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Characterisation of Genotypic and Phenotypic Differences between *Candida dubliniensis* and *Candida albicans*

A thesis submitted to the University of Dublin in fulfillment of the requirements for the degree Doctor of Philosophy by

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January 2001

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Declaration

I hereby declare that this thesis has not previously been submitted for a degree at this or any other University and that it represents my own unaided work, except where duly acknowledged in the text. I agree that this thesis may be lent or copied at the discretion of the librarian, Trinity College Dublin.

Samantha Donnelly

Samantha Donnelly

This thesis is dedicated to Sean

Thus I set pen to paper with delight,
And quickly had my thoughts in black and white.
For having now my method by the end,
Still as I pulled, it came; and so I penned
It down until it came at last to be,
For length and breadth, the bigness which you see.

John Bunyan
The Author's Apology for His Book
The Pilgrim's Progress

Summary

Candida dubliniensis is a recently described *Candida* species associated with oral candidosis in Human Immunodeficiency Virus (HIV)-infected and AIDS patients. The phylogenetic position of *C. dubliniensis* has previously been established on the basis of the sequence of rRNA genes. In order to establish that *C. dubliniensis* is a new yeast species and to confirm its relationship to other yeast species, particularly *C. albicans*, using non-rRNA gene sequences the *ACT1* gene was chosen for analysis. The *C. dubliniensis ACT1* gene (*CdACT1*) was cloned and sequenced from a genomic DNA λ library using PCR. Analysis of the sequence data revealed the presence of a 1131 bp ORF interrupted by a single 632 bp intron at the 5' extremity of the gene. Comparison of the *CdACT1* sequence with the *C. albicans* homologue (*CaACT1*) revealed that although the exons are 97.9 % identical the introns are only 83.4 % identical. A phylogenetic tree generated using *ACT1* exon sequences from a range of yeast species unequivocally confirmed the phylogenetic position of *C. dubliniensis* as a unique taxon within the genus *Candida*. Analysis of the *ACT1*-associated sequences from 10 epidemiologically unrelated *C. dubliniensis* isolates from disparate geographic locations showed a low level (0.002 %) of intraspecies sequence variation. In order to develop an accurate and rapid method to identify *C. dubliniensis* from primary isolation plates the significant divergence between the *C. dubliniensis* and *C. albicans ACT1* intron sequences was exploited by designing *C. dubliniensis*-specific PCR primers. Using a rapid boiling method to produce template DNA directly from colonies from primary isolation plates in 10 min, these primers were used in a blind test with 122 isolates of *C. dubliniensis*, 53 isolates of *C. albicans*, 10 isolates of *C. stellatoidea* and representative isolates of other clinically relevant *Candida* and other yeast species. Only the *C. dubliniensis* isolates yielded the expected *C. dubliniensis*-specific 288 bp amplimer. Use of this technique on colonies suspected to be *C. dubliniensis* allows their correct identification as *C. dubliniensis* in as little as 4 h.

A *C. dubliniensis* homologue of the *C. albicans* gene encoding the putative virulence factor Sap2 (*CaSAP2*) was cloned and sequenced from a recombinant phage isolated from a

genomic DNA λ library. Its nucleotide sequence was found to be 89.6 % identical to the corresponding *CaSAP2* sequence. A comparison of the *CdSAP2* nucleotide sequence with corresponding *SAP* gene sequences from *C. albicans*, *C. tropicalis* and *C. parapsilosis* revealed that the *CdSAP2* sequence is most closely related to that of *CaSAP2*. *CdSAP2* is predicted to encode a protein of 397 amino acids and this protein is 93.9 % identical to the corresponding CaSap2 protein. The deduced amino acid sequence of the CdSap2 protein exhibited many of the features common to previously characterised Saps, including a signal sequence, a Lys-Arg peptidase cleavage site, and two conserved aspartic acid residues known to be catalytically active in this class of enzymes. Northern hybridisation analysis of 8 strains of *C. dubliniensis* and 3 strains of *C. albicans* grown in the *SAP2* inducing medium YCB/BSA revealed that expression of the *SAP2* gene occurred later in the growth phase of *C. dubliniensis* than in *C. albicans* and the duration of expression of this transcript was found to be longer in *C. dubliniensis* than in *C. albicans*. Analysis of proteinase activity in the culture supernatants of the *C. dubliniensis* and *C. albicans* strains revealed that *C. dubliniensis* produced a higher level of proteinase activity than *C. albicans*.

The results of this study demonstrate unequivocally that *C. dubliniensis* is a discrete taxon within the genus *Candida*, phylogenetically distinct from but most closely related to *C. albicans*. Analysis of the *C. dubliniensis* and *C. albicans ACT1*-associated introns have allowed the development of a reliable and rapid definitive method of distinguishing between these two closely related species which will facilitate the in-depth epidemiological analysis of *C. dubliniensis*. The molecular analysis of the putative virulence factor gene *SAP2* revealed considerable differences at both the genotypic and phenotypic level, which warrant further investigation.

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Abbreviations

AIDS	Acquired Immunodeficiency Syndrome
A_{595}, A_{600}	absorption at 600_{nm}
ATP	adenosine 5'-triphosphate
bp	base pairs
BSA	bovine serum albumin
Bq	becquerel
c.f.u	colony forming units
Ci	curie
cm	centimeters
d.p.m	disintegrations per minute
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	dideoxynucleoside
dTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
e.g.	for example
et al.	and others
EtOH	ethanol
g	gram
g	gravity
h	hour(s)
HIV	human immunodeficiency virus
IPTG	isopropyl-β-D-thiogalactopyransoide
i.e.	that is
kb	kilobase pairs
kDa	kildalton
kg	kilogram
l	litre
log	logarithm (common)
M	molar
Mb	megabase
mg	milligram
µg	microgram
ml	millilitre
µl	microlitre
MIC	minimum inhibitory concentration

min	minute
mol wt	molecular weight
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
nm	nanometer
no.	number
OD	optical density
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PFGE	pulsed-field gel electrophoresis
p.f.u.	plaque forming units
RAPD	randomly amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNase	ribonuclease
mRNA	messenger ribonucleic acid
r.p.m.	revolutions per minute
s	seconds
SDS	sodium dodecyl sulphate
Tris	tris (hydroxymethyl) aminoethane
UK	United Kingdom
USA	United States of America
UV	ultraviolet
v/v	% "volume in volume" expresses the number of millilitres of an active constituent in 100 millilitres of solution
w/v	% "weight in volume" expresses the number of grams of an active constituent in 100 grams of solution or mixture
X-gal	5-bromo-4-chloro-indoyl-β-D-galactoside
~	approximately
>	greater than
<	less than

Publications

Some of the original work presented in this thesis has been published in refereed international publications as listed below. Offprints of published manuscripts are included at the end of the thesis.

Donnelly, S. M., Sullivan, D. J., Shanley, D. B., & Coleman, D. C. (1999). Phylogenetic analysis and rapid identification of *Candida dubliniensis* based on analysis of *ACT1* intron and exon sequences. *Microbiology* **145**, 1871-1882.

Sullivan D. J., Moran, G., Donnelly, S., Gee, S., Pinjon, E., McCartan, B., Shanley, D. B. & Coleman, D. C. (1999). *Candida dubliniensis*: An update. *Revista Iberoamericana de Micologia* **16**, 72-76.

Polacheck, I., Strahilevitz, J., Sullivan, D., Donnelly, S., Salkin, I. F. & Coleman, D. C. (2000). Recovery of *Candida dubliniensis* from non-human immunodeficiency virus-infected patients in Israel. *Journal of Clinical Microbiology* **38**, 170-174.

Chapter 1
General Introduction

1.1 General Introduction

1.1.1 Non-*Candida albicans Candida* infection

In recent years the frequency of opportunistic fungal infection has steadily increased, although *Candida* species remain the most common cause of this type of infection in the hospital setting (Eisenstein, 1990; Pfaller *et al.*, 1994 and 1996; Beck-Sague & Jarvis, 1993; Hazen *et al.*, 1995; Jarvis, 1995; Fridkin & Jarvis, 1996; Perfect & Schell, 1996). There are a number of reasons for this shift in the epidemiology of human fungal disease. Firstly, the numbers of immunocompromised patients have risen sharply over the last two decades. This patient group comprises the Human Immunodeficiency Virus (HIV)-infected population, individuals with chemotherapy-induced neutropaenia, immuosuppressed patients following organ transplantation or severe burns, diabetics, and premature low birth weight infants. Another contributory factor is the increasing use of invasive medical procedures, prosthetic devices, indwelling venous catheters, dentures and the widespread use of broad-spectrum antibiotics and corticosteroids (Klein *et al.*, 1984; Holmberg & Meyer, 1986; Bodey, 1988; Spencer & Jackson, 1989; Schaberg *et al.*, 1991; Wingard *et al.*, 1991; Dupont *et al.*, 1992; Samaranayake, 1992; Fox, 1993; Hazen *et al.*, 1995).

Candida albicans is the most frequent member of the genus *Candida* to be isolated from clinical specimens (Odds, 1988; Coleman *et al.*, 1993), but non-*C. albicans Candida* infections are increasingly being diagnosed as the cause of opportunistic infection (Wingard *et al.*, 1991 and 1993; Coleman *et al.*, 1995 and 1998; Fisher-Hoch *et al.*, 1995; Nguyen *et al.*, 1996; Pfaller *et al.*, 1996). In addition to the increase in the immunocompromised patient population, a second factor in the rise of *Candida* infections is the widespread therapeutic and prophylactic use of antifungal drugs such as fluconazole, which has led to the emergence of antifungal drug resistance (Powderly, 1992; Warnock *et al.*, 1992; Sullivan *et al.*, 1993; Coleman *et al.*, 1995; Denning, 1995; Johnson *et al.*, 1995; Boschman *et al.*, 1998). Furthermore, many of the non-*C. albicans Candida* species, such as *C. glabrata*, *C. tropicalis* and *C. krusei*, are inherently less susceptible than *C. albicans* to the azole drugs.

All of these aspects have contributed to the increasing importance of fungal infections, particularly infections caused by *Candida* species. Not surprisingly, several new fungal species have been described in recent years, some of which have been associated with disease in humans (Pfaller, 1994; Hazen, 1995; Perfect & Schell, 1996).

1.1.2 Atypical *Candida albicans* isolates

The identification and classification of fungal species has depended to a large extent on the analysis of a limited number of physiological and morphological traits, particularly those structures involved in sexual reproduction. The vast majority of species contained within the genus *Candida* are asexual and of simple morphology and subsequently *Candida* taxonomy is inherently problematic (Odds, 1988). It is not surprising, therefore, that coinciding with the dramatic increase in fungal opportunistic infections in the immunocompromised patient group came reports of the isolation of unusual *Candida* species that were referred to as "atypical *C. albicans*".

There were many reports of "atypical isolates" from laboratories around the world in the early 1990's and the majority of these were isolated from the oral cavity of HIV-positive individuals and AIDS patients (Odds *et al.*, 1990; Asakura *et al.*, 1991; Schmid *et al.*, 1992; Coleman *et al.*, 1993; Sullivan *et al.*, 1993; McCullough *et al.*, 1994 and 1995; Anthony *et al.*, 1995; Boerlin *et al.*, 1995; Le Guennec *et al.*, 1995; Tietz *et al.*, 1995). Phenotypically, these organisms were similar to *C. albicans* in that they produced germ tubes and chlamydospores. However, many of these isolates were found to yield atypical carbohydrate- and nitrogen-source substrate assimilation profiles when analysed with commercially available yeast identification systems such as the API 20C AUX and ID 32C systems, which did not correspond to any known *Candida* species. When these unusual isolates were tested they were found to agglutinate *C. albicans* serotype A antiserum and were sucrose-positive, two factors which distinguished these isolates from the closely related *C. stellatoidea*. *Candida stellatoidea* is closely related to *C. albicans* and this species has been divided into type I and type II *C. stellatoidea*. Both types react with *C. albicans* serotype B antigenic factor and,

unlike *C. albicans* are incapable of assimilating sucrose. Fingerprinting profiles of *C. stellatoidea* type II with the 27A probe are similar to those exhibited by *C. albicans* and subsequently type II is referred to as "sucrosed-negative *C. albicans*". Although 27A fingerprint profiles for type I are different from those of *C. albicans* it is now generally accepted that it is a variant *of C. albicans*. In addition, DNA fingerprint analysis of some of the atypical isolates with the *C. albicans*-specific repetitive-sequence containing fingerprinting probes 27A and Ca3 yielded fingerprint profiles unlike those of *C. albicans* or *C. stellatoidea* (Odds *et al.*, 1990; Asakura *et al.*, 1991; *Schmid et al.*, 1992; Sullivan *et al.*, 1993; Anthony *et al.*, 1995; Boerlin *et al.*, 1995; Le Guennec *et al.*, 1995; McCullough *et al.*, 1995). These atypical isolates also yielded unusual karyotype profiles (Mahrous *et al.*, 1990; Asakura *et al.*, 1991; Boerlin *et al.*, 1995; McCullough *et al.*, 1995; Tietz *et al.*, 1995).

In their report of 1993 Sullivan *et al.* postulated that atypical isolates recovered in Ireland were either sucrose-positive, serotype A variants of *C. stellatoidea*, or comprised a hitherto undescribed species of *Candida*. In order to resolve the taxonomic position of these atypical organisms, Sullivan *et al.* (1995) carried out an exhaustive study of the phenotypic and molecular characteristics of atypical isolates recovered in Ireland and Australia. The authors concluded that these organisms represented a distinct taxon within the genus *Candida* phylogenetically distinct from *C. albicans* and other *Candida* species, for which the name *C. dubliniensis* was proposed. Subsequent studies demonstrated that *C. dubliniensis* has a widespread geographic distribution especially in HIV-infected patient groups (Sullivan *et al.*, 1997, 1998 and 1999). A complete review of the characteristics of *C. dubliniensis* is provided below.

1.1.3 Phenotypic characteristics of *C. dubliniensis*

Candida dubliniensis shares many phenotypic characteristics in common with *C. albicans* (Sullivan *et al.*, 1995). It grows well at 30 °C and 37 °C on media used routinely for the isolation and culture of yeast such as Potato Dextrose Agar (PDA), Sabouraud Dextrose Agar (SDA) and Yeast Peptone Dextrose broth (YPD). On solid media its colonial

morphology is almost identical to that of *C. albicans* with both species producing colonies of similar size, shape and colour. Furthermore, both species are capable of the phenomenon of phenotypic switching. *Candida dubliniensis* strains that exhibit phenotypic switching have been observed to form small petite colonies and others have been observed to produce small, wrinkled colonies.

Candida dubliniensis is not as capable as *C. albicans* of growth at higher temperatures. Isolates of *C. dubliniensis* grow poorly or not at all at 42 °C, and fail to grow at 45 °C (Sullivan *et al.*, 1995; Pinjon *et al.*, 1998). In contrast, the majority of *C. albicans* isolates grow well at both temperatures. Growth at a temperature of 45 °C was described as the basis of a simple test to distinguish between the two species (Pinjon *et al.*, 1998), however, some isolates of *C. albicans* have been found that do not grow at this elevated temperature (Kirkpatrick *et al.*, 1998; Gales *et al.*, 1999). Generally, *C. albicans* appears to have a growth advantage over *C. dubliniensis*. In rich media such as YPD broth, the doubling times of *C. dubliniensis* strains are longer than those of *C. albicans* (S. Donnelly, unpublished data). Furthermore, in mixed cultures *C. albicans* shows a competitive advantage over *C. dubliniensis* (Kirkpatrick *et al.*, 2000).

Candida dubliniensis produces a distinctive colonial appearance on particular differential agars. A commonly used medium for the identification of medically important *Candida* species is the commercially available CHROMagar Candida medium (Odds & Bernaerts, 1994). On this media *C. albicans* colonies are a light blue-green colour, whereas *C. dubliniensis* colonies are a dark green colour (Schoofs *et al.*, 1997; Sullivan *et al.*, 1998; Koehler *et al.*, 1999). However, this medium is only useful for the preliminary isolation of *C. dubliniensis* following primary culture from clinical specimens as its distinctive colony colouration may be lost following storage and subculture (Schoofs *et al.*, 1997). Other differential media include methyl blue Sabouraud agar (Schoofs *et al.*, 1997). *Candida albicans* colonies grown on this medium fluoresce with a yellow colour upon exposure to UV light, but *C. dubliniensis* colonies fail to do so. However, an absence of fluorescence has been observed in some isolates of *C. albicans* (Schoofs *et al.*, 1997). Other researchers have

discussed the use of various differential agars for *C. dubliniensis* such as Pagano Levin agar (a medium that contains 2,3,5-triphenyltetrazolium chloride incorporated into Sabouraud agar; Velegraki *et al.*, 1998) and Staib agar (Staib & Morschhäuser, 1999). *Candida dubliniensis* has been reported to reduce the compound 2,3,5-triphenyltetrazolium chloride and, subsequently, forms purple colonies on Pagano-Levin agar. In contrast, the colour of *C. albicans* colonies on this medium varies from white to pale pink. However, examination of the colonial morphology of 50 isolates each of *C. dubliniensis* and *C. albicans* revealed that the colour of the *C. dubliniensis* colonies ranged from white through pink to purple, and were indistinguishable from *C. albicans* colonies (S. Donnelly & D. Coleman, unpublished data). Staib agar is widely used for the identification of *Cryptococcus neoformans* in clinical specimens from AIDS patients (Staib *et al.*, 1987; Polacheck, 1991). A recent study has reported that this agar may be used to differentiate between *C. albicans* and *C. dubliniensis* as the latter species forms hyphae and abundant chlamydospores following incubation on this agar at 30 °C (Staib & Morschhäuser, 1999). A more exhaustive study showed that 97.7 % of C. *dubliniensis* isolates produced rough colonies and all *C. albicans* colonies produced smooth colonies. However, only 85.4 % of *C. dubliniensis* isolates tested produced chlamydospores. Therefore, discrimination between these two species using Staib agar should be based on colonial morphology alone (Al Mosaid *et al.*, in press).

The ability of *C. albicans* to produce germ tubes and chlamydospores were traits previously considered diagnostic for this species. However, *C. dubliniensis* is also capable of germ tube production upon incubation in serum (Sullivan *et al.*, 1995), although, unlike *C. albicans*, it does not produce germ tubes when incubated in N-acetyl glucosamine-containing medium (Gilfillan *et al.*, 1998). *Candida dubliniensis* also produces chlamydospores when grown on media such as rice Tween 80 agar (RAT), Tween 80-oxgall-caffeic acid (TOC) or cornmeal agar (Sullivan *et al.*, 1995; Jabra-Rizk *et al.*, 1999; Koehler *et al.*, 1999). These structures are thick walled refractile cells of unknown function. In *C. albicans* they are usually produced singly and attached terminally to hyphae and pseudohyphae via single suspensor cells. In contrast, *C. dubliniensis* tends to produce abundant numbers of chlamydospores and

these are often arranged in contiguous pairs or triplets or sometimes in greater multiples (Sullivan *et al.*, 1995). As many as seven chlamydospores have been observed attached to a single suspensor cell. The chlamydospores are found not only terminally attached to short pseudohyphae but unilateral and bilateral attachment has also been found. However, this unusual chlamydospore arrangement has not been found to be reproducible in all laboratories with all *C. dubliniensis* isolates tested and, therefore, chlamydospore production may not be used to differentiate between *C. albicans* and *C. dubliniensis* (Schoofs *et al.*, 1997; Kirkpatrick *et al.*, 1998).

All isolates of *C. dubliniensis* tested to date have been found to react with *C. albicans* serotype A antiserum as determined by agglutination reactions with polyvalent antibodies raised against *Candida* antigenic factor No. 6, and with serotype A-specific antisera using flow cytometry (Sullivan *et al.*, 1995; Mecure *et al.*, 1996).

Several studies have shown that *C. dubliniensis* yields unusual carbohydrate assimilation profiles with commercial API yeast identification systems. These systems are the most commonly used for identifying *Candida* species and are used routinely in diagnostic laboratories. The method is based on the ability or inability of an isolate to grow on a range of specific substrates. The pattern of substrate assimilation yields a numerical code, which is then compared to a database and leads to the identification of the isolate. However, the atypical substrate assimilation profiles generated by *C. dubliniensis* isolates with either system yielded numerical codes that did not correspond with any known species in either database. Furthermore, some isolates generated codes that gave low discrimination profiles which corresponded to poor identification of seldom isolated species such as *C. stellatoidea*, *C. sake* and *C. colliculosa* (Boerlin *et al.*, 1995; McCullough *et al.*, 1995; Sullivan *et al.*, 1995; Coleman *et al.*, 1997a; Schoofs *et al.*, 1997; Sullivan *et al.*, 1997; Kirkpatrick *et al.*, 1998; Salkin *et al.*, 1998; Gales *et al.*, 1999; Jabra-Rizk *et al.*, 1999; Tintelnot *et al.*, 2000). In 1998, the API system database was updated to include limited *C. dubliniensis* profile data. A study by Pincus *et al.* (1999) has suggested further modifications of this database to include more

extensive *C. dubliniensis* profile data would result in the ability of these systems to correctly identify *C. dubliniensis* isolates.

1.1.4 Genotypic characteristics of *C. dubliniensis*

Despite the phenotypic similarity between *C. albicans* and *C. dubliniensis*, the differences at the genetic level are considerable. It was these genetic differences that originally led to *C. dubliniensis* being designated as a separate species (Sullivan *et al.*, 1995).

The technique of DNA fingerprinting provides evidence of the genetic differences between *C. dubliniensis* and *C. albicans*. When genomic DNA from *C. dubliniensis*, *C. albicans* and *C. stellatoidea* was digested separately with *Eco*RI and *Hin*fI and the fragments separated by gel electrophoresis, a direct visual analysis of the patterns obtained allowed *C. dubliniensis* isolates to be separated from *C. albicans*, and *C. stellatoidea* type I and type II isolates. These differences were more readily discernable when the digested DNA preparations were transferred to nylon membranes and hybridised to the probe 27A. The *C. albicans*-specific fingerprinting probe 27A corresponds to a repetitive DNA sequence, which is dispersed throughout the *C. albicans* genome, and is closely related to the Ca3 fingerprinting probe (Scherer & Stevens, 1988; Coleman *et al.*, 1993; Sullivan *et al.*, 1993). This probe generates fingerprint patterns consisting of 10-15 strongly hybridising bands with *C. albicans* genomic DNA that has been digested with *Eco*RI. The probe is useful in the epidemiological analysis of a wide range of infections caused by *C. albicans*. In contrast, *C. dubliniensis* *Eco*RI restricted genomic DNA yields a fingerprint pattern consisting of 4-7 weak hybridisation bands that is very distinct to the fingerprint pattern of *C. albicans*. *Candida dubliniensis* 27A fingerprint profiles are also distinct from those obtained with *C. stellatoidea* type I and type II isolates. Type II *C. stellatoidea* (i.e. sucrose-negative *C. albicans*) patterns are similar to *C. albicans* patterns, but type I *C. stellatoidea* patterns are somewhat different from *C. albicans*. Other distinct 27A fingerprint profiles were generated when the *C. dubliniensis* genomic DNA was digested with the restriction endonuclease *Hin*fI. These profiles are characterised by one or more very large bands of approximately 20 kb that

hybridise to the 27A probe (Odds *et al.*, 1990; Schmid *et al.*, 1992; McCullough *et al.*, 1994; Anthony *et al.*, 1995; Boerlin *et al.*, 1995; McCullough *et al.*, 1995; Boucher *et al.*, 1996; Schoofs *et al.*, 1997; Sullivan *et al.*, 1997).

Fingerprinting patterns obtained with the five synthetic oligonucleotide probes $(GGAT)_4$, $(GACA)_4$, $(GATA)_4$ $(GT)_8$ and $(GTG)_5$ with *Eco*RI and *Hin*fI digested DNA showed that *C. dubliniensis* profiles were, overall, very similar to each other, yet distinct from *C. albicans* and *C. stellatoidea* type I profiles (Sullivan *et al.*, 1993 and 1995). These five oligonucleotide probes have also been used in RAPD analysis of *C. dubliniensis* DNA. Again the RAPD profiles of *C. dubliniensis* isolates were similar to each other but distinct from those obtained with *C. albicans* and *C. stellatoidea* isolates. Further RAPD profiles employing four different oligonucleotide primers also resulted in the generation of RAPD patterns for *C. dubliniensis* isolates which were similar for all *C. dubliniensis* isolates tested but were different from those produced by *C. albicans* and *C. stellatoidea* isolates (Sullivan *et al.*, 1995 and 1997; Coleman *et al.*, 1997a). Furthermore, *C. dubliniensis* can be readily distinguished from *C. albicans* and both type I and type II *C. stellatoidea* on the basis of significant differences in *Hin*fI-generated RFLP patterns (Sullivan *et al.*, 1995, 1996 and 1997; Sullivan & Coleman, 1997; Kirkpatrick *et al.*, 1998; McCullough *et al.*, 1999).

The *C. dubliniensis* karyotype profile is also very distinct from that of *C. albicans* (Sullivan *et al.*, 1995 & 1997; Coleman *et al.*, 1997a; Jabra-Rizk *et al.*, 1999). *Candida albicans* isolates tend to produce karyotype profiles consisting of seven distinct chromosome sized bands (*C. albicans* contains 8 chromosomes although only 7 chromosome sized bands are visible in routine PFGE gels). In contrast, *C. dubliniensis* profiles consist of 9 or 10 individual chromosome sized bands. A characteristic feature of *C. dubliniensis* karyotype profiles is the presence of one or more chromosome-sized bands less than 1 Mb in size. This is a feature also displayed by the reference *C. stellatoidea* type I strain ATCC 11006 (Sullivan *et al.*, 1995 & 1997; Coleman *et al.*, 1997a). The hybridisation of chromosome-specific probes to karotype blots suggests that the genomes of many *C. dubliniensis* isolates may have undergone numerous rearrangements such as translocations and fragmentation (Sullivan *et al.*, 1996).

