adherence of the four C. dubliniensis isolates was compared to that of the two control C. albicans strains (Fig. 3) using the adherence of yeast form Candida cells to human BECs as described by Douglas *et al.* (1981). The Candida strains were grown in both glucose- and galactose-based media. Adherence was studied in both control strains of C. albicans and with C. dubliniensis CD36 and CD57, and the experiments repeated on 3 consecutive days. Adherence values were similar on each of the three days. C. albicans 3153 showed adherence values similar to that reported previously (Douglas *et al.*, 1981) and similar to that of the C. albicans clinical isolate SC5314, the parental strain of genetically marked strains used in most Candida gene disruption experiments (Fonzi & Irwin, 1993). Adherence of all these strains was approximately five times higher in cells grown in galactose medium over those grown in the glucose medium. Adherence of C. dubliniensis CD57 was similar to that of the C. albicans isolates. C. dubliniensis CD36 showed no significant difference in
adherence compared to the \textit{C. albicans} isolates when grown in galactose medium, but was significantly more adherent ($P < 0.01$) when grown in glucose medium. Adherence of two other \textit{C. dubliniensis} oral isolates, CD41 and CD43, to BECs was examined for 1 d only. Both exhibited increased adherence in the presence of glucose equivalent to that determined for CD36.

**Homologues to \textit{C. albicans} SAP genes**

SAP activity of strains now identified as \textit{C. dubliniensis} has been reported previously as significantly greater than that of reference \textit{C. albicans} isolates (McCullough \textit{et al.}, 1995). We therefore chose to examine \textit{C. dubliniensis} for the presence of homologues of the known \textit{C. albicans} SAP genes, using probes derived from seven members of the SAP multigene family. The ORFs of \textit{C. albicans} SAP4, 5 and 6 are 90\% identical (Monod \textit{et al.}, 1994) and cannot readily be distinguished by hybridization to probes derived from any of their sequences. A SAP5 sequence was therefore chosen to be used as probe for all three of the above genes and is taken to indicate the presence of one or more members of the SAP4-6 gene family.

Four \textit{C. dubliniensis} isolates were compared with the control \textit{C. albicans} strain 3153 using PFGE with chromosome blotting and probe hybridization (Fig. 4a). Each probe hybridized to a single chromosome. The same chromosome was recognized in all strains of the same species.

In addition to the PFGE results, DNA from \textit{C. albicans} 3153 and each of the four \textit{C. dubliniensis} strains was digested with EcoRI and BglII, separated by agarose gel electrophoresis and examined by Southern analysis. Fig. 4(b) shows the results obtained when such a filter was probed with SAP2. In all cases the restriction fragments for \textit{C. dubliniensis} strains differed to those from \textit{C. albicans} 3153 (data not shown). \textit{C. dubliniensis} strains CD36, CD41 and CD57 gave identical restriction frag-
Table 4. Hybridization of C. albicans SAP gene probes to restriction-endonuclease-digested C. dubliniensis genomic DNA

<table>
<thead>
<tr>
<th>SAP gene probe</th>
<th>Digest</th>
<th>C. dubliniensis bands (kb) hybridizing to probe*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CD36, CD41 &amp; CD57</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD43</td>
</tr>
<tr>
<td>1</td>
<td>EcoRI</td>
<td>4.3, (3.8)</td>
</tr>
<tr>
<td></td>
<td>BglII</td>
<td>(10.5), (4.1), (0.5)</td>
</tr>
<tr>
<td>2</td>
<td>EcoRI</td>
<td>3.8, 3.3</td>
</tr>
<tr>
<td></td>
<td>BglII</td>
<td>(9.5), 1.7</td>
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<tr>
<td>3</td>
<td>EcoRI</td>
<td>4.3, (3.8)</td>
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<tr>
<td></td>
<td>BglII</td>
<td>9.6, (2.1)</td>
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<tr>
<td>4-6</td>
<td>EcoRI</td>
<td>3.8, (3.3)</td>
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<tr>
<td></td>
<td>BglII</td>
<td>(8.0), (2.2)</td>
</tr>
<tr>
<td>7</td>
<td>EcoRI</td>
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</tr>
<tr>
<td></td>
<td>BglII</td>
<td>7.0</td>
</tr>
</tbody>
</table>

*Numbers in parentheses indicate weak hybridization. Identical hybridization patterns were seen between CD36, CD41 and CD57. CD43 produced a different pattern for all but SAP4-6.