1.1.5 Phylogenetic analysis of *C. dubliniensis*

Candida dubliniensis is phenotypically very similar to *C. albicans*. However, there are considerable differences between the two species at the genetic level. A combination of phenotypic and genotypic differences between *C. albicans* and what was originally considered to be "atypical" *C. albicans* isolates led to the conclusion that these isolates could be either variants of *C. stellatoidea* type I or an entirely new species (Sullivan *et al.*, 1993; Sullivan *et al.*, 1995). It was considered unlikely that these atypical isolates were variants of *C. stellatoidea* as, unlike *C. stellatoidea*, they were capable of assimilating sucrose. Furthermore, *C. stellatoidea* belongs to *C. albicans* serotype B, whereas the atypical isolates belonged to serotype A.

The degree of genetic difference between *C. albicans* and the atypical isolates needed to be quantified in order to determine the phylogenetic relationship between the atypical isolates and other *Candida* species, including *C. albicans*. In order to achieve this a phylogenetic analysis of the large and small ribosomal subunit genes was carried out. Initially the V3 variable regions of the large subunit gene from atypical *C. albicans* isolates and various other *Candida* species were compared (Sullivan *et al.*, 1995). In this study a 600 bp fragment spanning this region was amplified using PCR from 8 separate isolates of *C. dubliniensis*, including 3 Irish isolates, 3 Australian isolates and a single UK isolate, and from reference strains of *C. albicans*, *C. stellatoidea*, *C. tropicalis*, *C. glabrata*, *C. kefyr* and *C. krusei*. The DNA sequence of each amplimer was obtained and compared using a variety of computer software programs. This analysis demonstrated that all of the *C. dubliniensis* isolates were completely identical to each other but distinct from the other species tested.

These data were then used to construct a phylogenetic tree from which *C. dubliniensis* was found to form a totally distinct cluster, clearly separate from the other species examined. The most closely related species to *C. dubliniensis* was *C. albicans* with a sequence divergence in this region of 2.25-2.48 %. This study also indicated that *C. albicans* and *C. stellatoidea* were so closely related (0-0.02 % sequence divergence) as to be considered a single species. This relationship was confirmed by the analysis of the large subunit rRNA V3

region from a further 5 *C. dubliniensis* isolates from Ireland, UK, Argentina and Switzerland (Sullivan *et al.*, 1997).

The phylogenetic position of *C. dubliniensis* has since been confirmed by DNA sequence analysis of the entire small subunit rRNA gene (SSU rRNA) of the *C. dubliniensis* type strain CD36 and comparison of this with the SSU rRNA genes from *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. lusitaniae*, *C. krusei* and *Saccharomyces cerevisiae* revealed that the *C. albicans* small subunit rRNA gene was most closely related to that of *C. dubliniensis* with a nucleotide sequence divergence of 1.4 % between the two genes (Gilfillan *et al.*, 1998). An evolutionary tree generated from these sequence comparisons indicated that *C. dubliniensis* is phylogenetically distinct from other *Candida* species including *C. albicans*.

Kurtzman & Robnett (1997) analysed a 600 bp region of the D1/D2 variable region of the large subunit rRNA gene (LSU rRNA) from all known clinically significant yeasts. They found *C. dubliniensis* formed a discrete taxon within the genus *Candida* based on their data. Furthermore, an analysis of the self-splicing intron in the LSU rRNA gene of *C. albicans*, *C. stellatoidea* and *C. dubliniensis* provided further evidence of the distinct phylogenetic position of *C. dubliniensis* (Boucher *et al.*, 1996). The sequence of this intron from *C. albicans* and *C. dubliniensis* revealed that, despite a considerable degree of homology between the two introns, there were significant differences present also. This observed sequence homology is unusual, as self-splicing group I introns exhibit poor sequence conservation, and it is indicative of the close relationship between these two species. Furthermore, the sequence of the group I intron from a number of *C. dubliniensis* isolates revealed an intraspecies sequence conservation which was also observed in the introns sequenced from different isolates of *C. albicans* and *C. stellatoidea*. A comparison of the introns from *C. albicans* and *C. stellatoidea* also confirmed that these two organisms could be considered the same species (Boucher *et al.*, 1996).

All of these studies based upon comparisons of both small and large subunit rRNA nucleotide sequence data provided convincing evidence that *C. dubliniensis* was indeed a separate species clearly distinct from *C. albicans* within the genus *Candida*.

1.1.6 Isolation of *C. dubliniensis* from clinical specimens

The ability to identify a species in clinical samples as accurately and as rapidly as possible is essential in order to assess fully the clinical importance of that species and to carry out in depth epidemiological studies. The close relationship that exists between *C. dubliniensis* and *C. albicans* has been a significant factor in the methods used to identify these species.

The most pronounced differences between the two species are genetic and the most reliable methods capable of unequivocal differention between the two species are based on molecular techniques (Sullivan *et al.*, 1995). However, the techniques used to detect these genetic differences are generally time-consuming and expensive and not readily applicable to large numbers of isolates. Various methods have included hybridisation to the *C. albicans*-specific probe 27A or Ca3 probes (Boerlin *et al.*, 1995; McCullough *et al.*, 1995; Sullivan *et al.*, 1995; Coleman *et al.*, 1997a; Schoofs *et al.*, 1997; Sullivan *et al.*, 1997; Kirkpatrick *et al.*, 1998; Odds *et al.*, 1998), or hybridisation to the *C. dubliniensis*-specific probe Cd2 (Joly *et al.*, 1999), a *C. dubliniensis* species-specific molecular beacon (Park *et al.*, 2000) and a PCR-based line probe assay (Martin *et al.*, 2000). Other methods include oligonucleotide fingerprinting, karyotype analysis, multilocus enzyme electrophoresis, RAPD and RFLP and rRNA sequence analysis (Boerlin *et al.*, 1995; Sullivan *et al.*, 1995, 1996 and 1997; Kirkpatrick *et al.*, 1998; Jabra-Rizk *et al.*, 1999; McCullough *et al.*, 1999). There are a number of PCR-based techniques also described in the literature which detail either *C. dubliniensis*-specific primers or *C. albicans*-specific primers that fail to amplify *C. dubliniensis* DNA (Elie *et al.*, 1998; Manarelli & Kurtzman, 1998; Kurzai *et al.*, 1999; Tamura *et al.*, 2000).

In the diagnostic laboratory setting the identification of *C. albicans* is based upon a positive germ tube test and/or a positive chlamydospore test and other *Candida* species may be identified if required by commercial substrate assimilation systems. As *C. dubliniensis* is also germ tube- and chlamydospore-positive, this test does not distinguish it from *C. albicans*. Initially, the atypical substrate assimilation profiles yielded by *C. dubliniensis* isolates were used for their identification (Boerlin *et al.*, 1995; McCullough *et al.*, 1995; Sullivan *et al.*, 1995; Coleman *et al.*, 1997a; Schoofs *et al.*, 1997; Sullivan *et al.*, 1997; Kirkpatrick *et al.*,

1998; Salkin *et al.*, 1998; Gales *et al.*, 1999; Jabra-Rizk *et al.*, 1999; Tintelnot *et al.*, 2000). Although the database has been updated recently, further modifications are required to enable these systems to correctly identify *C. dubliniensis* isolates (Pincus *et al.*, 1999).

The use of *C. dubliniensis* phenotypic characteristics as the basis of a definitive identification test has encountered numerous problems. Perhaps the most important aid in the analysis of *Candida* populations in clinical specimens has been the development of CHROMagar Candida medium (Odds & Bernaerts, 1994). Although *C. dubliniensis* produces distinctive dark green colonies on this agar on primary isolation, this property may be unstable following subculture or prolonged storage (Schoofs *et al.*, 1997). Furthermore, incubation conditions when using this medium are critical as prolonged growth of *C. dubliniensis* on this medium results in an appearance similar to *C. albicans* (Schoofs *et al.*, 1997; Pfaller *et al.*, 1999). Others have reported that *C. albicans* colonies may produce a similar dark colour to *C. dubliniensis* (Tintelnot *et al.*, 2000). For these reasons, it has been recommended that this agar be used only for the presumptive identification of *C. dubliniensis* on primary isolation from clinical specimens (Sullivan *et al.*, 1999).

Initially, poor growth or lack of growth at 42 °C was considered typical for *C. dubliniensis* (Sullivan *et al.*, 1995). However, isolates that exhibit poor growth or good growth at this temperature, have been reported (Coleman *et al.*, 1997a and 1997b; Schoofs *et al.*, 1997; Sullivan *et al.*, 1997; Sullivan & Coleman, 1997 and 1998; Kirkpatrick *et al.*, 1998; Pinjon *et al.*, 1998). Subsequently, the inability of *C. dubliniensis* to grow at the higher temperature of 45 °C has been cited as a simple and inexpensive method of distinguishing between the two species (Pinjon *et al.*, 1998). However, other studies have reported that some isolates of *C. albicans* fail to grow at this temperature also (Kirkpatrick *et al.*, 1998; Gales *et al.*, 1999). Nevertheless, lack of growth at 45 °C remains a simple screening test for *C. dubliniensis* and has been used as such by other researchers (Odds *et al.*, 1998; Jabra-Rizk *et al.*, 1999 and 2000).

Various other discriminatory tests for *C. dubliniensis* based upon phenotypic characteristics have been described in the literature. An immunofluorescence test based on the

antibody detection of differential antigen expression on *C. dubliniensis* blastospores and *C. albicans* germ tubes has been described by Bikandi and colleagues. (1998). This method takes less than 2 h to perform and correctly identified 85 isolates of *C. dubliniensis*. Unfortunately, these antibodies are not widely available and therefore this method is not yet suitable for widespread diagnostic use.

A simple 3-minute test based on the ability of *C. dubliniensis* to coaggregate with *Fusobacterium nucleatum* has been developed by Jabra-Rizk *et al.* (1999a). This method has been used in another study with success (Jabra-Rizk *et al.*, 2000), but it has not yet been evaluated by other researchers.

The lack of intracellular β-glucosidase activity was first described as characteristic of atypical isolates subsequently identified as *C. dubliniensis* in 1995 (Boerlin *et al.*, 1995). However, a more recent study has shown that while all *C. dubliniensis* isolates tested so far do not exhibit β-glucosidase activity a substantial proportion of *C. albicans* strains tested have also proved negative for this test (Odds *et al.*, 1998; Tintelnot *et al.*, 2000).

Pyrolysis-mass spectrometry and Fourier transform-infrared spectroscopy have been described to be as accurate as genotypic methods for the identification of *C. dubliniensis* (Timmins *et al.*, 1998; Tintlenot *et al.*, 2000). *Candida dubliniensis* may also be differentiated from *C. albicans* by fatty acid methyl ester analysis using gas liquid chromatography (Peltroche-Llacsahuanga *et al.*, 2000). However, these three techniques are not widely available and are unlikely to be used for routine screening in diagnostic laboratories.

In summary, the gold standard methods for the identification of *C. dubliniensis* are the molecular based techniques. However, these are not suitable for use in most diagnostic laboratories. The most suitable way of identifying *C. dubliniensis* in the diagnostic laboratory would be to presumptively identify the organism on a differential medium followed by species confirmation using a substrate assimilation system such as the API 20 C AUX or ID 32C. However, this process is relatively expensive and as isolation on CHROMagar takes 48 h and the commercial yeast systems take 48-72 h, the identification of *C. dubliniensis* based on phenotypic methods is very time consuming.

1.1.7 Incidence and clinical significance of *C. dubliniensis*

There is limited data on the incidence of *C. dubliniensis* due to its recent recognition as a distinct species within the genus *Candida* and the problems encountered in the definitive identification of this organism. However, since its description by Sullivan *et al.* in 1995 it has been identified by laboratories worldwide in a variety of clinical settings (Boerlin *et al.*, 1995; McCullough *et al.*, 1995; Boucher *et al.*, 1996; Hannula *et al.*, 1997; Pujol *et al.*, 1997; Sullivan *et al.*, 1997; Sullivan & Coleman, 1997; Bikandi *et al.*, 1998; Elie *et al.*, 1998; Kirpatrick *et al.*, 1998; Odds *et al.*, 1998; Rodero *et al.*, 1998; Salkin *et al.*, 1998; Velegraki *et al.*, 1998; Jabra-Rizk *et al.*, 1999b; Joly *et al.*, 1999; McCullough *et al.*, 1999; Meis *et al.*, 1999; Polacheck *et al.*, 2000). The precise role of *C. dubliniensis* as a cause of disease has still to be elucidated.

To date the majority of *C. dubliniensis* isolates have been recovered from the oral cavities of HIV-infected individuals, including intravenous drug users, homosexuals and hemophiliacs (Sullivan *et al.*, 1995; Coleman *et al.*, 1997a and 1997b; Sullivan & Coleman, 1998). The organism has also been recovered from the oral cavities of HIV-negative intravenous drug users and from healthy individuals (Sullivan *et al.*, 1995; Moran *et al.*, 1997; Odds *et al.*, 1998; Pinjon *et al.*, 1998; Meis *et al.*, 1999; Brandt *et al.*, 2000; Kamei *et al.*, 2000; Polacheck *et al.*, 2000). A number of studies have been carried out to determine the incidence of *C. dubliniensis*. The incidence of *C. dubliniensis* amongst HIV-infected individuals appears to be higher than that of HIV-negative individuals. The percentage of HIV-infected individuals and AIDS patients from whom *C. dubliniensis* has been recovered varies from 15 % (McCullough *et al.*, 1995) to 24.2 % (Sullivan *et al.*, 1993). The best available data on the incidence of *C. dubliniensis* comes from an Irish study showed that *C. dubliniensis* was recovered from 32 % of AIDS patients presenting with clinical symptoms of oral candidosis and from 25 % of asymptomatic AIDS patients (Coleman *et al.*, 1997b). A high incidence of recovery of *C. dubliniensis* was also recorded for Irish HIV-infected individuals both symptomatic (27 %) and asymptomatic (19 %). In contrast to these findings among the HIV and AIDS populations, the incidence of *C. dubliniensis* in the Irish HIV-

negative population without oral candidosis was found to be only 3 %, and in HIV-negative individuals with denture-associated oral candidosis the incidence of *C. dubliniensis* was found to be 14.6 % (Coleman *et al.*, 1997b). The available data suggests that *C. dubliniensis* forms a small part of the normal flora in the healthy individual. The majority of HIV-positive and AIDS patients (76 %) and HIV-negative individuals (83 %) from whom *C. dubliniensis* was recovered were also found to harbour other *Candida* species in the oral cavity (Coleman *et al.*, 1997b). The most commonly isolated of these was *C. albicans* followed by *C. glabrata*, *C. tropicalis* and *C. krusei*. In some cases two or more of these species were co-isolated with *C. dubliniensis*.

Other details on the incidence of *C. dubliniensis* have come from retrospective studies on culture collections. A study carried out on an Irish archival culture collection found the 1.82 % of *C. albicans* isolates from asymptomatic normal healthy individuals were in fact *C. dubliniensis*, and 16.46 % of *C. albicans* isolates recovered from HIV-positive individuals were *C. dubliniensis* (Sullivan *et al.*, 1997). Odds *et al.* (1998) found that of a stock collection of 2,588 yeasts originally identified as *C. albicans*, 2.1 % of these were isolates of *C. dubliniensis* that had been misidentified. The majority of these newly identified *C. dubliniensis* isolates were originally recovered from oral and faecal samples, whilst a single isolate was recovered from a vulvovaginal sample. Furthermore, a significant proportion (24.7 %) of *C. dubliniensis* isolates from this study were recovered from HIV-infected individuals. The incidence of *C. dubliniensis* from healthy individuals was 11.8 % (Odds *et al.*, 1998). A retrospective study by Jabra-Rizk and colleagues (2000) concluded that of a collection of 1,251 isolates from the USA, originally identified as *C. albicans*, 1.2 % of these were in fact *C. dubliniensis*. This study also found a close association of *C. dubliniensis* with immunocompromised patients, including HIV-positive and AIDS patients. Meiller *et al.* (1999) have found that *C. dubliniensis* was isolated in 25 % of HIV-positive patients. They believe that the presence of *C. dubliniensis* may be related to high viral load, rapid AIDS progression and /or concomitant oral disease. A prospective study by Jabra-Rizk and colleagues (1999) also found that 5/25 HIV-positive individuals were found to harbour *C.*

dubliniensis in their oral cavities. *Candida dubliniensis* would seem to be particularly prevalent in the oral cavities of HIV-infected or AIDS patients.

The presence of *C. dubliniensis* in the oral cavity has been associated with disease in some individuals. Coleman *et al.* (1997b) found that 6 % of HIV-positive patients presenting with symptoms of oral candidosis were found to harbor *C. dubliniensis* only. In patients with full-blown AIDS this figure rose to 10 %. Fluconazole treatment of these patients resulted in clinical resolution and the failure to recover any yeast species. This suggests that *C. dubliniensis* was responsible for the original symptoms of oral candidosis. Furthermore, Velegraki *et al.* (1999) found that *C. dubliniensis* may be implicated in an unusual form of linear gingival erythematous candidosis.

Despite its association with the oral cavity of HIV-positive individuals and AIDS patients the incidence of *C. dubliniensis* predates the AIDS pandemic. One of the earliest known isolates of *C. dubliniensis* was deposited in the British National Collection for Pathogenic Fungi as *C. stellatoidea* in 1957 (Sullivan *et al.*, 1995). Another isolate was deposited in the Centraal Bureau voor Schimmelcultures in Holland in 1952 as *C. albicans* (Meis *et al.*, 1999). *Candida dubliniensis* has also been isolated from a variety of other clinical specimens and anatomical sites including the vagina, urine and faecal samples, blood cultures, and abdominal wounds (Sullivan *et al.*, 1995; Odds *et al.*, 1998; Meis *et al.*, 1999; Brandt *et al.*, 2000; Kamei *et al.*, 2000; Polacheck *et al.*, 2000). It has also been isolated from other immunocompromised patient groups including paediatric AIDS cases, HIV-positive children, HIV-negative individuals with chemotherapy-induced neutropaenia and bone marrow transplantation patients (Redding *et al.*, 1999; Velgraki *et al.*, 1999; Brown *et al.*, 2000; Sano *et al.*, 2000). Furthermore, *C. dubliniensis* has been associated with carriage and disease in the oral cavities of insulin dependant diabetics (Willis *et al.*, 2000).

1.1.8 Antifungal drug resistance and virulence of *C. dubliniensis*

Candida dubliniensis is most frequently isolated from HIV-positive and AIDS patients, a cohort that is subject to frequent antifungal therapy. This led to the suggestion that the recent

emergence of *C. dubliniensis* as a human pathogen may be a result of selection due to antifungal drug resistance (Coleman *et al.*, 1997b). This would not appear to be the case as the majority of *C. dubliniensis* isolates are susceptible to the most commonly used antifungals and to novel antifungal drugs (Moran *et al.*,1997; Kirpatrick *et al.*, 1998; Odds *et al.*, 1998; Ryder *et al.*, 1998; Meiller *et al.*, 1999; Pfaller *et al.*, 1999; Jabra-Rizk *et al.*, 1999b and 2000; Brandt *et al.*, 2000; Brown *et al.*, 2000; Polacheck *et al.*, 2000). However, a number of studies have documented isolates that show a dose dependant susceptibility (Moran *et al.*, 1997; Kirkpatrick *et al.*, 1998; Odds *et al.*, 1998; Jabra-Rizk *et al.*, 2000). One study revealed that the MIC values of *C. dubliniensis* are significantly and consistently higher than those of *C. albicans* isolates (Odds *et al.*, 1998). Both *C. albicans* and *C. dubliniensis* have been shown to be capable of developing resistance to fluconazole following repeated exposure to the drug in patients (Moran *et al.*, 1998; Ruhnke *et al.*, 2000). Moran and co-workers characterised two isolates recovered 18 months apart from a single patient, one of which was susceptible to fluconazole and the other was fluconazole-resistant. Molecular typing showed that the fluconazole-susceptible and fluconazole-resistant isolates were in fact the same strain and this strain had developed *in vivo* resistance to fluconazole (Moran *et al.*, 1998). Ruhnke and colleagues isolated fluconazole-susceptible and -resistant *C. albicans* and *C. dubliniensis* strains from a single patient. Molecular typing revealed that the patient was persistently colonised by the same strain of both species and that both species developed resistance after 3 years of asymptomatic colonisation (Ruhnke *et al.*, 2000).

A stable resistance to both fluconazole and itraconazole may be induced in *C. dubliniensis* following exposure to these drugs *in vitro* (Moran *et al.*, 1997 and 1998; E. Pinjon, personal communication). Resistance to other antifungal drugs such as ketoconazole, amphotericin B, voriconazole and a range of novel agents, including triazoles and echinocandins, has not yet been reported in *C. dubliniensis* (Ryder *et al.*, 1998; Pfaller *et al.*, 1999). The mechanism of resistance to fluconazole appears to differ between the *C. albicans* and *C. dubliniensis*. In both *in vitro*-generated fluconazole-resistant derivatives and in clinically resistant *C. dubliniensis* isolates overexpression of the major facilitator protein Mdr1

appears to be largely responsible for the resistance phenotype (Moran *et al.*, 1998). In contrast, although the ABC transporters Cdr1 and Cdr2 and the major facilitator protein Mdr1 play important roles in reducing the intracellular fluconazole content in *C. albicans*, overexpression of the protein Cdr1 is the more common mechanism of resistance in *C. albicans* (Sanglard *et al.*, 1995; Albertson *et al.*, 1996).

The fact that *C. albicans* is the most common yeast pathogen in man and that *C. dubliniensis* has only recently been associated with disease would suggest that *C. albicans* is the more successful pathogen. *In vitro* competitive studies between the two species indicate that *C. albicans* has a competitive advantage over *C. dubliniensis* in broth culture and under biofilm growing conditions (Kirkpatrick *et al.*, 2000). However, the presence of a supporting structure for biofilm formation enables *C. dubliniensis* to tolerate more successfully the competitive pressures from *C. albicans*. This may parallel the *in vivo* situation as, in clinical samples where both species are co-isolated, *C. albicans* is usually the predominant species (Sullivan *et al.*, 1993; Coleman *et al.*, 1997a and 1997b).

Very few studies to date have been concerned with investigating the virulence factors of *C. dubliniensis*. One notable difference involves the kinetics of hyphal formation. In a limited study using four *C. dubliniensis* isolates the production of hyphae appeared to be slower in *C. dubliniensis* than in a reference *C. albicans* strain (Gilfillan *et al.*, 1998). In this study, *C. dubliniensis* did not produce hyphae following growth in N-acetyl-D-glucosamine medium whereas *C. albicans* did. It is possible that the slower kinetics of hyphal formation of *C. dubliniensis* may adversely affect its ability to invade tissue and contribute to its apparently lower virulence.

Only one animal model study has been reported which investigated the *in vivo* virulence of four *C. dubliniensis* isolates (Gilfillan *et al.*, 1998). These four isolates were less virulent than the reference *C. albicans* strain used when an inoculum size of $2x\ 10^6$ cells per mouse was used. When a higher inoculum was used (1×10^7) there was a wide variation in the mean survival times amongst the mice infected with *C. dubliniensis*.

Candida dubliniensis has been reported to be more adherent to buccal epithelial cells than *C. albicans* (McCullough *et al.*, 1995, Gilfillan *et al.*, 1998). Gilfillan and colleagues (1998) showed that *C. dubliniensis* was significantly more adherent when grown in glucose-containing medium than when grown in galactose-containing medium. In contrast, *C. albicans* has been shown to be more adherent to epithelial cells when grown in galactose-containing medium than when grown in glucose-containing medium (Douglas *et al.*, 1981). The effect of glucose on the adherence of *C. dubliniensis* may be relevant given the organism's predilection for the oral cavity. Furthermore, *C. dubliniensis* has been shown to be the second most abundant species after *C. albicans* isolated from the oral cavity of insulin-using diabetes mellitus patients for both carriers of and those affected by the yeast (Willis *et al.*, 2000). However, increased adherence in the presence of glucose may be a feature of orally adapted *Candida* isolates and may not be specific to *C. dubliniensis*.

Hydrophobic *C. albicans* cells may be induced by growth at 23 °C and these hydrophobic cells have been reported to be less sensitive to phagocytic killing than hydrophilic cells which result from growth at 37 °C (Antley *et al.*, 1988). In contrast, *C. dubliniensis* cells have been reported to be hydrophobic whether they were grown at 37 °C or 23 °C (Hazen & Masuoka, 2000). However, despite this difference in hydrophobicity at 37 °C, there was no difference in the levels of phagocytosis and induced oxidative burst and killing by human nuetrophils for both species (Peltroche-Llacsahuanga *et al.*, 2000b). It is possible that the cell surface hydrophobicity of *C. dubliniensis* may be involved in the increased adherence of this species to epithelial cells.

The secreted aspartic proteinases (Saps) are believed to be involved, amongst other factors, in adherence of *C. albicans* to epithelial mucosa (Ray & Payne, 1988; Borg & Rüchel, 1988; Ollert *et al.*, 1993; Klotz *et al.*, 1994; Watts *et al.*, 1998). *Candida dubliniensis* has been shown to possess homologues of *C. albicans* genes *SAPs 1-7* and a number of studies have reported that *C. dubliniensis* has significantly more proteolytic activity than *C. albicans* (McCullough *et al.*, 1995; Lischewski *et al.*, 1999). It is possible that this elevated production of Sap activity may be related to the increased adherence to epithelial cells exhibited by *C.*

dubliniensis. De Repentigny *et al.* (2000) have shown that *C. dubliniensis*, *C. tropicalis* and *C. albicans* are all capable of binding to intestinal mucin, and that this ability is mediated by Saps in *C. albicans*. The ability to bind to mucin is important if the organism is to traverse the mucin layer and bind to the mucosal epithelial surface. However, data on *C. dubliniensis* virulence is extremely limited. Given the multifactorial nature of virulence in *C. albicans* it is likely that virulence in *C. dubliniensis* is of an equally complex nature.

1.2 Study Aims

Candida dubliniensis is a novel species of the genus *Candida* primarily associated with the oral cavities of HIV-infected individuals and AIDS patients. Within the genus *Candida*, *C. dubliniensis* is most closely related to *C. albicans*. Despite this close relationship there are many differences between the two species particularly at the genetic level. The aims of the current study were threefold:

(1) to extend the phylogenetic analysis of *C. dubliniensis* by analysis of the nucleotide sequence of the highly conserved housekeeping *ACT1* structural gene which encodes the cytoskeletal protein actin, and comparison of this gene with the nucleotide sequence of the *C. albicans ACT1* gene (phylogenetic analyses and *ACT1* are discussed in Chapter 3).

(2) to analyse the *ACT1*-associated intron sequences from geographically and epidemiologically unrelated *C. dubliniensis* isolates and to compare these sequences with the *ACT1*-associated intron sequences of *C. albicans*, *C. stellatoidea* and *C. tropicalis*. It was envisaged that differences observed in the *ACT1*-associated intron sequence would facilitate the design of *C. dubliniensis*-specific primers for the development of a rapid PCR-based identification technique for *C. dubliniensis*. In-depth epidemiological analyses of *C. dubliniensis* have been hampered by the lack of a simple, rapid and definitive method capable of the unequivocal differentiation of *C. dubliniensis* and *C. albicans*. It was hoped that a PCR-based identification method based upon thoroughly characterised differences between *C. dubliniensis* and *C. albicans* would resolve this problem.

(3) to further characterise the genetic divergence between *C. dubliniensis* and *C. albicans* by analysis of the nucleotide sequence of the *SAP2* gene, encoding an aspartyl proteinase enzyme, a putative virulence factor of *C. albicans* (the *C. albicans SAP* multigene family is discussed in Chapters 5 and 6). The genotypic expression of this gene by *C. dubliniensis* will be investigated and compared to the expression of the *C. albicans SAP2* gene under identical conditions. Finally, phenotypic proteinase production of *C. dubliniensis* will be analysed and compared to that of *C. albicans* by a chemical enzyme assay.

Chapter 2
Materials and Methods

2.1 General Microbiological Methods

2.1.1 Culture media and growth conditions

All *Candida* strains and clinical isolates were routinely cultured on Potato Dextrose Agar (PDA) medium (Oxoid, Basingstoke, Hants., UK) at pH 5.6 at 37 °C for 48 h. For liquid culture, isolates were routinely grown in Yeast Peptone Dextrose (YPD) broth (per litre: 10 g yeast extract [Oxoid], 20 g peptone [Difco, Detroit, MI, USA], 20 g glucose, pH 5.5) at 37 °C for 18 h in a Gallenkamp (Model G25) orbital incubator (New Brunswick Scientific Company Inc., Edison, New Jersey, USA) set at 200 r.p.m.

The *Escherichia coli* strains DH5α (*supE* Δ*lac*U169 [ø80 *lacZ*ΔM15] *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1; Sambrook *et al.*, 1989) and XL2-Blue MRF' (D[*mcrA*]*183* Δ[*mcrCB-hsdSMR-mrr*]*173 endA1 supE44 thi-1 recA1 gyrA96* r*elA1 lac*[F'*proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^{r}) Amy Cam^{r}]^{c,d}; Jerpseth, *et al.*, 1992) were routinely cultured on Luria-Bertani agar (LB agar), pH 7.4 (Lennox, 1955), at 37 °C, and for liquid culture, in Luria-Bertani broth (LB broth), pH 7.4 (Lennox, 1955), at 37 °C for 18 h in an orbital incubator (Gallenkamp) at 200 r.p.m. *Escherichia coli* DH5α and XL2-Blue MRF' were used as the host strains for plasmid pBluescript II KS (-) (Stratagene, La Jolla, California, USA) and its recombinant derivatives and were maintained on LB agar supplemented with 100 µg ampicillin ml^{-1} as required. *Escherichia coli* strain LE 392 (*supE*44 *sup*F58 *hsdR*514 *galK*2 *galT*22 *metB*1 *trpR*55 *lacY*1, Sambrook *et al.*, 1989) and its P2 phage lysogenic derivative, P2 392 (Sambrook *et al.*, 1989) were used for propagating bacteriophage lambda recombinant derivatives and for construction of the *C. dubliniensis* library, respectively. These were maintained on LB agar. LE 392 organisms for phage infection were grown as follows: A single colony from an 18 h culture of LE 392 on LB agar was inoculated in 50 ml of LB broth supplemented with 500 µl of 1M $MgSO_4$ and 500 µl of 20 % (w/v) maltose (LM broth). This was grown overnight at 37 °C in an orbital incubator (Gallenkamp) set at 180 r.p.m. One millilitre of this culture was inoculated into a fresh 50 ml of LM broth and grown to an OD_{600} of approximately 0.6. These mid-exponential phase organisms were used for phage infection as described in section 2.2.2.

2.1.2 Chemicals, enzymes and radioisotopes

All chemicals used were of analytical-grade or molecular biology-grade and were purchased from the Sigma-Aldrich Chemical Co. (Poole, Dorset, UK), BDH (Poole, Dorset, U.K.) or from Roche Diagnostics Ltd. (Lewes, East Sussex, UK). Enzymes were purchased from the Promega Corporation (Madison, Wisconsin, USA.), Roche or New England Biolabs Inc. (Beverley, Massachusetts, USA). RNase solutions were prepared by dissolving pancreatic RNase (RNase A, Roche) at a concentration of 10 mg ml^{-1} in 10 mM Tris-HCl (pH 7.5), 15 mM NaCl. This solution was boiled for 15 min to inactivate any DNases, allowed to cool to room temperature and stored at -20 °C. Proteinase K (Roche) solutions were prepared in sterile distilled water at a concentration of 20 mg ml^{-1} and also stored at -20 °C. DNA molecular weight markers were purchased from Gibco BRL Life Technologies (Gaithersburg, Maryland, USA). Zymolyase 20T (21,600 U g^{-1}) was purchased from the Seikagaku Corporation (Tokyo, Japan). [α-^{32}P]dATP (3,000 Ci $mmol^{-1}$; 110 TBq $mmol^{-1}$) was purchased from Amersham International Plc. (Little Chalfont, Buckinghamshire, UK).

2.1.3 Buffers and solutions

Tris-EDTA (TE) buffer was used routinely in many experiments and consisted of 10 mM Tris-HCl, 1 mM EDTA, pH 8.0. Citrate phosphate buffer (CPB, 0.2 M) consisted of, per 100 ml, 58 ml 0.4 M Na_2HPO_4 and 42 ml 0.2 M citric acid.

TBE buffer was prepared at 5x concentration and consisted of 0.45 M Trizma base, 0.45 M boric acid, 0.01 M EDTA. This was diluted in distilled water to 0.5x concentration and was used as the buffer for agarose gel electrophoresis. Final sample buffer (FSB) was also prepared at 10x concentration and consisted of 30 % (v/v) glycerol, 0.25 % (w/v bromophenol blue and 0.1 M EDTA, pH 8.0. SSC buffer was prepared at 20x concentration and consisted of 3.0 M NaCl, 0.3 M tri-sodium citrate, pH 7.0.

MOPS buffer used for RNA electrophoresis was prepared at 10x concentration and consisted of 20 mM morpholinepropanesulphonic acid, 5 mM sodium acetate, 1 mM EDTA, pH 7.5.

Liquefied phenol washed in Tris-buffer was purchased from Fisher Scientific Ltd. (Bishop Meadow Road, Loughborough, UK) and used in the preparation of phenol chloroform (1:1), which was prepared by mixing an equal volume of liquefied phenol and chloroform. This solution was stored at 4 °C for up to two months in the dark.

2.1.4 Identification of *Candida* species

2.1.4.1 Chlamydospore production

All *C. dubliniensis* isolates were tested for their ability or inability to produce chlamydospores on rice-agar-Tween medium (RAT medium, bioMérieux, Marcy l'Etoile, France) as described by Sullivan *et al.* (1995). Test isolates were cultured on PDA for 24-48 h at 37 °C. Single colonies (3-4 mm diameter) were removed from PDA plates with a sterile wire loop and used inoculate the RAT medium by cutting shallow grooves in the surface of the agar medium. A glass coverslip was then placed over the inoculated area to create semi-anaerobic conditions and the plate was incubated at room temperature for 2-3 days in the dark. Plates were stained by spotting lactophenol cotton blue stain (Larone, 1993) directly onto an inoculated RAT agar plate, having gently prised up the glass cover slip covering the culture growth, and recovering the stained area by replacing the cover slip. Plates were then examined microscopically (x 40 objective lens) 30 min after staining for the presence of pseudohyphae, hyphae and chlamydospores. The *C. albicans* oral reference strain 132A (Gallagher *et al.*, 1992) and the *C. dubliniensis* type strain CD36 (Sullivan *et al.*, 1995) were used as positive controls for chlamydospore production in all tests.

2.1.4.2 Assimilation profiles

Biotyping was carried out using the API ID 32C yeast identification system (bioMérieux) which identifies *Candida* isolates to the species level using a series of standard, miniaturised assimilation tests contained in 32 separate cupules on a plastic strip with a specially adapted database (Pincus *et al.*, 1999). Tests were carried out according to the

manufacturer's instructions. An inoculum was prepared for each test isolate from 24-48 h old colonies cultured on PDA medium. Four colonies of 3-4 mm in diameter were resuspended in sterile water to a turbidity equivalent to a 2 McFarland barium sulphate opacity standard. This suspension was then used to inoculate an aliquot of 'C medium', which was supplied by the manufacturers. Each of the cupules in the strip was then inoculated with 135 µl of the C medium suspension and incubated for 48 h at 30 °C. Readings were made at 24 h and 48 h by visually assessing the growth of the test isolate in each of the cupules compare to that in the negative control cupule. The presence or absence of growth was recorded for each cupule on a result sheet supplied by the manufacturers, and the substrate assimilation profile of the isolate was converted into an eight-digit numerical profile. These profiles were then cross-referenced in the APILAB ID 32C analytical profile index. Each profile is listed along with a percentage of identification (% id), which is an estimate of how closely the profile corresponds to that of a particular taxon, relative to all the other taxa in the database and the T index, which is an estimate of how closely the profile corresponds to the most typical set of reactions for a particular taxon. Based on these parameters, a set of reactions which closely resemble those of a particular taxon will be classed as an 'excellent' or 'good' identification, and will yield an identification to the species level, whereas atypical results will be classed as having 'poor' or 'low' discriminatory powers and are usually unable to yield a positive identification.

2.1.4.3 Serotyping

Serotyping of *C. dubliniensis* isolates and *C. albicans* reference strains was carried out using antibodies raised against *Candida* antigenic factor number 6 (Iatron Laboratories, Tokyo, Japan). Test isolates were cultured on PDA for 24 h at 37 °C. Slide agglutination tests were carried out by emulsifying a 24 h-old single colony (3-4 mm diameter) of each test isolate in 10-20 µl of serum upon a clean glass slide. Sterile saline was used as a control against spontaneous agglutination. *Candida* isolates were recorded as serotype A if a positive agglutination reaction occurred with this serum, and as serotype B if no positive agglutination reactions were observed. Agglutination reactions were found to occur within 10-15 seconds

with serotype A isolates. The serotype A *C. albicans* isolate 179B (Gallagher *et al.*, 1992) and the serotype B *C. albicans* oral reference isolate 132A were used as positive and negative controls, respectively.

2.1.4.4 Growth at 45 °C

All isolates were tested for the ability to grow on PDA at 37 °C and 45 °C. *Candida albicans* isolates could be characterised by their ability to grow at both temperatures, whereas *C. dubliniensis* isolates were found to grow well at 37 °C, but not at 45 °C (Pinjon *et al.*, 1998).

2.1.4.5 Growth on CHROMagar® Candida medium

CHROMagar Candida (CHROMagar® Candida, Paris, France) is a new commercially available agar medium containing chromogenic substrates, which allow colonies of several medically important *Candida* species to be presumptively identified on the basis of colony colour and morphology. Colonies of *C. albicans* (light green colonies), *C. glabrata* (pink colonies), *C. krusei* (rough, colourless colonies) and *C. tropicalis* (purple) can easily be distinguished from each other upon primary isolation, and the medium has been shown to be clinically useful in the presumptive identification of these species (Odds and Bernaerts, 1994). All putative *C. dubliniensis* isolates were inoculated on this medium along with control *C. dubliniensis* and *C. albicans* isolates, and incubated for 48 h at 37 °C. *Candida dubliniensis* isolates could be distinguished from *C. albicans* isolates on the basis of colour, with *C. albicans* colonies typically being light green, and *C. dubliniensis* colonies being dark green (Schoofs *et al.*, 1997; Coleman *et al.*, 1997a).

2.2 Isolation of DNA and DNA Hybridisation

2.2.1 Extraction of genomic DNA from *Candida* species

Genomic DNA was prepared from cells grown in 50 ml of YPD broth in a 250 ml flask (Erlenmyer) at 37 °C in an orbital incubator at 200 r.p.m. for 18 h. Cultures were then decanted into 50 ml Falcon tubes (Beckton Dickinson, New Jersey, USA) and centrifuged in a bench top centrifuge (Sepatech Megafuge 1.0, Heraeus, Germany) at 2,500 x *g* for 5 min. The supernatant was decanted and the pellet was resuspended in 5 ml of a solution consisting of 20 mM CPB, 40 mM EDTA, 1.2 M sorbitol, pH 5.6. Cell walls were then digested by the addition of 15 mg Zymolyase 20T and incubation at 37 °C for 1 h in a shaking waterbath. The resulting protoplasts were harvested by centrifugation at 2,500 x *g* for 5 min and the pellet was resuspended in 7.5 ml 10x TE. The protoplasts were then lysed by the addition of 0.75 ml 10 % (w/v) sodium dodecyl sulphate (SDS) and protein was precipitated by the addition of 2.5 ml 5 M potassium acetate and incubation on ice for 30 min. The cell lysates were then decanted into 50 ml Sorvall Oak Ridge tubes (Dupont Co., Wilmington, Denver, USA) and centrifuged at 8,000 x *g* at 4 °C for 5 min in a Sorvall RC 5B refrigerated centrifuge (Dupont Co.). The cleared supernatant was then decanted into a fresh 50 ml Falcon tube and mixed gently with 10 ml ice-cold iso-propanol. The mixture was incubated at -20 °C for 5 min and the resulting precipitate was pelleted in a bench centrifuge at 2,500 x *g* for 5 min. The pellet was dried at 37 °C to remove any remaining iso-propanol, and resuspended in 1 ml TE buffer. The suspension was then incubated with 0.1 ml of an RNase A solution (10 mg ml^{-1} for 1 h at 37 °C followed by the addition of 0.1 ml of a proteinase K solution (20 mg ml^{-1}) and incubation for 1 h at 37 °C. The DNA solution was then extracted twice using an equal volume of a mixture of phenol:chloroform (1:1) and precipitated with the addition of two volumes of ice-cold ethanol. The resulting precipitate was spooled from the solution with a glass rod and transferred to a fresh tube. The DNA precipitate was then washed in 1 ml ice-cold 70 % (v/v) ethanol, dried briefly at 37 °C and resuspended in ~150 µl sterile distilled water. DNA suspensions were stored at 4 °C.

2.2.2. Extraction of recombinant lambda phage DNA

Recombinant phage stocks were maintained at a titre of approximately 1 x 10^{11} p.f.u. ml^{-1} in SM broth (50 mM Tris-HCl, pH 7.5, 100 mM NaCl and 10 mM $MgCl_2$) supplemented with 0.01 % gelatin. Phage lysates were prepared from these stocks by the plate method of Sambrook *et. al.* (1989). Briefly, 100 µl of phage stock were mixed with 100 µl of mid-exponential phage plating bacteria (LE 392, prepared as described in section 2.1.1) and incubated for 20 min at 37 °C. To this mixture was added 3 ml of molten (47 °C) TB top agar (Tryptone 10 g l^{-1}, NaCl 5 g l^{-1}, Bacto Agar 8 g l^{-1}). This was poured onto a 90 mm plate containing LM agar (LB agar supplemented with 2 % maltose (w/v) and 0.1M $MgSO_4$). The plate was incubated at 37 °C overnight until confluent lysis was achieved. Three millitres of SM broth were added to the plate and it was stored at 4 °C for 1 h. The SM and the soft top agar were scraped into a Corex tube using a sterile bent glass rod. The agar suspension was incubated with 0.1 ml of chloroform with shaking for 15 min at 37 °C. The tube was then centrifuged at 4000 x *g* for 10 minutes at 4 °C. Approximately 500 µl of phage stock were propagated to yield 10 ml of phage lysate. DNA was extracted from this lysate using the Wizard Lambda Preps DNA Purification System (Promega) according to the manufacturer's instructions. Ten milliliters of phage lysate yielded approximately 5 µg of recombinant phage DNA.

2.2.3 Restriction endonuclease digestion of genomic DNA and agarose gel electrophoresis

Restriction endonuclease digestions of genomic, phage and plasmid DNA were carried out with approximately 400 ng or less of DNA in a 10 µl volume containing 12 U of restriction enzyme and the appropriate restriction enzyme buffer according to the manufacturer's instructions. Horizontal 0.8 % (w/v) agarose gels were cast in 0.5x TBE buffer containing 0.5 µg ml^{-1} ethidium bromide into horizontal gel trays. Restriction endonuclease-generated DNA fragments in 1x final sample buffer were applied to the gel wells. The appropriate DNA size standards were loaded in the first well of each gel. Electrophoresis was performed at 50-100 volts (with constant current) until the bromophenol blue tracking dye had

reached the end of the gel. Following electrophoresis, gels were visualised on a UV transilluminator (wavelength 345_{nm}, UVP TMW 20 transilluminator, UVP Products, Cambridge, England) and the gel was photographed through a red filter with Polaroid 667 film.

2.2.4 Southern transfer of DNA from agarose gels

Following the separation of restriction endonuclease digest-generated DNA fragments by agarose gel electrophoresis the positions of DNA reference size standards were marked on the membrane using sterile Pasteur pipette The DNA was then depurinated by soaking the gels in 0.02 M HCl with gentle shaking. Following depurination, the DNA was denatured by soaking the gel in denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 45 min with gentle agitation, after which the gels were placed in a neutralisation solution (1 M Tris-HCl, pH 7.5, 1.5 M NaCl) for a further 45 min with shaking.

DNA fragments were transferred to MagnaGraph nylon membranes (MSI, Wesborough, Massachusetts, USA) by capillary transfer using 10x SSC as the transfer buffer according to the method of Southern (1975). Following transfer, the positions of DNA reference size standards were then marked on the membrane using a ball-point pen. The membrane was then rinsed in 2x SSC, dried and the DNA was fixed using a crosslinker (CL-508, UVI tec, Cambridge, England) set at 365_{nm} and 0.08 J cm^2.

2.2.5 Random primer labelling of DNA fragments with [α-^{32}P]dATP

For hybridisation experiments, including Southern hybridisations and Northern hybridisations, DNA fragments were labeled with [α-^{32}P]dATP by random primer labelling using the Prime-a-gene kit purchased from the Promega Corporation. DNA fragments that were present in plasmid vectors were excised by restriction endonuclease digestion and gel purified with NA45 DEAE membranes (described in section 2.4.3). Purified DNA fragments (10-200 ng in a 30 µl volume) were denatured by boiling for 2 min. Denatured DNA was added to a reaction mixture containing 1x labelling buffer, which was supplied by the kit

manufacturers and contained a random mixture of hexanucleotides, dNTP's (dTTP, dCTP and dGTP) and bovine serum albumin (BSA). This reaction mixture was completed with the addition 3 µl of [α-^{32}P]dATP (3,000 Ci mmol^{-1}; 110 TBq mmol^{-1}) and 5 U of Klenow DNA polymerase, and incubated at room temperature for 1-2 h. Unincorporated nucleotides were removed prior to hybridisation by passing the reaction mixture through a Nick column (Pharmacia Biotech, Sweden) containing sephadex G-50, according to the manufacturer's instructions. Probes were routinely labeled to a specific activity of >10^6 d.p.m. µg^{-1} DNA.

2.2.6 Southern hybridisation

Hybridisation reactions were carried out in a rotary hybridisation oven (Hybaid, Teddington, Middlesex, UK) in 25 x 3.5 cm bottles (Hybaid) by the method of Sambrook *et al.* (1989). Nylon membranes were rinsed in 2x SSC prior to hybridisation to remove excess salt. Membranes were then prehybridised in the oven at 65 °C in 10 ml of a solution containing 1x Denhardt's solution (1 % [w/v] Ficoll, 1 % [w/v] polyvinylpyrrolidone, 1 % [w/v] BSA), 6x SSC, 100 µg ml^{-1} denatured salmon sperm DNA and 0.5 % (w/v) SDS for 2 h.

Radiolabelled probe (> 2 x 10^6 d.p.m.) was denatured by boiling for five minutes followed by incubation on ice. The denatured probe was then added to the prehybridisation solution and incubated with the membrane at 65 °C for 18 h. Unbound probe was removed from the membranes following hybridisation by washing the membrane in the bottle with a solution of 2x SSC, 0.1 % (w/v) SDS at room temperature for 5 min, followed by a wash at 37 °C in 0.1x SSC, 0.5 % (w/v) SDS for 30 min, and finally a high stringency wash at 65 °C in 0.1x SSC, 0.5 % (w/v) SDS for 30 min. After washing, the membranes were wrapped in Saran wrap (Dow Chemical Co., Germany) and placed in an autoradiograghy cassette with an intensifying screen (Biomax TranScreen, Sigma) and exposed to X-Omat X-ray film (Dupont) overnight at -70 °C. Autoradiograms were developed with Kodak LX-24 developer and fixed in Kodak FX-40 fixer using the dilutions recommended by the manufacturer.

Bound probe was removed from membrane filters by immersing the membrane in a boiling solution of 0.1 % (w/v) SDS for 15 min, followed by a brief rinse in 2x SSC.

2.3 *Candida dubliniensis* Genomic Library Construction

2.3.1 Isolation of high molecular weight *C. dubliniensis* genomic DNA

High molecular mass total cellular DNA from the *C. dubliniensis* type strain CD36 was isolated using a modification of the method described by Sullivan *et al.* (1995) as follows: after zymolyase treatment, the resulting spheroplasts were harvested and washed once in TE buffer and resuspended in 1 ml of 25 % (w/v) sucrose in 50 mM Tris-HCl, 1 mM EDTA at pH 8.0. This solution was incubated at 37 °C for 10 min and then transferred to ice followed by the addition of 2 mg proteinase K and then 400 µl 0.5M EDTA, pH 8.0, and 250 µl 10 % (w/v) sodium N-lauroyl sarcosinate. After thorough mixing, the spheroplast suspension was incubated on ice for 90 min, after which it was transferred to a shaking waterbath at 50 °C and incubated for 16 h. Following incubation, to each lysate was added 8 ml of a CsCl solution consisting of 69.9g CsCl, 55.2 ml TE buffer and 50 µg PMSF ml^{-1}. This mixture was then transferred into 10 ml Quickseal ultra-clear centrifuge tubes (Beckman, Instruments Inc., Fullerton, Calif., USA) and centrifuged for 40 h at 160,000 x *g* at 10 °C in a Beckman 70.1 Ti fixed-angle rotor using an L8-60M ultracentrifuge (Beckman). After centrifugation, DNA was collected from the tubes by side-puncture with an 18 gauge syringe needle (Microlance 2, Becton-Dickson). Caesium chloride was removed from the DNA by dialysis overnight (16 h) against 5 l of 1x TE buffer at 4 °C using dialysis tubing (Sigma). DNA was recovered by ethanol precipitation, dried, resuspended in TE buffer at pH 8.0 and stored in aliquots at -20 °C.

2.3.2 Construction of a *C. dubliniensis* CD36 genomic DNA library

High molecular weight *C. dubliniensis* CD36 chromosomal DNA was partially digested with *Sau*3A. Fragments greater than 10 kb were ligated into *Bam*H1-generated lambda bacteriophage replacement vector EMBL3 arms (Promega), and then packaged *in vitro* using prepared phage heads and tails (Promega), according to the manufacturer's instructions. DNA fragments ranging in size from 9-23 kb can be cloned into the EMBL3 vector (Frischauf

et al., 1983). The packaged recombinant phage particles were propagated on *E. coli* lysogenic strain P2 392.

2.3.3 Screening of the *C. dubliniensis* genomic library

Approximately 10,000 recombinant phages were propagated on *E. coli* LE 392 to give approximately 1,000 p.f.u. per 90 mm Petri dish (as described in section 2.2.2 but using TB top agar with 1 % Bacto Agar). The phage particles were transferred from the plaques to nitro-cellulose filters (Schleicher & Schuell, Dassel, Germany) by overlaying the plaques with the filters for 30 s. The DNA was denatured by soaking in denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 3 min. The filters were then placed in neutralisation solution (1 M Tris-HCl, pH 7.5, 1.5 M NaCl) for 5 min, and rinsed briefly in 2x SSC. The filters were dried and the DNA fixed using a crosslinker as described in section 2.2.4. The filters were then screened by plaque hybridisation by the method of Sambrook *et al.* (1989) as described above using radiolabelled probes. Reactive recombinant phages were picked and purified by successive rounds of hybridisations. Isolated recombinant phages were hybridised to labeled digested (*Hin*dIII) λ2001 DNA to verify that no contaminating phage particles were present as follows: purified recombinant phage was propagated to give approximately 50 p.f.u. per 90 mm Petri dish. Double plaque lifts were carried out from one plate as described above. One filter was then screened using a radiolabelled specific probe and the other hybridised to the radiolabelled λ2001 DNA. If the recombinant phage was pure then all plaques would hybridise to both probes. Any contaminating plaques would hybridise to the λ2001 DNA probe only.

2.4 Recombinant DNA Techniques

2.4.1 Small scale isolation of plasmid DNA from *E. coli*

Small scale preparations of plasmid DNA were prepared by the method of Sambrook *et al.* (1989). Briefly, *E. coli* cultures were grown overnight at 37 °C in LB medium in the presence of a selective antibiotic (100 μg ml^{-1} ampicillin in the case of pBluescript II KS [-]). A 2 ml aliquot of this culture was pelleted at 10,000 x *g* for 30 s in a microfuge (Centrifuge 5417C, Eppendorf, Hamburg, Germany) and resuspended in 100 μl ice cold solution 1 (50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA, pH 8.0). Cell were lysed by the addition of 200 μl solution 2 (0.2 N NaOH, 1 % [w/v] SDS) and left on ice for 5 min. Protein was then precipitated by the addition of 150 μl solution 3 (5 M potassium acetate, 11.3 % (v/v) acetic acid). The mixture was vortexed and centrifuged at 10,000 x *g* for 5 min in a microfuge. The supernatant was then transferred to a fresh microfuge tube and extracted once with an equal volume of phenol:chloroform (1:1), and the DNA precipitated by the addition of 2 volumes of ice-cold ethanol. The precipitate was pelleted again at 10,000 x *g* for 5 min and resuspended in 49 μl sterile distilled water and 1 μl of 0.1 mg ml^{-1} RNase A.