Table 5. In vivo virulence in mouse systemic candidosis model

Mice used were CR CD1 SPF females injected into the tail vein with the doses stated and followed for 8 d post-infection. For the inoculum size of $2 \times 10^6$, there was no statistical difference in the survival times of C. albicans 3153 and C. dubliniensis CD57. The other C. dubliniensis strains were significantly less virulent than C. albicans ($P<0.01$). For the inoculum size of $1 \times 10^7$, CD41 was significantly less virulent than all of the other strains ($P<0.05$), but there was no statistically significant difference between any of the other strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Inoculum size (cells per mouse)</th>
<th>Mean survival time (d)</th>
<th>No. of survivors at day 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. albicans 3153</td>
<td>$1 \times 10^7$</td>
<td>1.66</td>
<td>0/3*</td>
</tr>
<tr>
<td></td>
<td>$2 \times 10^8$</td>
<td>4.75</td>
<td>0/4</td>
</tr>
<tr>
<td>C. dubliniensis CD36</td>
<td>$1 \times 10^7$</td>
<td>0.5</td>
<td>0/4</td>
</tr>
<tr>
<td></td>
<td>$2 \times 10^8$</td>
<td>&gt;8.25</td>
<td>3/4</td>
</tr>
<tr>
<td>C. dubliniensis CD41</td>
<td>$1 \times 10^7$</td>
<td>&gt;6.0</td>
<td>1/3</td>
</tr>
<tr>
<td></td>
<td>$2 \times 10^8$</td>
<td>&gt;8.5</td>
<td>3/4</td>
</tr>
<tr>
<td>C. dubliniensis CD43</td>
<td>$1 \times 10^7$</td>
<td>0.5</td>
<td>0/4</td>
</tr>
<tr>
<td></td>
<td>$2 \times 10^8$</td>
<td>&gt;9.0</td>
<td>3/4</td>
</tr>
<tr>
<td>C. dubliniensis CD57</td>
<td>$1 \times 10^7$</td>
<td>3.0</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td>$2 \times 10^8$</td>
<td>&gt;6.25</td>
<td>2/4</td>
</tr>
</tbody>
</table>

*All mice died by day one.

ment sizes (Table 4). Strain CD43 gave different restriction patterns to the other three strains for SAP1, SAP2 and SAP3, but identical hybridizing fragments for SAP4-6 and SAP7 (Table 4). The C. dubliniensis genome therefore has homologues of all C. albicans SAP genes that were examined and polymorphisms may exist at certain SAP loci in some C. dubliniensis strains.

In vivo virulence tests

The relative virulence of C. albicans and C. dubliniensis was assayed using a mouse model of systemic candidosis (Table 5). At the higher inoculation density of $10^8$ cells per mouse, there was a wide variation in mean survival times amongst the mice infected with C. dubliniensis. At a lower inoculum density of $2 \times 10^6$ cells per mouse, mice survival times were 1.5–2 times greater for infections by C. dubliniensis strains compared to those caused by C. albicans 3153. Therefore, in this model infection C. dubliniensis had lower in vivo virulence than C. albicans.

DISCUSSION

Based on preliminary phylogenetic evidence, C. dubliniensis is the species most closely related to C. albicans. It is therefore not surprising that the two species share many phenotypic traits, such as the ability.
to form true germ tubes, adhere to epithelial surfaces, secrete a range of aspartic proteinases and form chlamydospores. \textit{C. albicans} is the \textit{Candida} species most frequently recovered from patients presenting with candidosis; consequently it is generally accepted that it possesses significantly greater pathogenic potential than other members of the genus. In this study, we have further established the phylogenetic relationship between \textit{C. dubliniensis} and other \textit{Candida} species, including \textit{C. albicans}, by comparing the nucleotide sequences of their small rRNA genes. In addition, we compared \textit{C. albicans} and \textit{C. dubliniensis} with respect to a number of parameters which have been postulated to play a role in the virulence of \textit{C. albicans}.

The small rRNA gene of the \textit{C. dubliniensis} type strain, CD36, was amplified using PCR primers specific for conserved sequences at the 5’ and 3’ ends of eukaryotic small rRNA genes. When the sequence of this 1791 bp product was compared to that previously described for the \textit{C. albicans} small rRNA gene, the two sequences were found to differ by 1-4%, the same level of difference found between \textit{C. albicans} and \textit{C. tropicalis}. In addition, when an evolutionary tree was generated by comparing the \textit{C. dubliniensis} small rRNA gene sequence with the corresponding sequences from a variety of other \textit{Candida} species it was evident that \textit{C. dubliniensis} formed a separate branch, thus confirming earlier data generated from comparisons of the V3 region of the large rRNA subunit genes (Sullivan et al., 1995; Kurtzman & Robnett, 1997).