2.4.2 Polymerase chain reaction (PCR)

Specific sequences of *C. dubliniensis* genomic DNA were amplified by PCR and cloned into pBluescript (described in section 2.4.4). Oligonucleotide primers were synthesised by Genosys Biotechnologies (Europe) Ltd. (Cambridge, UK) and stored at a stock concentration of 1 mM in sterile water at -20 °C. Amplification reactions were carried out in 0.5 ml microfuge tubes (Eppendorf) in a Perkin Elmer Cetus DNA thermal cycler in 100 μl volumes containing 1x *Taq* reaction buffer, 2 mM $MgCl_2$, 250 μM (each) dATP, dTTP, dCTP, dGTP (Promega), 10 pM (each) of a forward and reverse primer, 10 ng genomic DNA template and 2.5 U *Taq* DNA polymerase (Promega). The mixture was overlaid with 40 μl of sterile mineral oil. Amplification conditions and specific primers will be described in the relevant sections.

2.4.3 Purification of restriction endonuclease-generated DNA fragments and PCR amplimers from agarose gels

Restriction endonuclease-generated DNA fragments were purified from agarose gels using NA45 DEAE membranes (Schleicher and Schuell).The NA45 DEAE membranes were pre-treated by soaking 1 cm strips in 2 M NaCl for 5 min, followed by 3 washes in sterile distilled water for 5 min each. The strips were then stored at 4 °C in 1 mM EDTA, pH 8.0. Fragments were electrophoresed as described in section 2.3.2 in agarose gels and viewed on a UV transilluminator (345_{nm}). Using a clean scalpel blade, a small rectangular trough was excised from the gel immediately ahead of the fragment of interest, and a piece of NA45 DEAE paper was placed in the trough and the excised fragment of gel was replaced to hold the paper in place. The electrophoresis was allowed to continue until the fragment had run onto the paper, which could be verified by the fluorescent staining of the paper with ethidium bromide. The paper was then placed in 0.5 ml 1 M NaCl and placed in a water bath at 37 °C for at least 1 h to elute the fragment. The DNA solution was then extracted twice with iso-butanol to remove the ethidium bromide, and once with phenol:chloroform (1:1). The DNA was precipitated with two volumes of ice-cold ethanol, pelleted at 10,000 x *g* in a microfuge and resuspended in 5-10 µl sterile distilled water.

2.4.4 Ligation of DNA fragments

Agarose gel-purified DNA fragments were ligated to pBluescript II KS (-) phagemid digested with the appropriate restriction endonuclease. Ligation of PCR products to pBluescript was carried out either *via* restriction sites, which had been designed within the oligonucleotide primers, used in the amplification reactions, or *via* the addition of adenosine residues to the 3' ends of PCR products, mediated by the terminal transferase activity of *Taq* polymerase. These adenosine overhangs could be ligated to a pBluescript T-overhang vector. T-overhang vectors were created by incubating pBluescript DNA that had been cleaved with a restriction enzyme that generates 'blunt' ends (e.g. *Eco*RV) in a PCR reaction containing 1 x *Taq* reaction buffer, 2 mM $MgCl_2$, 250 mM dTTP and 5 U *Taq* polymerase (Promega). The

reaction was incubated at 70 °C for 2 h. Under these conditions *Taq* polymerase adds a single thymidine to the 3' end of the vector, which allows ligation to PCR products with adenosine overhangs.

Gel purified DNA fragments were ligated directly into the appropriate restriction endonuclease generated site in the cloning vector. However, if no appropriate restriction endonuclease cleavage site was present in the vector, the ends of the DNA fragment were blunted using the 5'-3' exonuclease activity of Klenow DNA polymerase, and cloned into a blunt site in the vector, usually generated with the restriction endonuclease *Eco*RV.

Ligation reactions were carried out in a 20 µl volume, with a 3:1 ratio of insert to vector DNA in 1x ligase buffer, with 1 U of T4 DNA ligase (Promega). Reactions were carried out for 18 h at 4 °C for 'blunt' ends, and 22 °C for 3 h for 'sticky' ended reactions.

2.4.5 Transformation of competent *E. coli* prepared using $CaCl_2$

Transformation of *E. coli* with $CaCl_2$ was carried out by the method of Sambrook *et al.* (1989). *E. coli* DH5α or XL2-Blue MRF' were inoculated from an overnight broth culture into 100 ml LB and grown at 200 r.p.m. in an orbital incubator at 37 °C for 3 h to an OD_{600} of ~0.5. The culture was then decanted into ice-cold 50 ml Sorval tubes and chilled on ice for 10 min. Cells were then pelleted by centrifugation at 5,000 x *g* in a Sorvall SS34 rotor (Dupont) at 4 °C for 10 min. Each pellet was resuspended in 10 ml of ice-cold 0.1 M $CaCl_2$, and recentrifuged as before. The pellets were then resuspended in a volume of 2 ml 0.1 M $CaCl_2$ for each 50 ml of original culture.

A 0.2 ml aliquot of this cell-suspension was transferred to a sterile microfuge tube on ice for each transformation experiment. Plasmid DNA (up to 50 ng) was added to each tube and incubated on ice for 30 min. A known amount of a standard plasmid preparation was added to a separate tube as a positive control, and a second control tube was also included which contained no plasmid DNA at all. A ligation control consisting of digested vector religated on itself was included for each batch of T4 DNA ligase. The tubes were then heat shocked at 42 °C for exactly 90 s and rapidly transferred to an ice bath. The cells were then

incubated at 37 °C in a water bath in the presence of 800 µl LB medium to allow the cells to recover and express the antibiotic resistance marker (ampicillin resistance in the case of pBluescript II KS [-]). A 0.1 ml aliquot of this suspension was then spread on LB plates containing antibiotic (100 µg ml^{-1} ampicillin in the case of pBluescript II KS [-]), 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Roche) and 100 µg (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal, Roche) and incubated for 20 h at 37 °C. Recombinants were identified using blue-white selection as described by Sambrook *et al.* (1989).

2.5 Northern Analyses

2.5.1 RNase-free conditions

All solutions used in the preparation of total RNA were rendered RNase-free by the addition of 0.1 M diethylpyrocarbonate (DEPC, Sigma). DEPC was dispersed in all solutions, which were then left to incubate at room temperature for 3-4 h, before autoclaving, which inactivates DEPC. Solutions containing amines (i.e. Tris and EDTA) were prepared with DEPC-pretreated water and autoclaved. Plasticware such as microfuge tubes (Eppendorf) and Falcon tubes, which were assumed to be free of RNase contamination, were handled only when wearing latex gloves. Bottles and other glassware were baked overnight at 200 °C. Glass beads (Sigma) used in RNA extractions were 450-600 microns in diameter and were treated in hydrochloric acid, washed in distilled water, and baked overnight.

2.5.2 Total RNA extraction from *Candida* isolates

Candida cells were harvested at mid-exponential phase (OD600: 0.6, unless otherwise stated in specific sections) from 50 ml YPD broth cultures for RNA extractions by centrifugation at 2,500 x *g* for 5 min. Pellets were resuspended in 2.5 ml extraction buffer (0.1 M LiCl, 0.01 M dithioreitol [DTT], 0.1 M Tris-HCl, pH 7.5) at 4 °C. In a Falcon tube at 4 °C a slurry consisting of 6 g glass beads, 5 ml phenol:chloroform (1:1) and 0.5 ml 10 % (w/v) SDS was prepared for each sample. The resuspended pellet was mixed with the slurry and vortexed

continuously for 5 min. The cell slurry was then centrifuged at 2,500 x *g* for 5 min and the upper aqueous phase (~2 ml) was transferred to microfuge tubes on ice. The aqueous phase was then extracted twice with an equal volume of phenol:chloroform (1:1) and transferred to a fresh microfuge tube and precipitated with 2 volumes of ethanol at -20 °C for 2 h. The precipitated RNA was collected by centrifugation at 3000 x *g* for 10 min, the supernatant removed and the pellet briefly air dried. The pellets were dissolved in 50 µl DEPC-treated water, and the separate fractions for each sample pooled. In order to remove DNA, the RNA was precipitated with LiCl. RNA samples were mixed with 2 volumes of 6 M LiCl and placed at -20 °C for 2 h. The samples were centrifuged at 11,600 x *g* for 10 min and the RNA pellet was resuspended in 0.2 ml DEPC-treated water. Finally the RNA was precipitated again by the addition of 20 µl of 3 M sodium acetate, pH 5.2 and two volumes of ethanol at -20 °C for 2 h. The RNA was pelleted at 11,600 x *g*, and resuspended in ~120 µl DEPC-treated water. Samples were stored at -70 °C.

2.5.3 RNA electrophoresis

The concentration of each RNA sample was assessed by measuring the A_{260} (1 unit of A_{260} = 42 µg RNA) and 10 µg were loaded on each gel. A test 1 % (w/v) agarose gel was prepared initially to assess the accuracy of concentration determinations and to assess the integrity of each sample. It was found that more accurate determinations of RNA loading could be made by comparison of ethidium bromide staining of the RNA on a test gel. The integrity of each sample could then be assessed and adjustments made to ensure equal loading of the samples if necessary.

Each RNA sample was mixed with 35 µl MFF solution (50 % [v/v] formamide, 6 % [v/v] formaldehyde, 0.8 µg ml^{-1} ethidium bromide, 1x MOPs buffer). The samples were heated at 70 °C for 15 min to denature the RNA, then placed on ice and mixed with 4 µl 10x FSB. Samples were loaded onto 1.2 % (w/v) agarose gels containing 6 % (v/v) formaldehyde, and 1x MOPS. Electrophoresis was carried out at 60 V in 1x MOPS buffer. Fractionated RNA was transferred to MagnaGraph nylon membranes (MSI) by capillary transfer. Gels were rinsed

briefly in DEPC-treated water to remove excess formaldehyde and then soaked in 0.05 N NaOH for 20 min, and then equilibrated in 20x SSC for 45 min. Capillary transfer was carried out in 20x SSC. RNA was then fixed to the membrane by baking at 80 °C for 30-45 min followed by UV crosslinking of the RNA to the membrane in a crosslinker as described earlier.

2.5.4 Northern hybridisation

Hybridisations were carried out in 15 ml hybridisation buffer (4x SSC, 1 % (w/v) SDS, 10 % (w/v) dextran sulphate, 50 % (v/v) formamide, 100 μg ml^{-1} denatured salmon sperm DNA). Membranes were prehybridised for 4 h at 42 °C. The radiolabelled probe was then denatured by boiling for 5 min followed by incubation on ice, added to the prehybridisation solution and hybridised with the membrane overnight for 18 h at 42 °C. The membrane was then washed for 15 min at 42 °C with 5x SSC, 0.1 % (w/v) SDS followed by a wash in 1x SSC, 0.5 % (w/v) SDS at 42 °C for 15 min also. The membrane was then exposed to BioMax Ms X-ray film (Eastman Kodak Company, Rochester, NY.) at -70 °C with an intensifying screen for 24-72 h. All membranes were hybridised with a probe homologous to either the *C. albicans* (Di Domenico *et al.*, 1992) or *C. dubliniensis TEF3* genes.

2.6 DNA Sequencing

2.6.1 Sequencing

DNA sequencing was performed by the dideoxy chain-termination method of Sanger *et al.* (1977) as described by Sambrook *et al.* (1989) using an automated Applied Biosystems 370A DNA sequencer and dye-labeled primers (ABI PrismTM Dye Terminator Cycle Sequencing Ready Reaction Kit, Applied Biosystems, the Perkin-Elmer Corporation, Warrington, UK). Reactions were carried out in a 10 μl volume using 3.2 pmoles of M13 forward and reverse control primers or specific primers and 400 μg of DNA. Plasmid DNA for

sequencing was prepared using the Quantum Prep® Plasmid Miniprep kit (Bio-Rad Laboratories, Los Angeles, Calif.).

2.6.2 Sequence analysis

Sequence analysis was performed using a variety of computer programs including Seqed version 1.0, DNA Strider version 1.2, and Gene Jockey version 1.0, for Macintosh computers. Searches of the EMBL and GenBank databases for nucleotide and amino acid sequence similarities were performed using the BLAST family of computer programs (Altschul *et al.*, 1990). DNA sequence alignments and phylogenetic analysis were carried using the CLUSTAL sequence alignment program (Thompson *et al.*, 1994), which was provided via TELNET, Genetics department, TCD. Analysis of amino acid sequences and peptide structure were carried out using the GCG Wisconsin package of computer programs (Genetics Computer Group, 1994), also accessed via TELNET, Genetics Department, TCD.

Chapter 3
The Actin Gene of *Candida dubliniensis*

3.1 Introduction

3.1.1 The molecular clock

Our current system of classification of living organisms, developed and refined through the years, has been based upon physical or phenotypic characteristics. This is a system that works well for plants and animals, both of which possess complex morphological characteristics and fossil record information. In contrast, the fossil record is very poor for bacteria and the lower eukaryotic groups. Furthermore, the morphological and physiological characteristics of bacteria and lower eukaryotic groups may be too few to be useful, or too unstable to be reliable indicators of phylogenetic relationships. Classification of organisms based upon the same or similar physical characteristics is inherently problematic as these phenotypic characteristics may take a continuous range of values. Any comparisons then made may range from identity to various degrees of dissimilarity, and the observation of these degrees of dissimilitude may be reliant upon the subjectivity of the observer. The modern solution to this problem is molecular phylogenetics, where evolutionary relationships are defined by the DNA sequence of an organism's genes. The concept that a molecular sequence could be representative of the evolutionary history and phylogenetics of an entire organism was first proposed by Zuckerkandl & Pauling in 1965. This gave rise to a new way of evaluating evolutionary relationships between organisms by comparison of their nucleotide and protein sequences. New molecular approaches were devised to evaluate these molecular relationships including DNA base ratios, nucleic acid hybridisation studies, cell wall analyses and protein sequencing. With the advent of PCR and automated nucleotide and protein sequencing techniques large quantities of sequence information became available and this in turn led to the development of software capable of performing complex sequence analyses. Subsequently, the science of molecular phylogeny has come to prominence and revolutionised our concept of phlyogeny and evolution.

The use of genotypic information to infer phylogenetic relationships has a number of advantages over phenotypic comparisons. The number of variables at the genotypic level is far

greater than that at the phenotypic level as each gene that codes for a single phenotypic characteristic, e.g. a particular enzyme, consists of tens to thousands of evolutionary independent variables. Each of these variables is precisely defined in that there are four nucleotide bases and twenty amino acids, and this enables divergence between homologous genes of different species to be mathematically defined. There is constant change at the genotypic level although not all genotypic changes manifest as phenotypic changes. Therefore, although similar phenotypic characteristics may not be useful at inferring phylogenetic relationships, the genes that code for them are useful due to this constant change at the genetic level. Alternatively, the absence of a particular phenotypic characteristic may be due to different and unrelated genotypic changes. As a result, grouping of organisms based upon absence of this characteristic may be incorrect. Comparative sequence analysis overcomes this problem as homologous sequences give rise to homologous proteins.

Evolutionary differences are a result of continuous and constrained drift rather than of innovative changes. This gives rise to the concept of an evolutionary clock with both tempo (rate of change) and mode (phenotypic changes). In their landmark paper in 1965, Zuckerkandl & Pauling introduced the notion of molecular chronometers, i.e. a molecular sequence whose tempo and mode may be used to define evolutionary relationships. In the early years of molecular phylogeny researchers depended upon the sequences of proteins such as ferredoxins and cytochromes, which provided insights into microbial evolution (Schwartz & Dayhoff, 1978). In the 1970's, however, Woese and colleagues began assembling a massive database on small subunit ribosomal RNA sequences (SSU rRNA), and used this information to generate the "universal tree", a hierarchical classification of all groups going back to the dawn of life (Woese, 1987 and 1990; Brown & Doolittle, 1997; Doolittle, 1999). Instead of defining whole organism phylogeny we refer now to a molecular phylogeny.

In order to achieve a molecular phylogeny particular genes or molecules must be capable of defining the organism. A molecular chronometer may be defined as a molecule whose sequence changes randomly over time. If a molecule is to be used as a chronometer then it must fulfill certain criteria. Firstly, the changes that occur in its sequence should occur

as randomly as possible and they should occur slowly. Subsequently, the best chronometers are molecules that are subjected to a high level of evolutionary constraint as their slow rate of change allows the inference of phylogenetic relationships over broad evolutionary distances, i.e. they have a large range. The functional constraints themselves must remain constant over the distance being measured otherwise selected sequence changes accumulate over random changes. The accumulation of non-random, i.e. selected, sequence changes over random changes results in artificially increased phylogenetic distances. Sequences that are subject to little evolutionary constraint change rapidly and this reduces their range. These sequences are useful for inferring relationships over restricted distances. Finally, the molecule must be sufficiently large to provide enough information. A large molecule consisting of a number of domains makes for a more accurate chronometer. Functional domains are somewhat independent of each other and subsequently minimise the effect of nonrandom changes. Molecules that fulfill these criteria include rRNAs, RNA polymerases (Puhler *et al.*, 1989), elongation factors Tu and G (Iwabe *et al.*, 1989), proton-translocating ATPases (Gogarten *et al.*, 1989), cytochrome C (Fitch & Margoliash, 1967) and actin (Hennessey *et al.*, 1993).

To date, the most frequently used molecules in phylogenetic analysis are the rRNAs. The larger RNAs may be used to elucidate relationships that span the full universal tree to intra-species relationships. Both the large and small subunit ribosomal genes have proved useful in phylogenetic studies. The small subunit rRNA genes (SSU rRNA) have been extensively studied and there is a massive database assembled on these sequences (Van de Peer *et al.*, 1994). This molecule is considered to be superior to other molecules for many reasons. It is abundant, it is present in both organellar as well as nuclear and prokaryotic genomes, it has slow- and fast-evolving portions, and it has a universally conserved structure. Its function is ancient and fundamental to the cell and it interacts with many other cellular RNAs and proteins (Woese, 1987). The current universal tree is based upon SSU rRNA sequence data (Woese, 1987 and 1990; Brown & Doolittle, 1997; Doolittle, 1999). Large subunit rRNA (LSU rRNA) sequence data has also been useful for inferring evolutionary relationships. All eukaryotes contain variable regions in their LSUs and they do not occur in

prokaryotes, an important factor in the development of eukaryote identification techniques (Hancock & Dover, 1989). Of particular use has been the V3 region (Raue *et al.*, 1988). It has been found to be sufficiently conserved to demonstrate phylogenetic differences among genera and species in groups of organisms such as ascomycetous (Gaudet *et al.*, 1989; Kurtzman, 1989; Peterson & Kurtzman, 1991) and basidiomycetous fungi (Guého *et al.*, 1989 and 1990; Yamada *et al.*, 1990a and 1990b). The 5S rRNA species has also been used to elucidate phylogenetic relationships (Hori & Osawa, 1979).

3.1.2 General actin information

The cytoskeleton is a complex network of protein filaments that extends throughout the cytoplasm of the eukaryotic cell. There are three cytoskeletal elements – microtubules, actin microfilaments and intermediate filaments. Of these the actin cytoskeleton constitutes a central organiser of the cell and is responsible for a variety of diverse functions such as cell structure and cell motility, intracellular transport, cytoplasmic streaming, cytokinesis, endocytosis, exocytosis, chromosomal condensation and mitosis. Actin is the most abundant intracellular protein in a eukaryotic cell. In muscle cells, actin comprises 10 % by weight of the total cell protein, and in nonmuscle cells actin makes up 1 – 5 % of the cell's protein. It is a moderate-sized protein consisting of approximately 375 amino acid residues. At least six different isoforms of actin have been identified in eukaryotes. Three are called alpha-actins: each one is unique to a different type of muscle. Two other actins, termed nonmuscle beta-actin and gamma-actin, are found in nearly all nonmuscle cells. The sixth actin, another gamma-actin occurs in smooth muscles that line the intestine (Korn, 1978; Pollard, 1990; Reisler, 1993; Welch *et al.*, 1994; Small *et al.*, 1999).

3.1.3 Structure of actin

Actin exists in two forms, G-actin, which is the globular monomer form of actin that exists at low ionic strengths, and F-actin the filamentous polymer of G-actin subunits. F-actin is a helix of uniformly oriented monomers and is the major component of the cytoskeleton.

They have a polar structure and this polarity from one end to the other is crucial for cell motility. G-actin normally binds one molecule of ATP. When it polymerises into F-actin however, the ATP is hydrolysed to ADP, which continues to be bound to the F-actin. In the G-actin form, the 375-residue monomer actin is folded into two large domains, each comprised of two sub-domains. The large domains are organised to form a hinged molecule with a deep cleft. Within the cleft are actin's essential cofactors – an adenine nucleotide and a divalent metal ion. They are bound within the cleft by ionic and hydrogen bonds to amino acid side chains and are predicted to make extensive contacts with the domains on either side of the cleft, thus increasing connectivity between them. The floor of the cleft acts as a hinge that allows the lobes of the proteins to flex relative to one another. When ATP is bound to the cleft, it becomes a latch that holds the two lobes together. It has been predicted that the residues surrounding the cleft region are likely to be involved in binding or hydrolysing nucleotide and possibly stabilising monomer structure. Other regions of the protein are likely to be involved in making contacts essential to filament formation or for the interactions with a number of binding proteins (Korn, 1978; Pollard, 1990; Reisler, 1993; Welch *et al.*, 1994; Small *et al.*, 1999).

3.1.4 Actin as a molecular clock

Although most molecular phylogenetic analyses have been carried out using the SSU rRNA gene and to a lesser extent the LSU rRNA gene these are not the only genetic sequences to have been employed in this manner. Proteins such as actin (Hennessey *et al.*, 1993), RNA polymerases (Pühler *et al.*, 1989), elongation factor G (Iwabe *et al.*, 1989), proton-translocating ATPases (Gogarten *et al.*, 1989), and cytochrome C (Fitch & Margoliash, 1967) have all been used in the construction of phylogenetic trees. Actin sequences are conserved throughout the eukaryotic kingdom. Indeed the high level of conservation suggests that there are constraints throughout the entire sequence, rather than individual sites of greater conservation. Many eukaryotic trees have been constructed using actin protein or nucleotide sequence and they tend to concur with the trees produced by rRNA comparative sequence

analysis. Actin genes arose by duplication and divergence from common ancestral genes and evolved early in eukaryotic evolution. Phylogenetic analysis using the actin gene has become established in the literature in recent years, mainly to confirm phylogenetic data already determined by rRNA analysis (Hightower & Meagher, 1986; Hennessey *et al.*, 1993; Fletcher *et al.*, 1994; Wery *et al.*, 1996;). It has been suggested that actin may be particularly useful in studies of fungi as it appears that all fungi have single copies of the gene as opposed to other organisms that may have many different isoforms of actin (Cox *et al.*, 1995). *Pneumocystis carinii* had been a taxonomic challenge for many years and was originally classified as a protozoan. 18s rRNA comparative sequence analysis established this organism as a member of the fungi, showing greatest homology with fungi such as *S. cerevisiae* and *Neurospora crassa* (Cushion *et al.*, 1988). Edman *et al.* (1988) showed that rRNA phylogenetic analysis indicated that *P. carinii* was closely related to the fungi. This classification was confirmed by phylogenetic analysis using the *ACT1* gene (Fletcher *et al.*, 1994). In their analysis of the actin gene of *Phaffia rhodozyma*, Wery *et al.* (1996) found that their phylogenetic tree was in accordance with earlier findings based on rRNA/rDNA sequencing studies which divided basidiomycete and ascomycete taxa. Their actin analysis also showed that the ascomycetous yeast except *Schizosaccharomyces pombe* formed a cluster distinct from the filamentous ascomycetous fungi. Indeed, the actin data suggest a distant relationship between *S. pombe* and the other ascomycetous yeasts. This confirms 18s rRNA studies showing that *S. pombe* is only remotely related to budding ascomycetous yeasts (Kurtzman *et al.*, 1989).

Within the genus *Candida* the actin genes (*ACT1*) of *C. albicans* (accession no. X16377) and *C. glabrata* (accession number AF069746) have been cloned and sequenced. The aim of this part of the present study was to clone and sequence the *C. dubliniensis ACT1* gene and to perform phylogenetic analysis on the coding region in order to confirm the phylogenetic position of *C. dubliniensis* as previously determined by rRNA sequence analysis. There are many *Candida* species closely related to *C. albicans* e.g. *C. clausenii*, and *C. langeronii* whose designation as a species distinct from *C. albicans* has long been questioned. Many of these species have subsequently been demonstrated to be synonymous to *C. albicans*

(Wickes *et al.*, 1992). *Candida stellatoidea* exhibits both phenotypic and genotypic differences from *C. albicans*, however these differences are not sufficient warrant species status (Odds *et al.*, 1998). Further evidence in support of this comes from rRNA sequence analysis (Sullivan *et al.*, 1995). Therefore, confirmation of the phylogenetic of *C. dubliniensis* is necessary to confirm that *C. dubliniensis* warrants species status within the genus *Candida*, distinct from *C. albicans*.

3.2 Materials and Methods

3.2.1 Yeast reference strains and clinical isolates

The reference strains used in this study included the *C. dubliniensis* type strain CD36 (Sullivan *et al.*, 1995), which has been lodged with the British National Collection of Pathogenic Fungi, Bristol, UK, under the accession number NCPF 3949 and with Centraalbureau Voor Schimmelcultures, Baarn, the Netherlands, under the accession number CBS 7987, which was used to construct the genomic library described in section 2.3.2. The other reference strains and clinical isolates used in the phylogenetic analysis of *C. dubliniensis* are listed in Table 3.1.

Table 3.1 Yeast species and strains used in the phylogenetic analysis of *C. dubliniensis*

Yeast Strain [1]	*ACT1* intron sequence [2]	Reference
C. albicans		
132A (serotype B)	This study and Donnelly *et al.*, 1999	Gallagher *et al.* (1992)
179B (serotype A)	This study and Donnelly *et al.*, 1999	Gallagher *et al.* (1992)
ATCC 10123	X16377	Losberger & Ernst (1989)
C. dubliniensis		
CD36 (Ireland)	AJ236897; this study and Donnelly *et al.*, 1999	Sullivan *et al.* (1995)
CD91 (Ireland)	This study and Donnelly *et al.*, 1999	This study and Donnelly *et al.*, 1999
CD70 (UK)	This study and Donnelly *et al.*, 1999	Sullivan *et al.* (1997)
NCPF 3108 (UK)	This study and Donnelly *et al.*, 1999	Sullivan *et al.* (1995)
CD93 (Finland)	This study and Donnelly *et al.*, 1999	This study and Donnelly *et al.*, 1999
94191 (Spain)	This study and Donnelly *et al.*, 1999	Pinjon *et al.* (1998)
P2 (Switzerland)	This study and Donnelly *et al.*, 1999	Boerlin *et al.* (1995)
CD71 (Argentina)	This study and Donnelly *et al.*, 1999	Sullivan *et al.* (1997)
CM2 (Australia)	This study and Donnelly *et al.*, 1999	Sullivan *et al.* (1995)
CD92 (Canada)	This study and Donnelly *et al.*, 1999	This study and Donnelly *et al.*, 1999
C. glabrata		
ATCC 90876	AF069746	Unpublished data submitted to GenBank
C. stellatoidea		
ATCC 11006	AJ237919; this study	Kwon-Chung *et al.* (1989)
303530	This study and Donnelly *et al.*, 1999	bioMérieux[3]
303531	This study and Donnelly *et al.*, 1999	bioMérieux[3]
C. tropicalis		
NCPF 3111	AJ237918; this study	NCPF catalogue
K. lactis		
J7	M25826	Deshler *et al.* (1989)
S. cerevisiae		
A364A	L00026	Gallwitz & Sures (1980)

[1] Abbreviations: ATCC, American Type Culture Collection, (Manassas, VA, USA); NCPF, National Collection of Pathogenic Fungi, Bristol, UK. The country of origin of the *C. dubliniensis* isolates is shown in parentheses.
[2] Accession numbers are for the EMBL/GenGank nucleotide sequence databases.
[3] From the culture collection of bioMérieux, St Louis, MO, USA, courtesy of D. Pincus.

3.2.2 Cloning of the *C. dubliniensis ACT1* gene

The *C. dubliniensis* genomic library was screened using a radioactively labelled probe consisting of the entire *C. albicans ACT1* (*CaACT1*) gene on a *Eco*RI/*Hin*dIII fragment cloned into pBR322 (p1002, a gift from B. Magee, University of Minnesota) as described in sections 2.2.5 and 2.2.6. Hybridising plaques were identified and one of these was selected for further analysis. The recombinant phage purified from this plaque was termed λCDACT1. Genomic DNA was purified from λCDACT1 as described by Sambrook *et al.* (1989) and was mapped with restriction endonucleases. Attempts to subclone specific fragments failed and it was decided to amplify the *C. dubliniensis ACT1* gene (*CdACT1*) from λCDACT1 by PCR using a mixture containing *Taq* DNA polymerase and the proof-reading polymerase *Pwo* (Expand high-fidelity PCR system, Roche) and three primer sets homologous to regions of the *CaACT1* gene and flanking sequences, including 5'F/5'R, ACTF/ACTR and 3'F/3'R (Table 3.2).

Table 3.2 PCR primers used to clone the *CdACT1* gene from λCDACT1

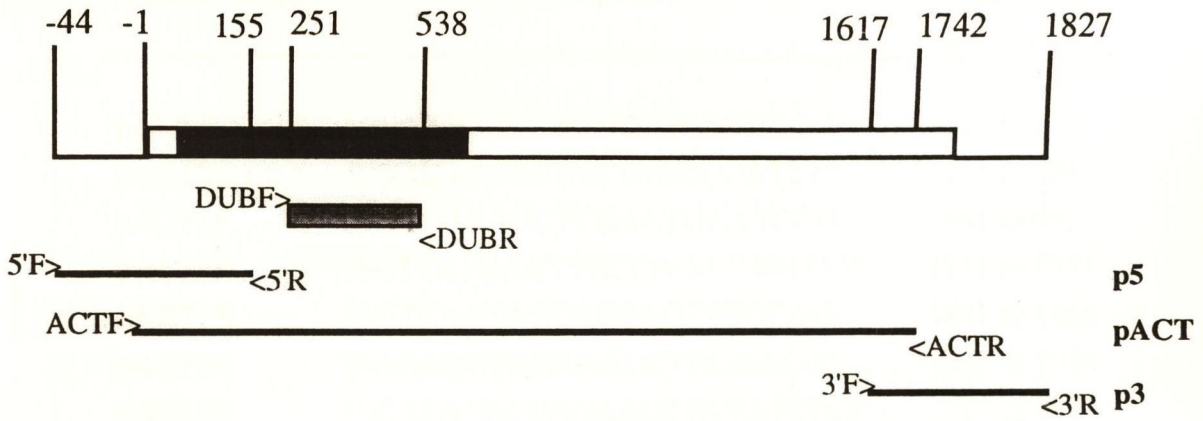
Primer	Sequence	Nucleotide co-ordinates [1]	RE site [2]
5'F	5'- CGGAATTCCTTAGAAACATTATCTCGAT -3'	-49 to -30	*Eco*RI
5'R	5'- GCTCTAGAGAGAAATATTATGTCGACAA -3'	126 to 145	*Xba*I
ACTF	5'-CGGAATTCAATGGACGGTGGTATGTT-3'	-1 to 17	*Eco*RI
ACTR	5'-CGGAATTCAATGGATGGACCAGATTCGTCG-3'	1746 to 1767	*Eco*RI
3'F	5'-CGGAATTCTAAGATTATTGCTCCACCAG-3'	1641 to 1660	*Eco*RI
3'R	5'-GCTCTAGACCAGATTTCCAGAATTTCAC-3'	1792 to 1811	*Xba*I
INTF	5'- CGGAATTCAATGGACGGTGGTATGGT -3'	-1 to 17	*Eco*RI
INTR	5'- CGGAATTCGAGCGTCGTCACCGGC -3'	724 to 739	*Eco*RI
AF	5'- CGGAATTCCTTCTTCTCAATCTTCTGCCA -3'	1346 to1366	*Eco*RI
AR	5'- CGGAATTCAATGGATGGACCAGATTCGTCG -3'	1746 to 1767	*Eco*RI

[1] Primers were complementary to the *C. albicans ACT1* gene, accession no. X16377 (Losberger & Ernst, 1989). Nucleotide co-ordinates shown are numbered in the 5' to 3' direction with the first base of the translation start codon being +1.

[2] Restriction endonuclease recognition sequence included within the primer sequence (underlined).

The PCRs were carried out in a final 100 µl volume as described in section 2.4.2 with 10 ng of recombinant phage DNA. Cycling conditions were as follows: 30 cycles of 1 min at 94 °C, 1 min at 52 °C and 1 min at 72 °C, followed by 72 °C for 10 min. Amplification products were purified and cloned into pBluescript as described in section 2.4 to yield the recombinant clones p5, pACT and p3 containing overlapping *CdACT1* homologous sequences as indicated in Fig. 3.1

Figure 3.1 Schematic diagram of the *C. dubliniensis ACT1* gene (*CdACT1*) [1]



[1] The *C. dubliniensis ACT1* gene is represented by the large horizontal box. The black area corresponds to the position of the intron at the 5' end of the gene. The thin horizontal lines in the lower part of the figure represent sequences amplified from the *CdACT1*-encoding recombinant phage λCDACT1 using the primer pairs 5'F/5'R, ACTF/ACTR and 3'F/3'R (Table 1). The names of the recombinant plasmids obtained when these amplimers were cloned in pBluescript are shown on the right of the figure. The location of sequences amplified with the *C. dubliniensis*-specific primer pair DUBF/DUBR is indicated by the shaded horizontal box in the central part of the figure. The nucleotide co-ordinates of the sequences contained in each amplimer relative to *CdACT1* sequences are shown in the upper part of the figure (numbering of *CdACT1* is in the 5' to 3' direction from the first base (+1) of the ATG translation start codon.).

3.2.3 Sequencing of the *C. dubliniensis ACT1* gene

The inserts contained within the recombinant clones p5 and p3 were sequenced fully in both directions using the M13 forward and reverse primers as described in section 2.6.1. The pACT recombinant clone was sequenced in both directions using both the M13 forward and reverse primers and the additional specific primers listed in Table 3.3.

Table 3.3 Additional internal primers used in sequencing the clone pACT

Primer Name	Sequence	Nucleotide co-ordinates [1]
pACT1F	5'-GATTGATCTGTCGGCAATGG-3'	301 to 320
pACT2F	5'-GACTGTCGTACTAACCCATT-3'	615 to 634
pACT3F	5'-ACCGAAGCTCCAATGAATCC-3'	951 to 970
pACT4F	5'-CTTATGAATTGCCAGATGGTC-3'	1351 to 1371
pACT1R	5'-GTCAATACCAGCAGCTTCCA-3'	1441 to 1460
pACT2R	5'-AAACGTAGAAAGCTGGAAC-3'	1020 to 1038
pACT3R	5'-CACATACCAGAACCGTTATCG-3'	665 to 685
pACT4R	5'-CCATTGCCGACAGATCAATC-3'	301 to 320

[1] Primers are complementary to the *C. dubliniensis ACT1* gene sequence, accession no. AJ236897 (this study and Donnelly *et al.*, 1999). Nucleotide co-ordinates shown are numbered in the 5' to 3' direction with the first base of the translation start codon being +1.

3.2.4 Cloning and sequencing of *Candida ACT1*-associated introns

Amplification of *ACT1*-associated intron sequences from the *Candida* strains listed in Table 3.1 was achieved using the primer pair INTF/INTR (Table 3.2). These primers were complimentary to sequences flanking the *C. albicans ACT1*-associated intron. The cycling conditions consisted of 30 cycles of 1 min at 94 °C, 1 min at 52 °C and 1 min at 72 °C, followed by one cycle of 72 °C for 10 min. The amplimers were purified using the Wizard PCR Preps DNA Purification system (Promega) and cloned into pBluescript as previously described (Section 2.4). The clones were sequenced fully in both directions using the universal M13 forward and reverse primers.

3.2.4 Sequence analysis of the *C. dubliniensis ACT1* gene and *Candida ACT1*-associated introns

DNA sequence alignments, predicted protein sequence analysis and phylogenetic analysis of the *C. dubliniensis ACT1* gene were carried out as described in Section 2.6.2.

3.3 Results

3.3.1 Identification of a *C. dubliniensis ACT1* homologue

In order to identify the *C. dubliniensis ACT1* gene (*CdACT1*) a library of *C. dubliniensis* CD36 genomic DNA cloned in the lambda replacement vector EMBL3 was screened by plaque hybridisation with a radioactively labelled probe consisting of the entire *C. albicans ACT1* gene (*CaACT1*). Ten reactive plaques were identified and one of these was selected for further study. Subsequent analysis of the recombinant phage isolated from this plaque indicated that the 5' end of the *CdACT1* gene was not present. DNA isolated from this recombinant phage used as a template in a PCR with the INTF/R primer set failed to yield any product. However, primers designed to amplify a 600 bp section of the gene close to the 3' end did yield the expected size product. These results indicated that a portion of the *CdACT1* gene was missing from the cloned insert contained within this recombinant phage.

A second *CaACT1*-reactive plaque was selected and the recombinant phage purified from it (λCDACT1) contained the entire *CdACT1* gene. λCDACT1 was found to contain a cloned DNA insert of approximately 15 kb. Southern hybridisation analysis of restriction endonuclease-generated fragments using the *C. albicans ACT1* gene as a probe identified two strongly hybridising fragments, a 4 kb *Pst*I/*Eco*RI fragment and a 2.2 kb *Spe*I/*Eco*RI fragment, both of which appeared to contain the entire *C. dubliniensis ACT1* gene. Attempts were made to subclone these fragments using a 'shotgun' cloning method whereby the entire *Pst*I/*Eco*RI or *Spe*I/*Eco*RI λCDACT1 genomic DNA digest was cloned into the vector pBluescript and the resultant recombinants screened for *CaACT1* hybridising fragments. This approach was used as the quantity of DNA obtained from the extraction method of Sambrook *et al.* (1989) was very low with this particular recombinant phage and gel purification of individual fragments for cloning proved unsuccessful. When specific clones containing the appropriately sized inserts were sequenced they were found to contain both *ACT1*-homologous and EMBL3 vector-homologous sequences. No recombinant plasmids harbouring *ACT1*-homologous DNA

only were obtained. It was concluded that the *ACT1*-homologous insert DNA fragments from λCDACT1 was unstable when cloned in pBluescript in *E. coli* DH5α.

It was decided to amplify *CdACT1* directly from the recombinant phage by PCR. The primer pair ACTF/ACTR (Table 3.2) was designed to amplify a 1743 bp fragment from the *CaACT1* gene. This fragment contained most of the coding sequence of *CaACT1*. A similar sized fragment was obtained when λCDACT1 DNA was used as a template. The 5F/5R primer pair (Table 3.2) was designed to amplify approximately 200 bp from the 5' end of *CaACT1* containing the ATG start codon. A similar sized fragment was obtained with the λCDACT1 DNA. The λCDACT1 amplification products achieved with the primer pairs ACTF/ACTR and 5F/5R were cloned into pBluescript to generate the recombinant plasmids pACT and p5, respectively (Fig 3.1). The 3F/3R primer pair (Table 3.2) was designed to amplify approximately 200 bp from *CaACT1* surrounding the TAA stop codon. A number of different fragments were obtained when λCDACT1 DNA was used as the template, including a product of similar size to that obtained when *C. albicans* genomic DNA was used as a template in the PCR. This amplimer was cloned into pBluescript to yield the recombinant plasmid p3 (Fig. 3.1). The insert DNA cloned in p5 and p3 was sequenced fully in both directions using universal primers, while the insert DNA cloned in pACT was sequenced fully by primer walking. These three overlapping sequences yielded a contiguous sequence of 1827 bp revealing an ORF of 1131 bp interrupted by a single 632 bp intron at the 5' end (Fig. 3.2). The overall nucleotide sequence identity between the *CdACT1* and *CaACT1* sequences was 90.6%. Further analysis of the sequence showed that the region of greatest divergence is contained within the introns located at the 5' end of both genes (Fig. 3.3). The identity that exists between the introns is 83.4 %, whilst the identity between the spliced coding sequences is 97.9 %. The spliced coding sequences are identical in length (1131 bp) and the predicted proteins are both 375 aa in length. However, there are a total of 24 base changes between the two sequences, but only one of these base changes (A -> G, at position 661 in the *C. dubliniensis* sequence; Fig. 3.2) results in an amino acid change in the predicted *C. dubliniensis* protein. This change in the tenth amino acid from isoleucine to valine is a

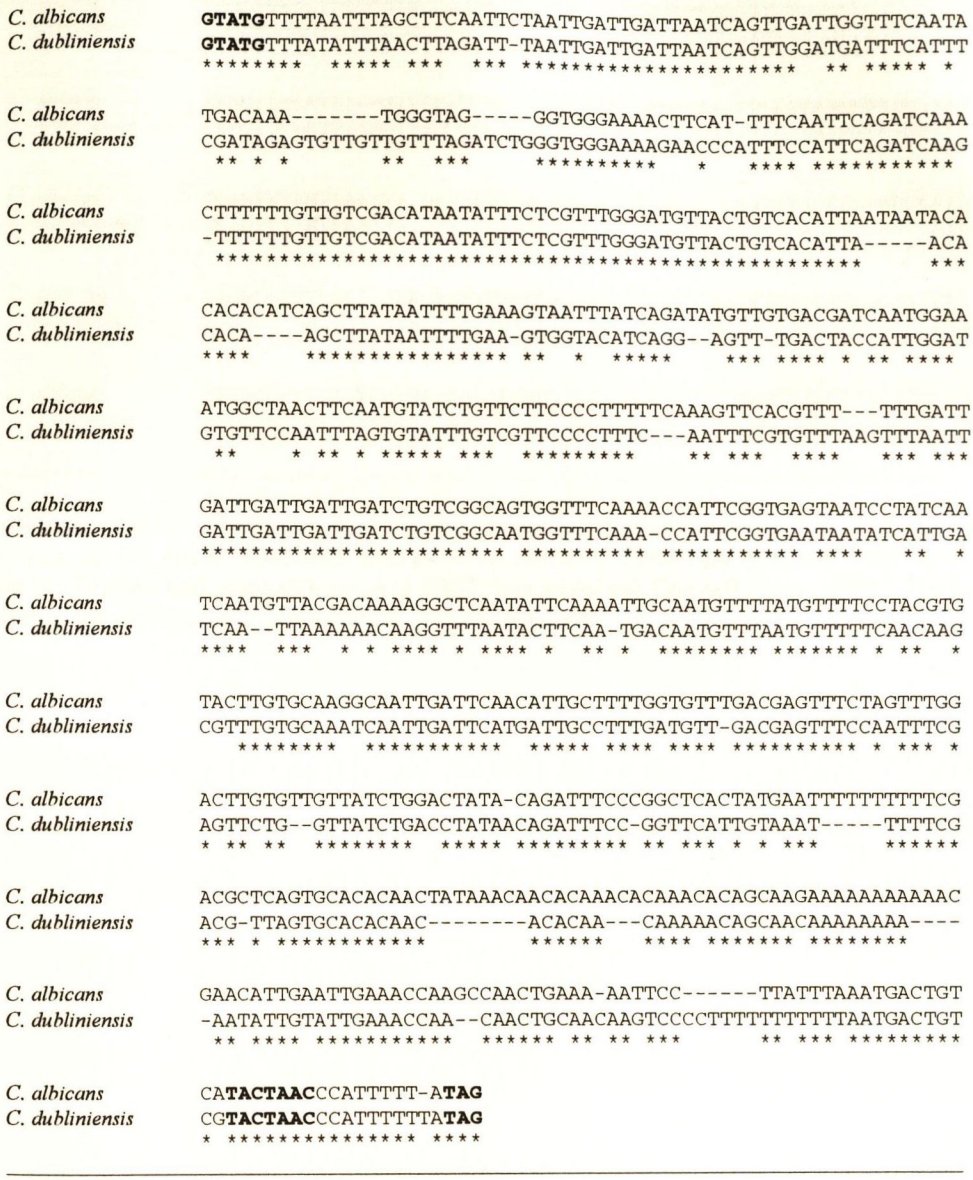
conservative change as both residues are neutral and hydrophobic. The *C. dubliniensis* predicted Act1 protein is otherwise identical to that of *C. albicans* (Fig. 3.4). The *CdACT1* intron is 632 bp long which is 25 bp shorter than the corresponding sequence in *CaACT1*; however, it is situated in exactly the same position at the 5' end of the gene and is recognisable by the presence of yeast intron consensus sequences. These are the 5' consensus sequence GTATG, the 3' consensus sequence YAG where Y is a variable nucleotide representing either T or C, and the branchpoint sequence TACTAAC located near the 3' end which is essential for efficient splicing (Fig. 3.3).

Figure 3.2 Nucleotide sequence and deduced amino acid sequence of the *C. dubliniensis ACT1* gene [1]

```
-44                                                                 CTTAGAAACATTATCTCGATATTAATATAAAATAATCATTAAAA
  1     ATG GAC GGT GGT ATG TTT ATA TTT AAC TTA GAT TTA ATT GAT TGA TTA ATC AGT TGG ATG
  1     M   D   G   E
 61     ATT TCA TTT CGA TAG AGT GTT GTT GTT TAG ATC TGG GTG GGA AAA GAA CCC ATT TCC ATT
121     CAG ATC AAG TTT TTT GTT GTC GAC ATA ATA TTT CTC GTT TGG GAT GTT ACT GTC ACA TTA
181     ACA CAC AAG CTT ATA ATT TTG AAG TGG TAC ATC AGG AGT TTG ACT ACC ATT GGA TGT GTT
241     CCA ATT TAG TGT ATT TGT CGT TCC CCT TTC AAT TTC GTG TTT AAG TTT AAT TGA TTG ATT
301     GAT TGA TCT GTC GGC AAT GGT TTC AAA CCA TTC GGT GAA TAA TAT CAT TGA TCA ATT AAA
361     AAA CAA GGT TTA ATA CTT CAA TGA CAA TGT TTA ATG TTT TTC AAC AAG CGT TTG TGC AAA
421     TCA ATT GAT TCA TGA TTG CCT TTG ATG TTG ACG AGT TTC CAA TTT CGA GTT CTG GTT ATC
481     TGA CCT ATA ACA GAT TTC CGG TTC ATT GTA AAT TTT TCG ACG TTA GTG CAC ACA ACA CAC
541     AAC AAA AAC AGC AAC AAA AAA AAA ATA TTG TAT TGA AAC CAA CAA CTG CAA CAA GTC CCC
601     TTT TTT TTT TTA ATG ACT GTC GTA CTA ACC CAT TTT TTA TAG  AA GAA GTT GCT GCT TTA
  5                                                             E   V   A   A   L
661     GTT ATC GAT AAC GGT TCT GGT ATG TGT AAA GCC GGT TTT GCC GGT GAT GAC GCT CCA AGA
 10     V   I   D   N   G   S   G   M   C   K   A   G   F   A   G   D   D   A   P   R
721     GCT GTG TTC CCA TCT CTC GTT GGT AGA CCA AGA CAT CAA GGT ATC ATG GTT GGT ATG GGT
 30     A   V   F   P   S   L   V   G   R   P   R   H   Q   G   I   M   V   G   M   G
781     CAA AAA GAT TCT TAC GTC GGT GAC GAA GCC CAA TCC AAA AGA GGT ATT TTG ACC TTG AGA
 50     Q   K   D   S   Y   V   G   D   E   A   Q   S   K   R   G   I   L   T   L   R
841     TAC CCA ATT GAA CAC GGT ATT GTT TCC AAC TGG GAC GAT ATG GAA AAA ATC TGG CAT CAC
 70     Y   P   I   E   H   G   I   V   S   N   W   D   D   M   E   K   I   W   H   H
901     ACT TTC TAC AAC GAA TTG AGA GTT GCT CCA GAA GAA CAT CCA GTT TTG TTG ACC GAA GCT
 90     T   F   Y   N   E   L   R   V   A   P   E   E   H   P   V   L   L   T   E   A
961     CCA ATG AAT CCA AAA TCT AAC AGA GAA AAG ATG ACT CAA ATC ATG TTT GAA ACA TTC AAT
110     P   M   N   P   K   S   N   R   E   K   M   T   Q   I   M   F   E   T   F   N
1021    GTT CCA GCT TTC TAC GTT TCC ATT CAA GCT GTT TTG TCT TTG TAC TCT TCT GGT AGA ACC
130     V   P   A   F   Y   V   S   I   Q   A   V   L   S   L   Y   S   S   G   R   T
1081    ACC GGT ATT GTT TTG GAT TCT GGT GAT GGT GTT ACT CAC GTT GTT CCA ATT TAC GCT GGT
150     T   G   I   V   L   D   S   G   D   G   V   T   H   V   V   P   I   Y   A   G
1141    TTC TCC TTA CCA CAT GGT ATT TTG AGA ATT GAT TTG GCT GGT AGA GAC TTG ACC AAC CAC
170     F   S   L   P   H   G   I   L   R   I   D   L   A   G   R   D   L   T   N   H
1201    TTG TCC AAG ATT TTG TCT GAA CGT GGT TAC AGT TTC ACT ACT AGT GCT GAA AGA GAA ATT
190     L   S   K   I   L   S   E   R   G   Y   S   F   T   T   S   A   E   R   E   I
1261    GTC AGA GAC ATT AAA GAA AGA TTA TGT TAC GTT GCT TTG GAT TTC GAA CAA GAA ATG CAA
210     V   R   D   I   K   E   R   L   C   Y   V   A   L   D   F   E   Q   E   M   Q
1321    ACT TCT TCT CAA TCT TCT GCT ATT GAA AAA TCT TAT GAA TTG CCA GAT GGT CAA GTT ATC
230     T   S   S   Q   S   S   A   I   E   K   S   Y   E   L   P   D   G   Q   V   I
1381    ACT ATT GGT AAC GAA AGA TTC AGA GCT CCA GAA GCT TTG TTC AGA CCA GCT GAT TTG GGT
250     T   I   G   N   E   R   F   R   A   P   E   A   L   F   R   P   A   D   L   G
1441    TTG GAA GCT GCT GGT ATT GAC CAA ACC ACT TTC AAC TCC ATC ATG AAG TGT GAC ATG GAT
270     L   E   A   A   G   I   D   Q   T   T   F   N   S   I   M   K   C   D   M   D
1501    GTT AGA AAG GAA TTA TAC GGT AAC ATT GTT ATG TCT GGT GGT ACT ACC ATG TTC CCA GGT
290     V   R   K   E   L   Y   G   N   I   V   M   S   G   G   T   T   M   F   P   G
1561    ATT GCT GAA CGT ATG CAA AAG GAA ATT ACT GCT TTG GCT CCA TCT TCT ATG AAA GTT AAG
310     I   A   E   R   M   Q   K   E   I   T   A   L   A   P   S   S   M   K   V   K
1621    ATT ATT GCT CCA CCA GAA AGA AAA TAC TCT GTC TGG ATT GGT GGT TCT ATC TTG GCT TCA
330     I   I   A   P   P   E   R   K   Y   S   V   W   I   G   G   S   I   L   A   S
1681    TTG TCT ACT TTC CAA CAA ATG TGG ATT TCA AAA CAA GAA TAC GAC GAA TCT GGT CCA TCT
350     L   S   T   F   Q   Q   M   W   I   S   K   Q   E   Y   D   E   S   G   P   S
1741    ATT GTT CAT CAC AAA TGT TTC TAA GTGAAATTCTGGAAATCTGG
370     I   V   H   H   K   C   F
```

[1] Nucleotide sequences are numbered in the 5' to 3' direction from the first base (+1) of the ATG translation start codon. Amino acid sequences are numbered from the initial methionine. The amino acid sequence is based on the predicted ORF as determined by intron 5' and 3' consensus sequences. The intron interrupts the fouth codon between the first and second nucleotides of this codon and is highlighted in bold. The single base change between the *C. albcians* and *C. dubliniensis* sequences which results in an amino acid change is located at postion 661 and is underlined. The amino acid change between *C. albicans* and *C. dubliniensis* is located at position 10 and is also underlined.