One of the putative virulence factors of \textit{C. albicans} is its ability to form true uniconstrued germ tubes. The clinical implications of this dimorphic behaviour are not clear as both yeast and hyphal forms have been observed in infected tissue. However, the hyphal form of \textit{C. albicans} is thought to be more adherent than the yeast form and hyphae are more likely to be able to penetrate tissue (Cutler, 1991). The hyphal induction experiments described in this study suggest that the kinetics of hyphal formation were slower in \textit{C. dubliniensis} compared to \textit{C. albicans}. Nevertheless, \textit{C. dubliniensis} formed unconstricted germ tubes that would pass the “germ tube test” used to differentiate \textit{C. albicans} from other non-\textit{C. albicans} species. None of the strains of \textit{C. dubliniensis} tested were able to form hyphae in GlcNAC. This raises the possibility that \textit{C. dubliniensis} may carry a mutation in the pathway controlling hyphal induction in response to GlcNAC. However, the inability to form hyphae on exposure to GlcNAC is also a feature of some \textit{C. albicans} isolates (Mattia et al., 1982; Gow et al., 1994). This suggests that \textit{C. dubliniensis} may be as potent as \textit{C. albicans} at penetrating the epithelial layers of host tissues and may relate to the primary association of \textit{C. dubliniensis} with superficial infections of the oral mucosa (Coleman et al., 1997a).

The finding that oral \textit{C. dubliniensis} isolates adhered to BECs to a greater extent than reference \textit{C. albicans} isolates when grown in the presence of glucose may also be relevant to the observation that \textit{C. dubliniensis} is found almost exclusively in the oral cavity (Coleman et al., 1997a). The increased adherence of what were originally described as atypical \textit{C. albicans} isolates, now known to be \textit{C. dubliniensis} (Sullivan et al., 1995, 1997), has been described before (McCullough et al., 1995). Interestingly, the vaginal \textit{C. dubliniensis} isolate CD57 showed no signs of increased adherence when grown in glucose-containing medium. It has not yet been established whether this increased adherence in relation to growth in glucose is a feature common to oral-adapted strains of \textit{Candida}, or is peculiar to \textit{C. dubliniensis}. Diabetes, antibiotic treatment and steroid therapy increase a patient’s level of salivary glucose – and all three conditions predispose a patient to candidosis (McCourtie & Douglas, 1981). It is possible that the increased adhesion of CD36 and other \textit{C. dubliniensis} oral isolates described by McCullough et al. (1995) compared with \textit{C. albicans} may be due to the elevated expression of receptors, such as the glucose-responsive \textit{C. albicans} analogue to the mammalian neutrophil integrin protein, CD11b/CD18 (Gustafson et al., 1991). Expression of the \textit{C. albicans} integrin analogue is elevated in the presence of 20 mM glucose and can be reversed if cells are treated with mAbs to the integrin analogue (Gustafson et al., 1991). However, the concentration of sugars employed in this standard adhesion assay protocol are likely greater than those found in vivo and so the resulting data must be interpreted cautiously.

SAPs are believed to be involved in the attachment and tissue invasion of \textit{Candida} cells during infection (Borg & Ruchel, 1988) and correlations between proteolytic activity and virulence of \textit{Candida} strains have been reported (Ruchel, 1992). To date at least seven SAP genes (SAP1–7) have been cloned and sequenced in \textit{C. albicans} and the presence of homologous multigene families in other \textit{Candida} species has also been suggested (Monod et al., 1994). The seven SAP genes of \textit{C. albicans} that were examined here are expressed differentially under a variety of conditions influenced by the medium composition, growth phase, morphological form and strain of the organism (Hube et al., 1994). Isolates originally identified as atypical \textit{C. albicans}, later confirmed as \textit{C. dubliniensis} strains, were demonstrated to be highly proteolytic, producing greater amounts of proteinase than reference \textit{C. albicans} strains (McCullough et al., 1995). In this study we showed that all the \textit{C. dubliniensis} isolates examined possessed homologues to each of the seven \textit{C. albicans} SAP genes tested. Whether the products of these genes have the same activities and pathological roles or are produced under the same environmental conditions as their \textit{C. albicans} homologues has yet to be determined.

The majority of \textit{C. dubliniensis} isolates have been recovered from HIV-positive patients with oral candidosis and the oral cavities of healthy individuals, suggesting that \textit{C. dubliniensis} may be adapted specifically to the oral cavity. However, in the absence of an established oral candidosis animal model, we used a systemic mouse model to investigate the relative virulence of \textit{C. dubliniensis} and \textit{C. albicans} strains. Under
these experimental conditions, the four C. dubliniensis isolates indicated that the new species may be less virulent than C. albicans.

Moran et al. (1997) recovered clinical C. dubliniensis isolates resistant to fluconazole and found that resistance could rapidly be developed on culture in vitro. Nevertheless, the majority of C. dubliniensis isolates are susceptible to fluconazole, as were the four isolates examined here (MIC range 0.19–0.78 μg ml⁻¹). Like those isolates examined previously, these isolates were also susceptible to itraconazole, ketoconazole and amphotericin B.

Clearly, C. dubliniensis and C. albicans are closely related and share many properties. The decreased ability of C. dubliniensis to form hyphae and its increased levels of proteinase production may help to provide an explanation as to why C. dubliniensis is almost exclusively associated with oral candidosis. The precise roles of these and other potential virulence factors, such as the acquisition of antifungal resistance have yet to be addressed.

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