Figure 3.3 Alignment of the *C. albicans* and *C. dubliniensis ACT1*-associated introns [1]



[1] The *C. albicans* sequence was from strain ATCC 10123 (GenBank accession no. X16377; Losberger & Ernst, 1989) and the *C. dubliniensis* sequence from strain CD36 (EMBL accession no. AJ236897; this study and Donnelly *et al.*, 1999). The 5' intron consensus sequence (GTATG), the 3' intron consensus sequence (TAG) and the branchpoint sequence (TACTAAC) are shown in bold type. Asterisks indicate identical nucleotides and dashes indicate deletions.

Figure 3.4 Alignment of the *C. albicans* and *C. dubliniensis* predicted Act1 proteins [1]

```
C. albicans      MDGEEVAALIIDNGSGMCKAGFAGDDAPRAVFPSLVGRPRHQGIMVGMGQKDSYVGDEAQ
C. dubliniensis  MDGEEVAALVIDNGSGMCKAGFAGDDAPRAVFPSLVGRPRHQGIMVGMGQKDSYVGDEAQ
                 *********:**************************************************

C. albicans      SKRGILTLRYPIEHGIVSNWDDMEKIWHHTFYNELRVAPEEHPVLLTEAPMNPKSNREKM
C. dubliniensis  SKRGILTLRYPIEHGIVSNWDDMEKIWHHTFYNELRVAPEEHPVLLTEAPMNPKSNREKM
                 ************************************************************

C. albicans      TQIMFETFNVPAFYVSIQAVLSLYSSGRTTGIVLDSGDGVTHVVPIYAGFSLPHGILRID
C. dubliniensis  TQIMFETFNVPAFYVSIQAVLSLYSSGRTTGIVLDSGDGVTHVVPIYAGFSLPHGILRID
                 ************************************************************

C. albicans      LAGRDLTNHLSKILSERGYSFTTSAEREIVRDIKERLCYVALDFEQEMQTSSQSSAIEKS
C. dubliniensis  LAGRDLTNHLSKILSERGYSFTTSAEREIVRDIKERLCYVALDFEQEMQTSSQSSAIEKS
                 ************************************************************

C. albicans      YELPDGQVITIGNERFRAPEALFRPADLGLEAAGIDQTTFNSIMKCDMDVRKELYGNIVM
C. dubliniensis  YELPDGQVITIGNERFRAPEALFRPADLGLEAAGIDQTTFNSIMKCDMDVRKELYGNIVM
                 ************************************************************
```

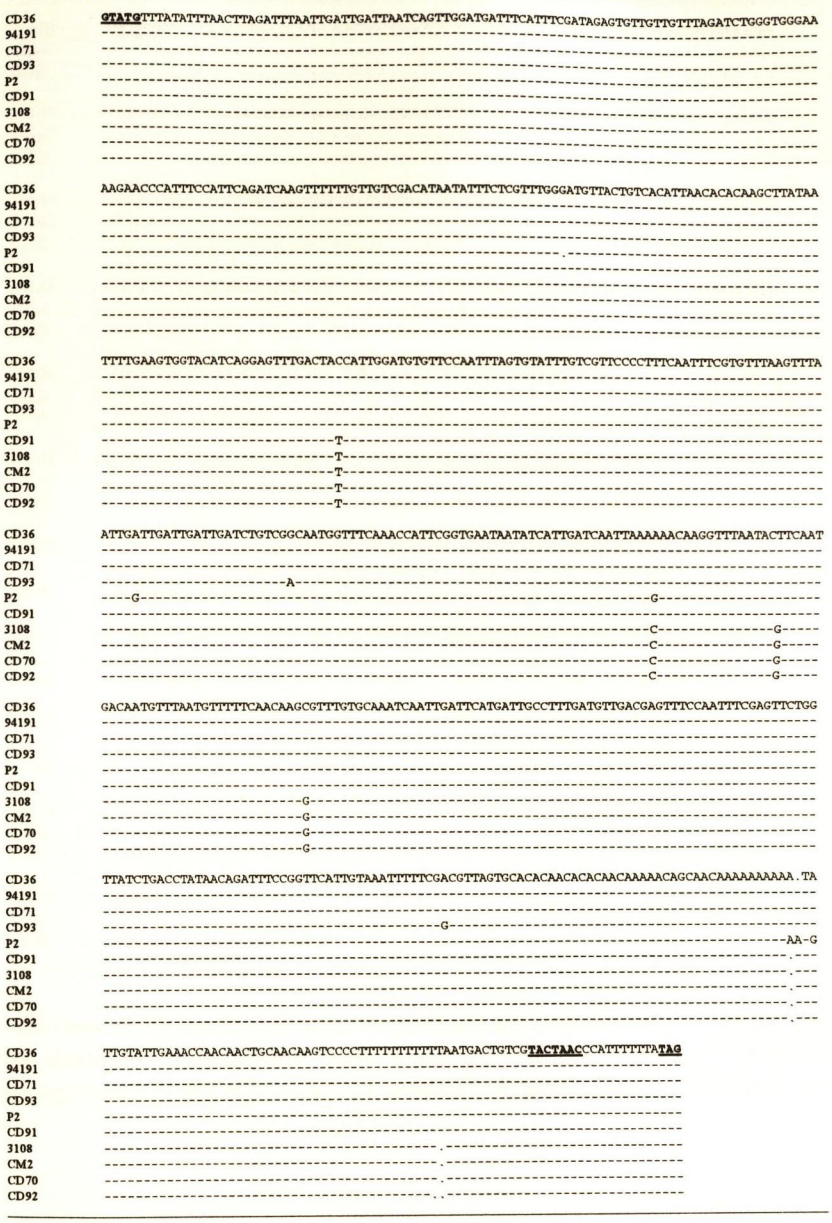
[1] The *C. albicans* and *C. dubliniensis* Act1 protein sequences are predicted from the nucleotide sequences of *C. albicans* strain ATCC 10123 (GenBank accession no. X16377; Losberger & Ernst, 1989) and *C. dubliniensis* type strain CD36 (GenBank accession no. AJ236897; this study and Donnelly *et al.*, 1999). The asterisks indicate indentical residues and the semi-colons indicate similar residues.

3.3.2 *Candida ACT1*-associated introns

The PCR primers INTF/R (Table 3.2), flanking the *C. albicans ACT1*-associated intron were used to amplify the *ACT1*-associated introns from 10 *C. dubliniensis* strains from geographically diverse locations and from 2 *C. albicans* isolates (strains 132A serotype B and 179B serotype A, Table 3.1), 3 *C. stellatoidea* isolates (strains ATCC 11006, 303530 and 303531, Table 3.1) and 1 *C. tropicalis* isolate (NCPF 3111, Table 3.1) using a high fidelity thermostable polymerase. Genomic DNA from the *C. albicans* and *C. stellatoidea* strains yielded similar size amplimers. All amplimers from the 10 *C. dubliniensis* strains were of the same size (~ 630 bp) and slightly smaller than that of *C. albicans* (~ 660 bp), while the *C. tropicalis* amplimer was slightly larger than that of *C. albicans* (~ 690 bp) as estimated by agarose gel electrophoresis. The amplimers were cloned into pBluescript and sequenced in both directions. All *Candida* introns show consensus sequence at the 5' (GTATG) and the 3' (YAG) ends and at the branch point (TACTAAC), which has been shown to be essential for efficient splicing as it is involved in the formation of the intron lariat intermediate. Analysis of the intron sequence from 10 *C. dubliniensis* isolates tested from disparate geographic locations (Table 3.1) revealed that they are almost identical to each other (Fig. 3.5). The introns from the type strain *C. dubliniensis* CD36 (Irish), the Spanish (94191) and the Argentinean (CD71) isolates are 632 bp long and identical to each other at the nucleotide sequence level. The Finnish isolate (CD93) is also 632 bp long and it differs from CD36 by two base changes (G–>A, nt 304; A–>G, nt 510). The Swiss isolate (P2) intron is the same size as the intron of *C. dubliniensis* CD36 although there are 4 differences consisting of two base changes (A–>G, nt 284 and 351), one deletion (G, nt 153), and one insertion (A, nt 556). The intron from the Irish strain CD91 is 631 bp long. The differences here consist of one base change (C–>T, nt 227) and one deletion (A, nt 554). The introns from the two British strains, NCPF 3108 and CD70 and the Australian isolate, CM2, are identical to each other both in length (630 bp) and in sequence. They differ from the type strain by three base changes (C–>T, nt 227, T–>G, nt 367 and C–>G, nt 399) and two deletions (A nt 554; T, nt 602) in the same locations. Finally, the intron from the Canadian strain CD92 is 629 bp long. It is identical to the intron sequences

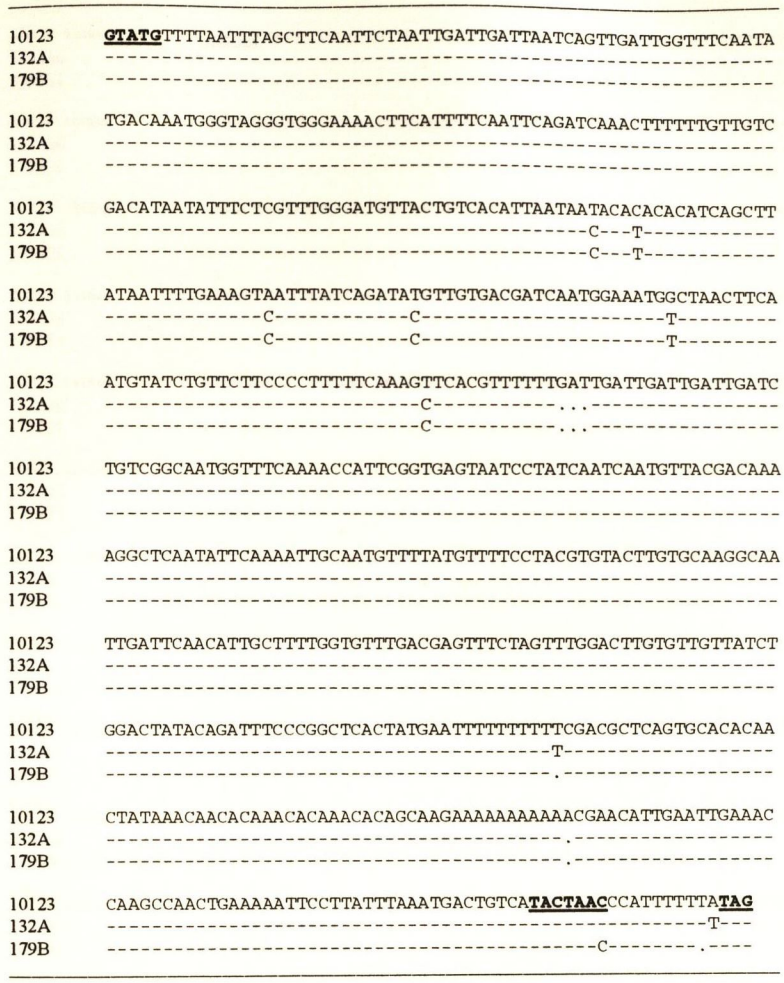
of NCPF 3108, CM2 and CD70 except for the deletion of an additional T (nt 601). These sequence alignments show that *C. dubliniensis* strains from disparate locations, isolated at different times (NCPF 3108 was recovered in 1957) show evolutionary constraint in the sequence of their *ACT1*-associated introns. Like *C. dubliniensis*, the intron sequence in both *C. albicans* and *C. stellatoidea* does not exhibit significant strain-to-strain variation (Figs. 3.6 and 3.7). The degree of homology between the *C. albicans* and *C. stellatoidea* introns (99.8 %) is indicative of the close relationship between these two organisms (Table 3.4). The divergence between the introns of *C. albicans* and *C. tropicalis* is 43.4 %. This shows that *C. tropicalis*, a well-established species closely related to *C. albicans* (1.4 % divergent based upon the V3 variable region of the large subunit ribosomal gene), is considerably more divergent from *C. albicans* than *C. dubliniensis*, the latter two species being 16.6 % divergent (Table 3.4) in their *ACT1*-associated intron sequences based on the data presented here.

Figure 3.5 Alignment of the *C. dubliniensis ACT1*-associated intron sequences [1]



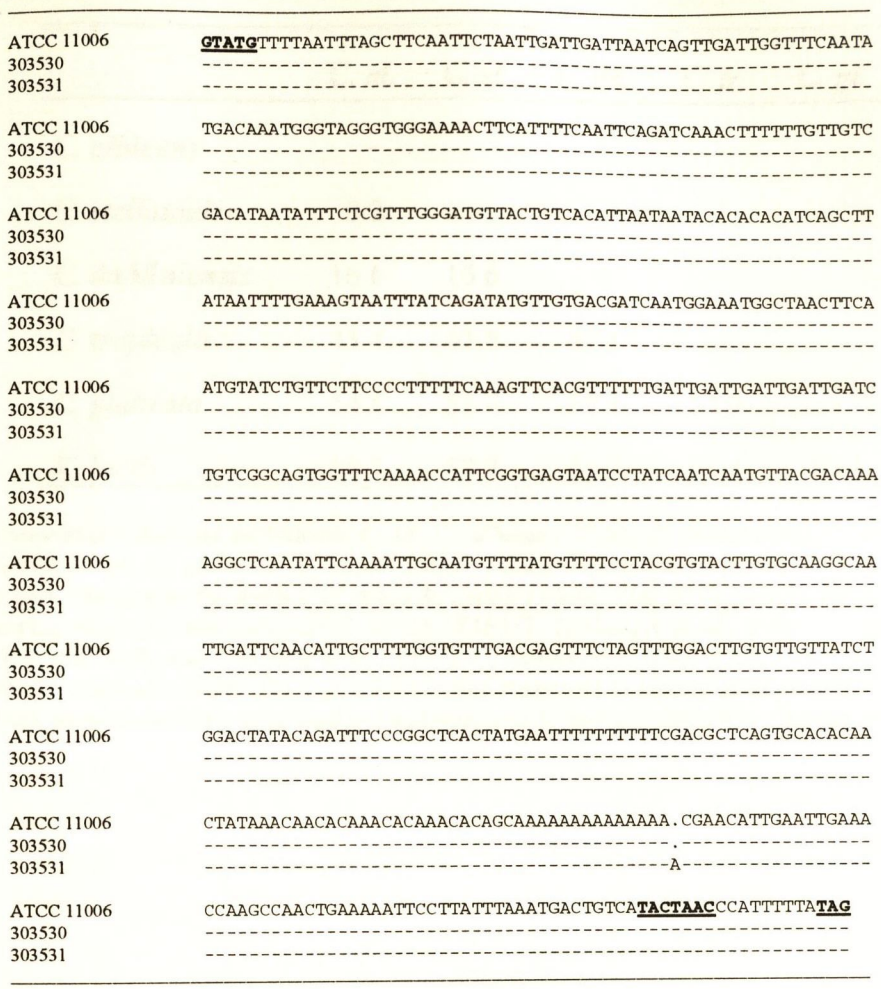
[1] The *ACT1*-associated intron sequences are from *C. dubliniensis* isolates CD36 (Irish), 94191 (Spanish), CD71 (Argentinean), CD93 (Finnish), P2 (Swiss), CD91 (Irish), NCPF 3108, (UK), CM2 (Australian), CD70 (UK), CD92 (Canadian). Conserved elements - branchpoint (TACTAAC), 5' (GTATG) and 3' (TAG) - are highlighted (bold and underlined typeface) in the CD36 sequence. Dashes represent identical nucleotides and dots represent deletions.

Figure 3.6 Alignment of *C. albicans ACT1*-associated introns [1]



[1] The *C. albicans ACT1*-associated intron sequences are from strains ATCC 10123, 132A and 179B (Table 3.1). Conserved elements - branchpoint (TACTAAC), 5' (GTATG) and 3' (TAG) - are highlighted (bold and underlined typeface) in the ATCC 10123 sequence. Dashes represent identical nucleotides and dots represent deletions.

Figure 3.7 Alignment of *C. stellatoidea ACT1*-associated introns [1]



[1] The strains represented are ATCC 11006, 303530 and 303531 (Table 3.1). Conserved elements - branchpoint (TACTAAC), 5' (GTATG) and 3' (TAG) - are highlighted (bold and underlined typeface) in the ATCC 11006 sequence. Dashes represent identical nucleotides and dots represent deletions.

Table 3.4 Genetic distance matrix based on comparison of *ACT1*-associated intron sequences [1]

	C. al.	*C. st.*	*C. du.*	*C. tr.*	*C. gl.*	*K. la.*
C. albicans	-					
C. stellatoidea	0.2	-				
C. dubliniensis	16.6	16.6	-			
C. tropicalis	43.4	43.5	47.1	-		
C. glabrata	54.8	55.0	57.1	54.0	-	
K. lactis	58.1	58.3	54.7	61.4	63.1	-

[1] The abbreviations used are as follows: *C. al.*, *C. albicans*; *C. st.*, *C. stellatoidea*; *C. du.*, *C. dubliniensis*; *C. tr.*, *C. tropicalis*; *C. gl.*, *C. glabrata*; *K. la.*, *K. lactis*. Values correspond to percentages of difference corrected for multiple base changes by the method of Jukes & Cantor (1969). The *ACT1* gene intron sequences used were from the following strain: *C. albicans* ATCC 10123 (X16377; Losberger *et al.*, 1989); *C. stellatoidea* ATCC 11006 (AJ237919; this study and Donnelly *et al.*, 1999); *C. dubliniensis* CD36 (AJ236897; this study and Donnelly *et al.*, 1999); *C. tropicalis* NCPF 3111 (AJ237918; this study and Donnelly *et al.*, 1999); *C. glabrata* NCPF 90876 (AF069746; unpublished data submitted to GenBank) and *K. lactis* J7 (M25826; Deshler *et al.*, 1989).

3.3.3 Phylogenetic analysis based on *ACT1* sequences

The *ACT1* gene has been used extensively to infer interspecies relationships across broad evolutionary distances (Fidel *et al.*, 1988; Fletcher *et al.*, 1994; Cox *et al.*, 1995; Wery *et al.*, 1996). This part of the study was undertaken to confirm the phylogenetic position of *C. dubliniensis* in relation to other yeast species using *ACT1* sequences. This is the first time that the phylogeny of *C. dubliniensis* has been investigated using non-ribosomal gene sequences. Since the *ACT1* gene of many yeast species contains highly conserved (i.e. exon) and less well-conserved (i.e. intron) sequences, these sequences were compared separately. Firstly, the *ACT1*-associated intron sequences from selected strains of *C. albicans*, *C. dubliniensis*, *C. stellatoidea*, *C. tropicalis*, *C. glabrata* and *Kluyveromyces lactis* (Table 3.1) were obtained either from the GenBank database or following amplification using the INTF/R primer set (Table 3.2) and compared using the CLUSTAL w sequence alignment software package. Secondly, the *ACT1* spliced coding sequences of *C. albicans*, *C. dubliniensis*, *C. glabrata*, *K. lactis* and *S. cerevisiae* (Table 3.1) were compared also using CLUSTAL W. *Kluyveromyces lactis* and *S. cerevisiae* sequences were included in the analyses to act as outliers. An evolutionary distance matrix for each group of sequences was generated incorporating corrections for multiple base changes according to the method of Jukes & Cantor (1969, Tables 3.4 and 3.5). These data indicate that the *C. dubliniensis* coding and intron sequences differ from the corresponding *C. albicans* sequences by 2.1 and 16.4 % respectively.

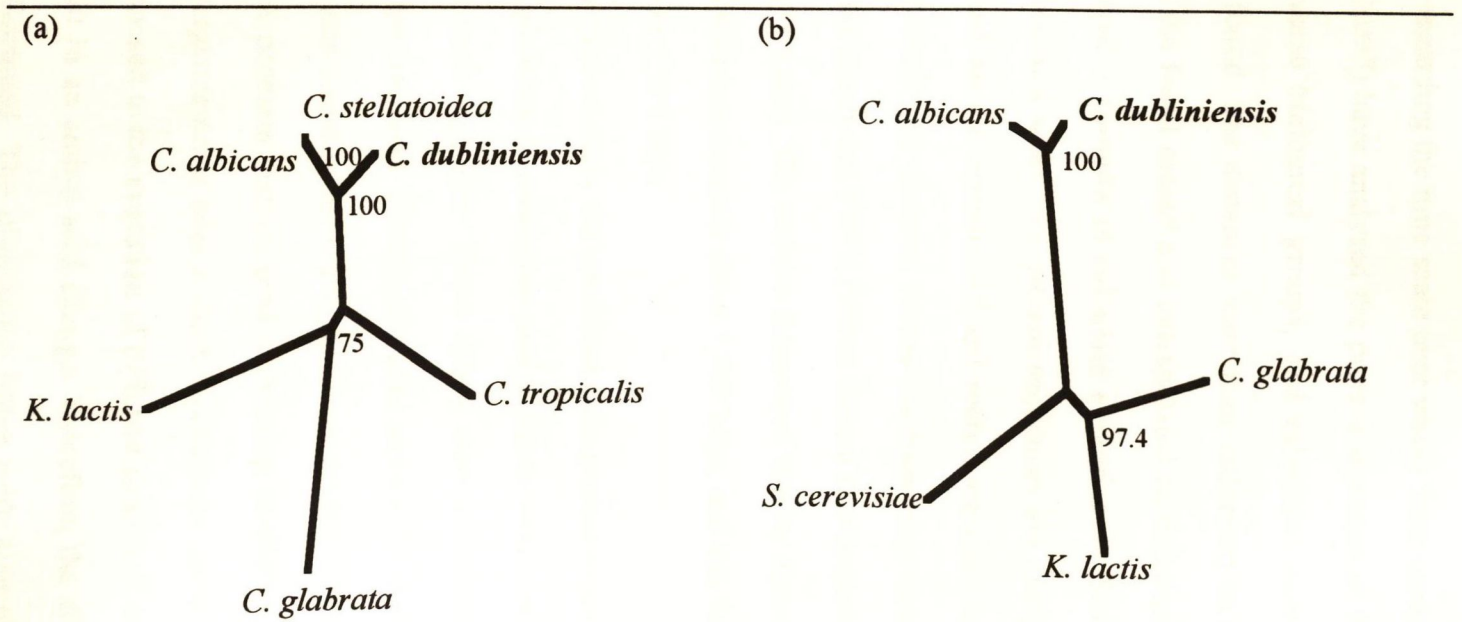
Table 3.5 Genetic distance matrix based on comparison of *ACT1* gene coding sequences [1]

	C. al.	*C. du.*	*S. ce.*	*C. gl.*	*K. la.*
C. albicans	-				
C. dubliniensis	2.1	-			
S. cerevisiae	12.9	12.6	-		
C. glabrata	13.2	12.3	9.8	-	
K. lactis	13.2	12.6	10.1	7.8	-

[1] The abbreviations used are as follows: *C. al.*, *C. albicans*; *C. st.*, *C. stellatoidea*; *C. du.*, *C. dubliniensis*; *C. tr.*, *C. tropicalis*; *C. gl.*, *C. glabrata*; *K. la.*, *K. lactis*. Values correspond to percentages of difference corrected for multiple base changes by the method of Jukes & Cantor (1969). The *ACT1* gene coding sequences used were as follows: *C. albicans* ATCC 10123 (X16377; Losberger & Ernst, 1989); *C. dubliniensis* CD36 (AJ236897; this study and Donnelly *et al.*, 1999); *S. cerevisiae* A364A (L00026; Gallwitz *et al.*, 1980); *C. glabrata* NCPF 90876 (AF069746; Kurzai and others, unpublished data) and *K. lactis* J7 (M25826; Deshler *et al.*, 1989).

Evolutionary trees based on these data were generated using the neighbour-joining method of Saitou & Nei (1987) and are shown in Figure 3.8. These trees and the bootstrap values determined for each node unequivocally confirm the phylogenetic position of *C. dubliniensis* as a separate taxon in relation to other yeast species, including *C. albicans*, as previously determined using ribosomal gene sequences. In addition, these data also confirm that *C. albicans* and *C. stellatoidea* are so closely related as to be considered a single species.

Figure 3.8 Unrooted phylogenetic neighbour-joining trees generated from the alignment of *ACT1*-intron (a) and -exon (b) sequences of *C. dubliniensis* and other yeast species [1]



[1] Numbers at each node were generated by boot-strap analysis (Felsenstein, 1985) and represent the percentage of times the arrangement occurred in 1000 randomly generated trees. The sequences used to construct the trees are indicated in the legends to Tables 3.4 and 3.5.

3.3.4 Divergence of *C. dubliniensis* and *C. albicans*

The construction of phylogenetic trees and the evaluation of evolutionary relationships between organisms on the basis of ribosomal or protein sequences has been very successful, and indeed has provided interesting insights. However, this approach does not provide any information regarding the time scale over which these evolutionary events occurred. Feng and colleagues (1997) have analysed the protein sequence of 57 different sets of enzymes taken from 15 diverse biological groups, and calculated evolutionary distances based on the similarities found. The distances were then calibrated on the basis of the divergence times drawn from the fossil record and extrapolated to encompass more distantly related groups. They employed a formula to calculate evolutionary distance based upon the fraction of unchanged residues when two protein sequences are compared. Six established divergence times all based on the vertebrate fossil record are used to calibrate the system. They then plotted the evolutionary distance versus the divergence times to yield the relationship where the slope of the graph was 0.0891D/Mya (where D = evolutionary distance and Mya = time in millions of years ago). The authors discovered that by direct extrapolation plants and animals last shared a common ancestor about 1,200 Mya, and that fungi diverged from either of those groups at about 1,275 Mya.

In the present study the predicted actin protein sequence data from *C. dubliniensis*, *C. albicans*, *C. glabrata*, *S. cerevisiae and K. lactis* were used to calculate D, the evolutionary distance, for each organism. These values were substituted into the above relationship to produce an evolutionary divergence time matrix for these species (Table 3.6). *Candida dubliniensis* was found to have diverged very recently from *C. albicans* (divergence time = 0.03 Mya). A problem encountered with using predicted protein sequence comparisons to calculate divergence times over a short evolutionary period (i.e. the evolution between yeast species as opposed to the evolution of different groups of organisms) is that not all nucleotide changes result in an amino acid change. Therefore, the divergence times predicted may be artificially shortened. The divergence times were also calculated using *ACT1* nucleotide sequence comparisons (Table 3.6) in order to establish a range of time over which *C.*

dubliniensis may have diverged from *C. albicans*. Using nucleotide sequences the divergence time of *C. dubliniensis* from *C. albicans* is shown to be 0.241 Mya. Given the close phylogenetic relationship between the two species it is likely that their divergence from each other is a recent event. The time frame of 30,000 to 241,000 years reflects this close relationship.

Table 3.6 Divergence times of yeast species based on comparison of *ACT1* nucleotide and predicted protein sequences [1]

	C. albicans **nucleotide sequence**	***C. albicans*** **protein sequence**
	Divergence times in millions of years ago	
C. dubliniensis	0.241	0.03
C. glabrata	1.517	0.63
S. cerevisiae	1.437	0.66
K. lactis	1.527	0.78

[1] Values were calculated based on the formula of Feng *et al.* (1997). The *ACT1* gene coding sequences used were as follows: *C. albicans* ATCC 10123 (X16377; Losberger & Ernst, 1989); *C. dubliniensis* CD36 (AJ236897; this study and Donnelly *et al.*, 1999); *S. cerevisiae* A364A (L00026; Gallwitz *et al.*, 1980); *C. glabrata* NCPF 90876 (AF069746; Kurzai and others, unpublished data) and *K. lactis* J7 (M25826; Deshler *et al.*, 1989).

3. 4 Discussion

The purpose of this study was to confirm the phylogenetic position of *C. dubliniensis* as previously determined by rRNA genes sequence data by using the *ACT1* gene sequence as the basis of comparison. An analysis of the phylogenetic position of *C. dubliniensis* using non-ribosomal DNA sequence was essential to confirm that *C. dubliniensis* is a species and not a variant of *C. albicans*. The *ACT1* gene was chosen firstly because it is conserved throughout the eukaryotic kingdom and has been used extensively to infer phylogenetic relationships and, secondly, because it has been shown to be useful in confirming phylogenetic data on the fungi in particular (Hightower & Meagher, 1986; Hennessey *et al.*, 1993; Fletcher *et al.*, 1994; Cox *et al.*, 1995; Wery *et al.*, 1996). Overall, the similarity between the *C. dubliniensis* and *C. albicans ACT1* genes was 90.6 %. However, analysis of the spliced coding nucleotide sequences indicates the percentage divergence between *C. albicans* and *C. dubliniensis* is 2.1 % (Table 3.5). This figure is comparable to the divergence between the two species for the V3 variable region of the large subunit RNA gene (2.25-2.48 %; Sullivan *et al.*, 1995 and 1997) and the small subunit RNA gene (1.4 %, Gilfillan *et al.*, 1998). The predicted *C. dubliniensis* Act1 protein sequence is identical to that of *C. albicans* with one difference, residue 10 is changed from isoleucine to valine (postion 661 of the *CdACT1* sequence, A–>G; Fig. 3.2). However, as both these amino acids are neutral and hydrophobic, substitution is unlikely to contribute to any significant structural or functional differences. Given the highly conserved nature of actin proteins this is to be expected for two organisms as closely related to each other as *C. dubliniensis* and *C. albicans*. A phylogenetic tree generated from the *ACT1* gene coding sequence shows that *C. dubliniensis* is grouped separately from *C. albicans* in 100 % of trees generated (Fig. 3.8b). This is the first phylogenetic analysis of *C. dubliniensis* based on non-ribosomal sequences and it confirms the phylogenetic position of *C. dubliniensis* as a distinct species within the genus *Candida* as determined previously by this laboratory and by others using RNA sequence analysis (Sullivan *et al.*, 1995 and 1997; Kurtzman & Robnett, 1997; Gilfillan *et al.*, 1998). This also confirms the usefulness of *ACT1* based phylogenetic analyses

for the fungi as shown previously by other researchers (Hightower & Meagher, 1986; Fletcher *et al.*, 1994; Cox *et al.*, 1995; Wery *et al.*, 1996).

The *ACT1* gene of the fungi in general is noteworthy because of the presence of intervening sequences (Deshler *et al.*, 1989; Wildeman *et al.*, 1988; Gallwitz & Sures, 1980; Ng *et al.*, 1980). At present, most known introns can be assigned unambiguously to one of four classes, depending on the intron structure and location (Krainer & Maniatis, 1988). The *ACT1*-associated intron belongs to class IV, which are nuclear pre-mRNA introns. The type strain CD36 *C. dubliniensis ACT1*-associated intron is located at the 5' end of the gene where it interrupts the fourth codon (Fig. 3.2). This position seems to be conserved amongst fungi, as all fungal actin genes containing an intron do so at the third, fourth or fifth codon (Bagavathi & Malathi, 1996). *Candida albicans*, *C. glabrata*, *S. cerevisiae* and *K. lactis* all contain introns located at this codon (Gallwitz & Sures, 1980; Deshler *et al.*, 1989; Losberger *et al.*, 1989). Three conserved sequence elements have been identified in the nuclear pre-mRNA of eukaryotes at the 5' and 3' splice sites and at a site within the intron near the 3' splice site, known as the branchpoint sequence. All three conserved elements have been shown to be important for the accurate and efficient splicing of introns in *S. cerevisiae* (Mount 1992; Langford *et al.*, 1984; Leer *et al.*, 1984; Molenaar *et al.*, 1984; Teem *et al.*, 1984). The *Candida ACT1*-associated introns presented in this study possess all three conserved elements, namely GTATG (5' consensus), YAG (3' consensus, where Y represents either C or T) and TACTAAC (branchpoint). *Candida albicans*, *C. dubliniensis*, *C. tropicalis* and *S. cerevisiae* all possess the 3' consensus sequence TAG, whilst *C. glabrata* and *K. lactis* have CAG.

The distance matrix generated by comparison of the *Candida ACT1*-associated introns provides interesting insight into the relationships that exist between the different *Candida* species. The *C. albicans* and *C. stellatoidea ACT1*-associated introns differ by one substitution, which corresponds to a 0.2 % difference (Table 3.4). This situation is analogous to that between the *S. cerevisiae* and *S. carlsbergensis*. The *ACT1*-associated introns of these two genes differ by one deletion and one substitution, and it is accepted that these two organisms are in fact the same species (Nellen *et al.*, 1981). Data from the present study

provides further evidence that *C. albicans* and *C. stellatoidea* should be classed as the same species. The *C. albicans* and *C. dubliniensis ACT1*-associated introns, however, differ by 16.6 % while the *C. tropicalis* intron differs from that of *C. albicans* by 43.4%. This is indicative of the closer relationship between *C. albicans* and *C. dubliniensis*. One striking feature of the *C. dubliniensis* introns was that they showed little intraspecies variation, even amongst strains from geographically diverse location (Fig. 3.5). These changes consisted of single base changes, some of which were shared by more than one strain, and deletions which occurred at the end of polyT and polyA runs and one deletion which occurred following two Gs. Introns containing these deletions were sequenced on separate occasions and using different amplimers to rule out the possibility of sequencing artefacts. It was concluded that these deletions are genuine and the result of slipped strand mispairing during replication. Similar intraspecies homology was observed with the *C. albicans* and *C. stellatoidea* isolates (Figs. 3.6 and 3.6). Boucher *et al.* (1996) observed similar results with their analysis of the Group I self-splicing intron present in the large ribosomal subunit gene. In that study the intron was present in a similar location in *C. albicans*, *C. stellatoidea* and *C. dubliniensis*. Again there was no significant intraspecies variation in the intron sequence. The *C. albicans* self-splicing intron and that of *C. stellatoidea* show a high degree of homology, differing only by three substitutions. They found that the homology between the *C. albicans* and *C. dubliniensis* group I introns (CaLSU and CdLSU, respectively) was relatively high except for two regions of divergence. These areas of difference were contained in two stem loop regions, both of which are much longer in *C. dubliniensis* than in *C. albicans*. These two regions lie outside the catalytic core, and although they are predicted to have a more complex secondary structure than those of *C. albicans*, they do not affect the self-splicing ability of the intron, and may be assigned into intron group IC, as is CaLSU. In the present study analysis of the *ACT1*-associated introns revealed that although conserved elements are present and identical in both *C. albicans* and *C. dubliniensis*, nucleotide differences were dispersed throughout the length of the intron. With group I introns, conservation of the nucleotide sequence may be important as it dictates the secondary structure of the intron and therefore its self-splicing ability.

However, with group IV introns the splicing event is mediated by the spliceosome, and although maintenance of the three conserved elements is important for splicing there does not appear to be any other constraints upon conservation of the nucleotide sequence. This may explain why divergence between *C. dubliniensis* and *C. albicans ACT1*-associated intron sequence is dispersed throughout the intron.

In conclusion, analysis of the actin gene has confirmed the phylogenetic position of *C. dubliniensis* as a separate species distinct from *C. albicans*. This confirmation of the previous rRNA analyses was to firmly establish *C. dubliniensis* as a distinct species and confirm that it is not a synonym of *C. albicans*. In addition, the analysis of the intron sequence, a sequence that does not have the same level of evolutionary constraint as the actin coding sequence, provides interesting information with respect to the three closely related organisms *C. albicans*, *C. dubliniensis* and *C. stellatoidea*. Despite the sequence variation found in the actin-associated introns within the *Candida* genus generally, the *C. albicans* and *C. stellatoidea* sequences are practically identical as would be expected for a single species as these two are now generally considered. In contrast, despite the close relationship between *C. albicans* and *C. dubliniensis*, their intron sequences have considerably diverged.

The calculation of divergence times of the yeast species in Table 3.6 is based upon the formulae of Feng *et al.* (1997). This formula has been used to calculate divergence times of major groups of organisms based upon protein sequence. The formula has been adapted here to calculate the divergence times between species using both protein and nucleotide sequences as the basis of comparison. Using both sequences *C. dubliniensis* is shown to have diverged from *C. albicans* over a time period of approximately 0.03 to 0.241 Mya. Comparing this figure to that obtained for those obtained for *C. glabrata* (0.63 to 1.517 Mya), *S. cerevisiae* (0.66 to 1.437 Mya) and *K. lactis* (0.79 to 1.527 Mya), *C. dubliniensis* is the most recently diverged species from *C. albicans*. This is to be expected for two species as closely related as *C. dubliniensis* and *C. albicans*.

Chapter 4
PCR Identification of *Candida dubliniensis*

4.1 Introduction

Candida dubliniensis was originally identified in samples taken from the oral cavities of HIV-infected individuals who had recurrent oral candidosis (Sullivan *et al.*, 1995). Since then it has been recovered from laboratories around the world and has been associated with both carriage and disease in the presence and absence of HIV infection (Coleman *et al.*, 1997b; Sullivan *et al.*, 1997; Sullivan & Coleman, 1998; Salkin *et al.*, 1998; Kirkpatrick *et al.*, 1998; Meiller *et al.*, 1999; Sano *et al.*, 2000; Brown *et al.*, 2000; Kamei *et al.*, 2000; Brandt *et al.*, 2000; Polacheck *et al.*, 2000; Willis *et al.*, 2000). Although it has been primarily associated with the oral cavity it has been isolated from vaginal and faecal samples (Sullivan *et al.*, 1995; Odds *et al.*, 1998), from an abdominal wound infection (Kamei *et al.*, 2000), from urine samples (Polacheck *et al.*, 2000), and it has been recovered in cases of systemic disease in both HIV- and non-HIV-infected individuals (Pinjon *et al.*, 1998; Meis *et al.*, 1999; Brandt *et al.*, 2000).

A thorough investigation of the epidemiology of *C. dubliniensis* has been hampered by the lack of a simple and reliable method capable of unequivocally differentiating between *C. dubliniensis* and *C. albicans* in the clinical laboratory. Both species share the ability to produce germ tubes and chlamydospores, features previously used for the definitive identification of *C. albicans*. Indeed, retrospective analyses on stored culture collections show that *C. dubliniensis* has been misidentified as both *C. albicans* and *C. stellatoidea* (Sullivan *et al.*, 1995; Coleman *et al.*, 1997b; Odds *et al.*, 1998; Jabra-Rizk *et al.*, 2000). An investigation of our own collection of stored oral isolates show a misidentification rate of 1.8 % of isolates recovered from asymptomatic normal healthy individuals, and 16.5 % of isolates recovered from HIV-infected individuals (Coleman *et al.*, 1997b). Other researchers have reported similar findings. Odds *et al.* (1998) reported that approximately 2 % of a stored archival culture collection of 2500 yeast isolates, originally identified as *C. albicans*, were in fact *C. dubliniensis* and the prevalence of *C. dubliniensis* was significantly higher among HIV-infected individuals than among HIV-negative individuals. Jabra-Rizk *et al.* (2000) found that

1.2 % of 1,251 isolates initially identified as *C. albicans* were *C. dubliniensis* and that the majority of these isolates came from immunocompromised individuals. The earliest known isolates of *C. dubliniensis* predate the HIV pandemic. One of these strains, NCPF 3108, was recovered in the UK in 1957 and was originally deposited in the British National Collection for Pathogenic Fungi as *C. stellatoidea* (Sullivan *et al.*, 1995). Another strain, CBS 2747, was isolated in the Netherlands in 1952 and was deposited in the Centraal Bureau fur Schimmelcultures as *C. albicans* (Meis *et al.*, 1999).

There are a number of tests based upon phenotypic characteristics that discriminate between *C. dubliniensis* and *C. albicans*, however most of these are not completely reliable. The phenotypic tests that are currently in use for the identification of *C. dubliniensis* include colonial colouration on the differential media CHROMagar Candida and methyl blue-Sabauroud agar (Sullivan *et al.*, 1995 and 1997; Coleman *et al.*, 1997; Schoofs *et al.*, 1997), lack of growth of *C. dubliniensis* at 45 °C (Pinjon *et al.*, 1998), and carbohydrate assimilation profiles using the commercially available yeast identification systems including the API 32C and 20C AUX systems (Sullivan *et al.*, 1995 and 1997). However, these methods have in one way or another been shown to be unreliable. Differential media have been shown to be useful only for the presumptive identification of *C. dubliniensis* from clinical specimens, as isolates of *C. dubliniensis/C. albicans* tend to lose their characteristic colour/fluorescence upon subculture and prolonged storage (Schoofs *et al.*, 1997; Sullivan & Coleman, 1998). Significant numbers of *C. albicans* isolates have been found to be unable to grow at 45 °C and hence this test may not be used on its own (Kirkpatrick *et al.*, 1998). The identification of *C. dubliniensis* has been improved recently by the addition of limited *C. dubliniensis* profiles to the APILAB database; however, further modifications are still required in order to correctly identify *C. dubliniensis* (Pincus *et al.*, 1999). This method still takes 48 h to perform and results may be difficult to interpret (Kirkpatrick *et al.*, 1998). Another phenotypic assay, which has been described in the literature, is the inability of *C. dubliniensis* to express β-glucosidase activity (Boerlin *et al.*, 1995; Schoofs *et al.*, 1997; Sullivan *et al.*, 1997). However, a significant proportion of *C. albicans* isolates have been shown to lack β-

glucosidase activity (Odds *et al.*, 1998; Tintelnot *et al.*, 2000). The discrepancies in the results of these tests observed by various researchers means that identification of *C. dubliniensis* must be carried out by a number of phenotypic methods in conjunction with each other. Other methods such as indirect immunofluorescence based on differential localisation of antigens on *C. dubliniensis* blastospores and *C. albicans* germ tubes (Bikandi *et al.*, 1998), the co-aggregation of *C. dubliniensis* with *Fusobacterium nucleatum* (Jabra-Rizk *et al.*, 1999), pyrolysis-mass spectrometry, Fourier transform infrared (FT-IR) spectroscopy (Timmins *et al.*, 1998; Tintelnot *et al.*, 2000) and fatty acid methyl ester analysis (Peltroche-Llacsahuanga *et al.*, 2000a) have been employed successfully by some researchers.

Since the most significant differences between the two organisms are at the genetic level the most discriminatory methods are molecular methods. These methods encompass a variety of techniques including pulsed-field gel electrophoresis, DNA fingerprinting with repetitive-sequence-containing probes, RAPD and RFLP analysis (Boerlin *et al.*, 1995; McCullough *et al.*, 1995; Sullivan *et al.*, 1995 and 1997; Coleman *et al.*, 1997a; Schoofs *et al.*, 1997; Kirkpatrick *et al.*, 1998; Odds *et al.*, 1998; Joly *et al.*, 1999), species-specific molecular beacons (Park *et al.*, 2000) and a PCR-based line probe assay (Martin *et al.*, 2000). Although these methods are reliable and accurate they are time consuming and costly to perform and therefore not suited to a high sample volume throughput diagnostic laboratory.

The aim of this section of the present work was to develop a specific and rapid test for the identification of *C. dubliniensis* based upon genetic differences. The polymerase chain reaction (PCR) was chosen as it is specific, easy to perform, and amenable to automation. It is also a technique that is increasingly available to the diagnostic laboratory. The sequencing of the *ACT1* gene of *C. dubliniensis* led to the identification of significantly divergent sequences within the non-coding portion of this gene and that of *C. albicans*. It was decided to exploit these sequence differences in the development of *C. dubliniensis*-specific primers for use in a rapid PCR test system.

4.2 Materials and Methods

4.2.1 Preparation of yeast DNA

Yeast genomic DNA was prepared as previously described in Section 2.2.1.

4.2.2 Rapid preparation of template genomic DNA

The rapid preparation of yeast template DNA for use in PCR identification experiments was as follows: a single colony from a culture grown for 48 h at 37 °C on PDA or CHROMagar Candida media was suspended in 50 µl sterile distilled water. Cell suspensions were boiled for 10 min and the lysed cells subjected to a clearing spin for 5 min at 20, 000 x *g* in an Eppendorf microfuge. Template DNA contained in 25 µl supernatant was used for PCR amplification.

4.2.3 PCR identification of *C. dubliniensis*

PCR identification of *C. dubliniensis* using the *C. dubliniensis*-specific primer pair DUBF/DUBR (Table 4.1) was carried out in a 50 µl final volume containing 10 pmol each of the forward and reverse primers, 2.5 mM $MgCl_2$, 10 mM Tris/HCl (pH 9.0 at 25 °C), 10 mM KCl, 0.1 % (v/v) Triton X-100, 2.5 U *Taq* DNA polymerase (Promega) and 25 µl template DNA containing cell supernatant (prepared as described above). Each reaction mixture also contained 10 pmol each of the universal fungal primers RNAF/RNAR (Fell, 1993; Table 4.1), which amplify approximately 610 bp from all fungal large-subunit rRNA genes and were used as an internal positive control. Cycling conditions consisted of 6 min at 95 °C followed by 30 cycles of 30 s at 94 °C, 30 s at 58 °C, 30 s at 72 °C, followed by 72 °C for 10 min. Amplification products were separated by electrophoresis through 2.0 % (w/v) agarose gels containing 0.5 µg ethidium bromide ml^{-1} and were visualized on a UV transilluminator.

Table 4.1 Primers used in the PCR identification of *C. dubliniensis* [1]

Primer	Sequence	Reference
DUBF	5'-GTATTTGTCGTTCCCCTTTC-3'	Donnelly *et al.*, 1999; this study
DUBR	5'-GTGTTGTGTGCACTAACGTC-3'	Donnelly *et al.*, 1999; this study
RNAF	5'-GCATATCAATAAGCGGAGGAAAAG-3'	Fell *et al.*, 1993
RNAR	5'-GGTCCGTGTTTCAAGACG-3'	Fell *et al.*, 1993

[1] The DUBF/DUBR primer pair was designed to amplify a 288 bp fragment from *C. dubliniensis* DNA only. The RNAF/RNAR primer pair was designed to amplify an approximately 610 bp fragment from the large subunit rRNA gene of all fungi.

4.2.4 Evaluation of *C. dubliniensis*-specific primers

The *C. dubliniensis*-specific primer pair DUBF/DUBR was tested in a blind trial using template DNA from the yeast isolates listed in Table 4.2 as follows: *C. albicans* (n=53), *C. dubliniensis* (n=122), *C. glabrata* (n=1), *C. parapsilosis* (n=4), *C. sake* (n=1), *C. stellatoidea* (n=10), *C. tropicalis* (n=1) and *Trichosporon beigelii* (n=1). All 196 yeast isolates had been identified using the methods described in Chapter 2. The yeast isolates were grown for 48 h on PDA agar and then number coded. The template DNA was prepared from the coded isolates as described above. Following the PCR with the specific and universal primer sets the isolates were decoded.

Table 4.2 Yeast species used in PCR identification experiments with the *C. dubliniensis*-specific primers DUBF/DUBR

Species	No. of isolates	Reference(s)
C. albicans	53	Pinjon *et al.* (1998); this study and Donnelly *et al.* (1999); Jabra-Rizk *et al.* (1999)
C. dublinienisis [1]	122	Sullivan *et al.* (1995 and 1997); Coleman *et al.* (1997); Moran *et al.* (1997 and 1998); Pinjon *et al.* 1998; Jabra-Rizk *et al.* (1999); this study and Donnelly *et al.* (1999)
C. glabrata	1	Haynes & Westerneng (1996)
C. kefyr	1	NCPF [2] 3234
C. krusei	1	Haynes & Westerneng (1996)
C. norvegensis	1	NCPF 3860
C. parapsilosis	4	This study and Donnelly *et al.* (1999)
C. sake	1	NCPF 8360
C. stellatoidea	1	ATCC [2] 11006
	9	This study and Donnelly *et al.* (1999)
C. tropicalis	1	NCPF 3111
T. beigelii	1	NCPF 3857

[1] One hundred and fourteen of the *C. dubliniensis* isolates were recovered from oral specimens, five were recovered from faecal specimens and one each was recovered from a vaginal, sputum and a post-mortem lung specimen. The isolates were recovered from different countries as follows: Argentina, 1 isolate; Australia, 2; Belgium, 5; Canada, 6; France, 4; Germany, 4; Greece, 1; Ireland, 48; Scandinavia, 4; Spain, 5; Switzerland, 4; UK, 17; USA 12.

[2] Abbreviations: ATCC, American Type Culture Collection, (Manassas, VA, USA); NCPF, National Collection of Pathogenic Fungi, (Bristol, UK)

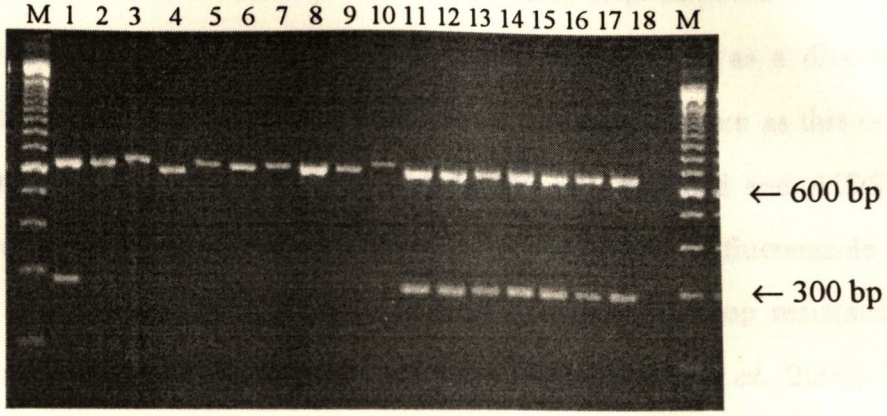
4.3 Results

An examination of a DNA nucleotide sequence alignment of the *ACT1*-associated introns of *C. dubliniensis* and *C. albicans* and the observation that they differ by 16.6% (Fig. 3.3 and Table 3.4) allowed the design of PCR primers specific for the *C. dubliniensis ACT1* intron (DUBF/R; Table 4.1 and Fig. 4.1). These primers were designed to amplify a DNA fragment of 288 bp from *C. dubliniensis* genomic DNA only. The primers were initially tested on high molecular weight genomic DNA from *Candida* strains to confirm that they amplified the correctly sized fragment from *C. dubliniensis* isolates only. Since the preparation of high molecular weight genomic DNA from *Candida* species is a time consuming process, it was decided to develop a more rapid method of template DNA preparation. This involved boiling a single 48 h-old colony in 50 µl water for 10 min and using the DNA containing supernatant as a template. The fungal universal primers RNAF/R (Fell, 1993; Table 4.1) were also incorporated into the PCR to serve as an internal positive control. While all fungal species should produce a product of approximately 610 bp with the RNAF/R primers, only *C. dubliniensis* isolates should yield the 288 bp amplimer with the DUBF/R primer set. The *C. dubliniensis*-specific primer pair DUBF/R were tested in a blind trial using template DNA prepared using the rapid method from the yeast isolates listed in Table 4.2. All 196 yeast isolates yielded an amplimer of approximately 610 bp, but only the *C. dubliniensis* isolates yielded the 288 bp amplimer. Fig. 4.2 shows examples of the PCR amplimers obtained with representative strains belonging to a variety of different yeast species, including *C. dubliniensis* isolates from disparate geographical locations. Use of this PCR test in conjunction with the rapid template DNA preparation procedure used here means that a *C. dubliniensis* isolate can be identified unequivocally in less than 4 h.

Figure 4.1 Alignment of the *C. albicans* and *C. dubliniensis ACT1*-associated introns showing the position of the DUBF/DUBR primer sequences [1]

[1] The position of the *C. dubliniensis*-specific DUBF/DUBR primers are underlined in the *C. dubliniensis ACT1* intron sequence.

Figure 4.2 Agarose gel showing the PCR products obtained with universal and *C. dubliniensis*-specific primers [1]



[1] The profiles shown correspond to: the *C. dubliniensis* type strain CD36 (lane 1); *C. albicans* (lane 2); *C. glabrata* (lane 3); *C. kefyr* (lane 4); *C. krusei* (lane 5); *C. norvegensis* (lane 6); *C. sake* (lane 7); *C. stellatoidea* (lane 8); *C. tropicalis* (lane 9); *Trichosporon beigelii* (lane 10); *C. dubliniensis* American isolate (lane 11); *C. dubliniensis* Argentinean isolate (lane 12); *C. dubliniensis* Australian isolate (lane 13); *C. dubliniensis* Canadian isolate (lane 14); *C. dubliniensis* French isolate (lane 15); *C. dubliniensis* German isolate (lane 16); *C. dubliniensis* UK isolate (lane 17); A negative control in which no template DNA was used in the PCR reaction was also included (lane 18). The 288 bp *C. dubliniensis*-specific amplimer generated by the DUBF/DUBR primers is present in lane 1 and lanes 11-17. Lanes marked M contain 100 bp size reference markers.

4.4 Discussion

As *C. dubliniensis* is a recently described species it is important to establish its epidemiology and its incidence of both asymptomatic carriage and as a disease-causing organism. Indeed, epidemiological studies so far show cause for concern as this organism is prevalent in immunocompromised groups, particularly HIV-infected and AIDS patients. *Candida dubliniensis* has been shown to rapidly develop resistance to fluconazole following exposure to the drug *in vitro*, and it has also been shown to develop resistance *in vivo* following fluconazole therapy (Moran *et al.*, 1997 and 1998; Ruhnke *et al.*, 2000). Therefore, it is essential to be able to definitively identify this organism in clinical samples by a simple, rapid and reliable method. As previously discussed, the most reliable methods for identification of *C. dubliniensis* are molecular methods based upon genetic differences. In general, these methods are not suitable for large-scale epidemiological investigation. In contrast, the PCR technique may be readily used to detect genetic differences and as it is a rapid procedure, easy to perform and relatively inexpensive. For these reasons it was chosen as a method to provide a discriminatory test between *C. dubliniensis* and *C. albicans*.

The *ACT1*-associated intron of *C. dubliniensis* was chosen as the basis for the design of species-specific primers as it exhibits a large sequence divergence from its *C. albicans* homologue (16.6 %, Table 3.4), and because it shows a high level of intraspecies sequence conservation (Fig. 3.5). A rapid method of template DNA preparation was developed in order to facilitate the processing of a large number of samples. With template DNA preparation taking approximately 15 min, PCR amplification 2.5 h and electrophoresis of amplimers 1 h, presumptive *C. dubliniensis* colonies may be identified in as little as 4 h. The method of rapid template preparation allows *C. dubliniensis* colonies to be identified directly from primary isolation plates without the need for additional subculture. The blind trial carried out with a total of 196 yeast isolates, including 122 *C. dubliniensis* and the closely related *C. albicans* (53 isolates) and *C. stellatoidea* (10 isolates), showed that the primers DUBF/R correctly identified the *C. dubliniensis* isolates with 100 % accuracy. The 122 isolates of *C. dubliniensis*

tested were a thoroughly characterised group of geographically and epidemiologically diverse strains.

Other researchers have developed PCR based identification techniques to distinguish between *C. dubliniensis* and *C. albicans*. Mannarelli & Kurtzman (1998) designed species-specific primers based upon the D1/D2 region of the large subunit rRNA gene. These primers were capable of distinguishing between *C. dubliniensis* and *C. albicans*, however, they were only tested against seven *C. dubliniensis* isolates. Elie *et al.* (1998) have also reported *C. dubliniensis*-specific primers that target the internal transcribed spacer region (ITS2) of the ribosomal gene cluster. Again their system has been tested against only five isolates of *C. dubliniensis*. The method itself involves a PCR-ELISA, which is a time consuming procedure. Similarly, the PCR-based line probe assay of Martin *et al.* (2000) is laborious and would not be routinely available in a diagnostic laboratory. More recently other groups have reported primers capable of distinguishing between the two species (Kurzai *et al.*, 1999; Tamura *et al.*, 2000). Kurzai *et al.* (1999) designed primers based upon the sequence of *PHR1*. These primers were specific for *C. albicans* but failed to amplify from *C. dubliniensis*. Although these primers were tested upon a larger number of isolates (n=19), the primers themselves do not definitively identify an isolate as *C. dubliniensis*; rather they identify the isolate as not being *C. albicans*. In this case a further test would be required for definitive identification of *C. dubliniensis*. Tamura and colleagues (2000) based the design of their *C. dubliniensis*-specific primers on the sequence of a RAPD band obtained with one oligonucleotide primer considered to be specific for *C. dubliniensis*. They tested their primer set on 58 isolates previously identified as *C. albicans* and re-identified one isolate as *C. dubliniensis*. They confirmed this by sequencing of the D1/D2 region of the large subunit ribosomal RNA gene.

Given that the reliability of many phenotypic tests for the identification of *C. dubliniensis* has come into question as larger numbers of isolates are tested, it is essential that any new method for the identification of this organism be rigorously tested. The *ACT1* intron sequence upon which the DUBF/R primer set was designed was investigated for intraspecies variation among epidemiologically and geographically diverse isolates of *C. dubliniensis*. It

was found that there was very little intraspecies variation in the intron sequence of *C. dubliniensis*, *C. albicans* and *C. stellatoidea*. Other researchers have observed this intraspecies sequence conservation in the self-splicing group I intron from the LSU rRNA genes of *C. dubliniensis*, *C. albicans* and *C. stellatoidea* (Boucher *et al.*, 1996), it was concluded that any future isolates of *C. dubliniensis* would probably exhibit this same level of sequence conservation. The sequences of the *ACT1*-associated introns of *C. dubliniensis* and *C. albicans* were sufficiently divergent to facilitate the easy design of primers capable of distinguishing between the two species. The *C. dubliniensis*-specific primers have been tested extensively against a large number of isolates from diverse geographical locations. The method is simple and rapid and capable of detecting suspected *C. dubliniensis* colonies from a primary isolation plate in as little as 4 h.

Chapter 5

Cloning, Nucleotide Sequence and Analysis of the *C. dubliniensis SAP2* gene

5.1 Introduction

5.1.1 Aspartic proteinases

The aspartic proteinases are a class of enzymes found in or secreted by a variety of eukaryotic organisms. They share a number of common features including a pH optimum in the acid range, inhibition by pepstatin, a hexapeptide produced by *Streptomyces*, and their mechanism of action is *via* two catalytically active aspartic acid residues which target extended peptides (Davies, 1990). These enzymes have a very broad range of substrate specificities and the individual enzymes perform many different functions. The best known members of this enzyme family are the enzymes pepsin, gastricin and chymosin (rennin), which are involved in digestion (Foltmann, 1981; Kay *et al.*, 1981). Cathepsin D is a lysosomal enzyme which acts on its target intracellularly (Shewale *et al.*, 1985; Blum *et al.*, 1991). The enzyme renin has a very specific target; it is responsible for the production of the precursor of angiotensin II which is a factor in the control of blood pressure (Davies, 1990). The most recently discovered members of this family are the retroviral aspartic proteinases and these are responsible for cleavage of the viral polyprotein during activation of the virus (Wlodawer *et al.*, 1989; Debouck & Metcalf, 1990). Aspartic proteinases are also produced by the fungi including, for example, the proteinase A from *S. cerevisiae*, which is involved in intracellular proteolysis (Dreyer *et al.*, 1985). Other characterised fungal proteinases include rhizopuspepsin, penicillopepsin and endothiapepsin which have been shown to be secreted into growth media to hydrolyse proteins for nutrient requirements (Sardinas, 1965; Hsu *et al.*, 1976; Subramanian, 1976).

The archetypal aspartic proteinase is pepsin whose structure was first described in 1934. The aspartic proteinases are mostly produced in zymogen form. In the inactive form the mature enzyme is preceded by an N-terminal propeptide of approximately 50 amino acids long which is cleaved upon activation. The propeptide is basic in nature and contains an invariant lysine at residue position 36 (using the residue numbering system employed for the enzyme pepsinogen; Davies, 1990). Activation of the zymogen may be carried out at low pH in an

autocatalytic manner involving cleavage of the propeptide. It has been suggested that the zymogen is inactive due to the blocking of the catalytic aspartic residues by the propeptide (Sielecki, 1986). Most of the aspartic proteinases are single chain enzymes with a molecular weight of ~ 35 kDa. They are approximately 327 amino acids long with ~ 5 % sequence identity between all members of the family (Davies, 1990). An exception to this are the retroviral enzymes, which are considerably shorter (< 130 amino acids long) and are thought to associate in pairs to form the active enzyme (Pearl & Taylor, 1987). The three-dimensional structure of individual members of the superfamily show a considerable degree of structural similarity. The typical three-dimensional structure is bilobal with two domains of similar structure. There is a central binding cleft that accommodates the peptide substrate. Each domain provides one of the two catalytic aspartic acid residues. These residues may be contained in either DTG or DSG motifs. The mixed pairing of both DTG and DSG motifs is seen in yeast and plant aspartic proteinases. Other fungal and mammalian enzymes have the DTG motif on both domains. Members of the chimeric viral enzyme family may have either DTG or DSG, paired symmetrically.

5.1.2 *Candida* secreted aspartic proteinases

Staib first reported extracellular proteolytic activity in *C. albicans* in 1965. Proteolytic activity may be induced *in vitro* in the more pathogenic species of *Candida*, including *C. albicans*, *C. dubliniensis*, *C. tropicalis* and *C. parapsilosis* by growing the yeasts in a medium that contains a complex protein such as BSA as the sole nitrogen source (Staib, 1965; Remold *et al.*, 1968; Rüchel & Borg, 1986; Ray & Payne 1990; Fusek *et al.*, 1993; Lerner & Goldman 1993; McCullough *et al.*, 1995; this study). A number of *Candida* aspartic proteinase genes have been cloned and sequenced including ten genes from *C. albicans* (Hube *et al.*, 1991; Wright *et al.*, 1992; Magee *et al.*, 1993; White *et al.*, 1993; Miyasaki *et al.*, 1994; Monod *et al.*, 1994 and 1998; Hube *et al.*, direct submission to GenBank), two proteinase genes from *C. parapsilosis* (de Viragh *et al.*, 1993) and four proteinase genes from *C. tropicalis* (Togni *et al.*, 1991; Zaugg & Monod, direct submission to GenBank). These genes are listed in Table 5.1.

Other *Candida* species such as *C. glabrata*, *C. kefyr* and *C. guillermondii* have been shown to be weakly or non-proteolytic when grown in the presence of complex protein as a nitrogen source (Macdonald, 1984; Ray & Payne, 1990).

Table 5.1 Currently known *Candida* secreted aspartic proteinase (*SAP*) genes

Gene name	Length of ORF [1]	Length of protein sequence [2]	Accession No.	Reference [3]
C. albicans				
SAP1	1176	391	X56867	Hube *et al.*, 1991
SAP2	1197	398	M83663	Wright *et al.*, 1992
SAP3	1197	398	L22358	White *et al.*, 1993
SAP4	1254	417	L25388	Miyasaki *et al.*, 1994
SAP5	1257	418	Z30191	Monod *et al.*, 1994
SAP6	1257	418	Z30192	Monod *et al.*, unpublished
SAP7	1767	588	Z30193	Monod *et al.*, unpublished
SAP8	1218	405	AF043330	Monod *et al.*, 1998
SAP9	1635	544	AF043331	Monod *et al.*, 1998
SAP10	1326	441	AF146440	Felk *et al.*, unpublished
C. tropicalis				
SAPT1	1185	394	X61438	Togni *et al.*, 1991
SAPT2	1269	422	AF115320	Zaugg & Monod, unpublished
SAPT3	1170	389	AF115321	Zaugg & Monod, unpublished
SAPT4	1185	394	AF115322	Zaugg & Monod, unpublished
C. parapsilosis				
SAPP1 (*ACPL*)	1239	412	Z11918	de Viragh *et al.*, 1993
SAPP2 (*ACPR*)	1209	402	Z11919	de Viragh *et al.*, 1993

[1] The length of the ORF in each gene was calculated from the ATG start codon to the stop codon and is given in bp.

[2] The length of the protein sequence in each case was predicted from the nucleotide sequence of the corresponding gene and refers to the protein before processing and is given in numbers of amino acid residues.

[3] The references listed are as per GenBank entry.

Candida secreted aspartic proteinases (Saps) all share a similar primary structure consisting of a hydrophobic signal sequence, a propeptide with putative Lys-Arg recognition sites for a Kex2-like proteinase, which is cleaved to produce the mature protein, and a conserved mature protein (Togni *et al.*, 1991; de Viragh *et al.*, 1993; Hube *et al.*, 1998). Monod and colleagues compared amino acid sequences of the then currently known *Candida* Saps including *C. albicans* Saps 1-9, *C. parapsilosis* AcpL and AcpR, *C. tropicalis* Acp and *S. cerevisiae* Yap3 (Monod *et al.*, 1998). They showed that *C. albicans* Saps 1-3 are closely related to each other and form a distinct group as do *C. albicans* Saps 4-6. *Candida albicans* Saps 8 and 9 are distinct from either group with Sap8 clustering with the Acp protein from *C. tropicalis* and Sap9 grouping with the GPI-anchored protease Yap3 from *S. cerevisiae*. The two proteins from *C. parapsilosis*, AcpL and AcpR are grouped together separately from the other *Candida* Saps (Monod *et al.*, 1998).

The crystalline structures of a number of fungal aspartic proteinases have been determined. These include the proteinases of *Rhizopus chinensis* (Subramanian *et al.*, 1977), *Endothia parasitica* (Tang *et al.*, 1978), *Penicillium janthinellum* (Hsu *et al.*, 1977), *C. tropicalis* (Symersky *et al.*, 1997) and *C. albicans* (Cutfield *et al.*, 1995; Abad-Zapapero *et al.*, 1996). The structure of the *Candida* secreted aspartic proteinases show a number of unique features when compared with other fungal proteinases which puts them into a subclass of their own (Abad-Zapapero *et al.*, 1998). The differences are as follows: (1) there is an 8 residue insertion near the first disulphide bridge (Cys 45-50) that results in a broad flap extending towards the active site. The glycine residue at position 54 is important for stabilising the flap. However, this residue is not conserved amongst the *C. albicans* SAPs 4-6 which may result in different conformations of this loop for different members of the *Candida* aspartic proteinase gene family; (2) a seven residue deletion (Ser110-Tyr114) which removes a helical structure present in other proteinases. This results in physical enlargement of the enzyme pocket S3 and probably affects substrate binding; (3) an extended polar region that joins the amino and carboxyl domains together. This changes the orientation of the domains to each other; (4) a twelve residue addition onto the carboxyl terminal end. It has been speculated that these

variations alter the specificity of this subclass of fungal aspartic proteinases (Abad-Zapapero *et al.*, 1998).

The overall aim of the present study was to characterise genetic differences between *C. dubliniensis* and *C. albicans* using a housekeeping gene (*ACT1*) and a gene encoding a putative virulence factor. There is much indirect evidence to suggest that the Sap enzyme family are virulence factors in *C. albicans*. Homologues of the *C. albicans SAP 1-7* genes have been detected in *C. dubliniensis* by Southern analysis (Gilfillan *et al.*, 1998), and *C. dubliniensis* Sap activity has been reported to be significantly greater than that of reference *C. albicans* isolates (McCullough *et al.*, 1995). Most biochemical studies relate to *C. albicans* Sap2, which is the major Sap produced *in vitro* when BSA is the sole nitrogen source. For this reason the *CaSAP2* homologue of *C. dubliniensis* was selected for cloning and sequencing and expression studies. The purpose of the studies described in this chapter was to characterise differences at the nucleotide and amino acid sequence level between *CaSAP2* and its *C. dubliniensis* homologue, *CdSAP2*.

5.2 Materials and Methods

5.2.1 Yeast reference strains

Three isolates of *C. albicans* and eight isolates of *C. dubliniensis* were included in this study (Table 5.2). The reference *C. dubliniensis* strain used in this study was the type strain CD36 (Sullivan *et al.*, 1995) which was used to construct the EMBL3 genomic library described in section 2.3.2. *Candida albicans* 132A was recovered from the oral cavity of a HIV-infected individual attending the Dublin Dental Hospital in 1992 (Gallagher *et al.*, 1992). *Candida albicans* CA411 was co-isolated with *C. dubliniensis* CD411 from the oral cavity of a HIV-infected intravenous drug user commencing triple therapy treatment in September 1999. This individual presented with symptoms of pseudomembraneous candidosis. The *Candida* culture isolated from this patient consisted predominantly of *C. albicans* (360 c.f.u for CA411 and 8 c.f.u. for CD411). *Candida dubliniensis* strains CM1 and CM2 are Australian isolates recovered from the oral cavity of a homosexual individual with AIDS (McCullough *et al.*, 1995; Sullivan *et al.*, 1995). Strain CM1 was recovered initially when the patient presented with symptoms of oral candidosis. Strain CM2 is a fluconazole-resistant isolate (MIC = 32 μg/ml) recovered at a later date at which time the patient was asymptomatic (Moran *et al.*, 1997). At both instances of isolation the patient had received previous fluconazole therapy. *Candida dubliniensis* isolates CBS 2747 and CBS 8500 are blood culture isolates recovered from non-HIV-infected individuals (Meis *et al.*, 1999). *Candida dubliniensis* CD57 is a fluconazole-susceptible isolate from a HIV-negative individual and $CD57^R$ is a fluconazole-resistant derivative of CD57 generated *in vitro* (Moran *et al.*, 1998).

Table 5.2 *Candida* isolates used in analysis of the *C. dubliniensis SAP2* gene

Yeast strain	Source and/or comments	Reference
C. albicans		
SC5314	Reference strain	Fonzi & Irwin, 1992
132A	Oral reference strain	Gallagher *et al.*, 1992
CA411	IVDU [2] with AIDS [2]	This study
C. dubliniensis [1]		
CD36	Type strain	Sullivan *et al.*, 1995
CBS 2747	Blood culture; HIV^- [2]	Meis *et al.*, 1999
CBS 8500	Blood culture; HIV^-	Meis *et al.*, 1999
CD 411	IVDU with AIDS	This study
CM1	Homosexual with AIDS	Sullivan *et al.*, 1995
CM 2	Homosexual with AIDS	Sullivan *et al.*, 1995
CD57	Vaginitis patient; HIV^-	Moran *et al.*, 1998
$CD57^R$	Fluconazole-resistant derivative of CD57	Moran *et al.*, 1998

[1] The *C. dubliniensis* isolates were recovered from the oral cavity, except for the blood culture isolates CBS 2747 and CBS 8500 and isolate CD57 which was recovered from a high vaginal swab.
[2] Abbreviations: IVDU, intravenous drug user; HIV^+, HIV-positive, HIV^-, HIV-negative.

5.2.2 Cloning of the *C. dubliniensis SAP2* gene

The *C. dubliniensis* genomic library was screened using a radioactively labeled probe consisting of the entire *C. albicans SAP2* gene (*CaSAP2*) on a 3010 bp *Xba*I/*Eco*RI fragment cloned into pBluescript (pAS2, a gift from B. Hube, University of Hamburg, Germany) using the procedure described in sections 2.2.5 and 2.2.6. Recombinant phage purified from a pAS2-hybridising plaque was termed λCDSAP2. Genomic DNA from λCDSAP2 was purified as described by Sambrook *et al.* (1989), and the cloned insert DNA mapped with restriction endonucleases. Subsequently, specific fragments were subcloned into pBluescript by conventional methods.

5.2.3 Sequencing of the *C. dubliniensis SAP2* gene

DNA sequencing was carried out using an automated technique by the dideoxy chain terminating method of Sanger *et al.* (1977) using an Applied Biosystems 370A DNA sequencer as described in section 2.6.1. DNA fragments cloned in the plasmid vector pBluescript were sequenced using the M13 forward and reverse primers. Additional primers were designed for sequencing internal regions of subcloned fragments (Table 5.3). Sequence analysis was performed as described in section 2.6.2.

Table 5.3 Additional internal primers used in sequencing the *C. dubliniensis SAP2* gene [1]

Primer	Sequence	Nucleotide co-ordinates
pCDS1		
S1a	5'-TACAGTCACAATGGAGTCTTC-3'	-526 to -506
S1b	5'-GACTGGAATTGAATAAGAACT-3'	-257 to -237
S1c	5-CGGATAAGTTGAATTGAACG-3'	-229 to -210
S1d	5'-ATTAGTCGATGCTACTCCAAC-3'	42 to 62
pCDS2		
S2a	5'-ATGTTGATTGCCAAGTCACC-3'	296 to 315
S2b	5'-TAATGTTGATTGCCAAGTCACC-3'	294 to 315
S2c	5'-CCAAGTAGTTCATCAGCTTC-3'	555 to 573
S2d	5'-TGTGTCTGGAGATGTGGTAT-3'	936 to 955
pCDS3		
S3a	5'-GCTTCTGAATTTGCTGCTCC-3'	991 to 1010
S3b	5'-TCTGAATCCAGCATTTCGGC-3'	1165 to 1184
pCDS4		
S4a	5'-CCAATGAAGCTGGTGGTGAT-3'	554 to 573
S4b	5'-CAATGCTGCTGCGGGACAA-3'	650 to 669

[1] Primers are complementary to the *C. dubliniensis SAP2* gene sequence (this study). Nucleotide co-ordinates shown are numbered in the 5' to 3' direction with the first base of the translation start codon being +1

5.2.4 Restriction analysis of the *C. dubliniensis SAP2* locus

High molecular weight genomic DNA was prepared from the strains listed in Table 5.1 as described in Section 2.2.1. For each strain, an aliquot containing approximately 4 µg of genomic DNA was digested separately with *Eco*RI and *Hin*fI in a final 30 µl volume in each case. Digested DNAs were separated in a 0.8 % (w/v) agarose gels (for *Eco*RI digests) or in a 2.0 % (w/v) agarose gels (for *Hin*fI digests) as described in section 2.2.3. Following electrophoresis the DNA in the gels was transferred to nitrocellulose membrane filters according to the method of Southern (1975) as described in section 2.2.4. These Southern blots were then probed with the radioactively labeled pAS2 (consisting of the entire *CaSAP2* gene cloned into pBluescript) as described in sections 2.2.5/6.

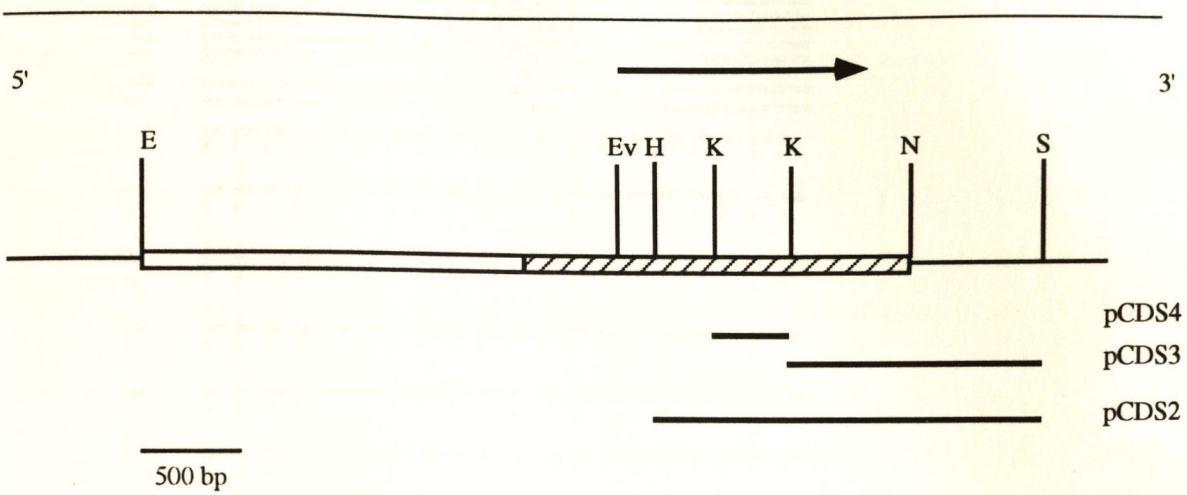
5.3 Results

5.3.1 Identification of a *C. dubliniensis SAP2* homologue

In order to identify the *C. dubliniensis SAP2* gene (*CdSAP2*), a library of *C. dubliniensis* CD36 genomic DNA, cloned in the lambda replacement vector EMBL3, was screened by plaque hybridisation with a radioactively labeled probe consisting of the entire *C. albicans SAP2* (*CaSAP2*) gene on a 3010 bp *Xba*I/*Eco*RI fragment cloned into pBluescript (pAS2, a gift from B. Hube, University of Hamburg). Six strongly hybridising plaques were identified and one of these was selected for further analysis. The recombinant phage purified from this plaque was termed λCDSAP2 and it was found to harbour a cloned DNA insert of approximately 20 kb. Southern hybridisation analysis of restriction endonuclease-generated fragments of λCDSAP2 cloned insert DNA with the pAS2 probe identified a strongly hybridising *Eco*RI/*Not*I insert DNA fragment of approximately 4 kb. This fragment was subcloned into pBluescript and the resulting recombinant plasmid termed pCDS1. Further restriction endonuclease mapping studies and Southern hybridisation analysis with the pAS2 probe identified 1.3 kb *Hin*dIII/*Sac*I, 600 bp *Kpn*I/*Sac*I and 400 bp *Kpn*I overlapping fragments contained within the cloned DNA insert of pCDS1. These fragments were purified and cloned into pBluescript to yield the recombinant plasmids pCDS2, pCDS3 and pCDS4, respectively (Fig. 5.1). The DNA inserts contained within these recombinant plasmids were sequenced fully in both directions using the M13 forward and reverse primers and the additional primers listed in Table 5.2. Sequence analysis of the DNA inserts contained within pCDS2, pCDS3 and pCDS4 revealed that they comprised most of the coding region and 3' flanking sequence of the *C. dubliniensis CaSAP2* homologue. The 5' coding region of the *C. dubliniensis CaSAP2* homologue and the 5' flanking sequence were sequenced by primer walking from the DNA insert in recombinant plasmid pCDS1 with a primer (S2a, Table 5.3) homologous to the sequence of the pCDS2 insert. In total, a 1996 bp region of the 4 kb DNA insert contained within pCDS1 was sequenced on both strands. Computer assisted analysis of this 1996 bp sequence revealed one significant uninterrupted ORF of 1194 bp with a single

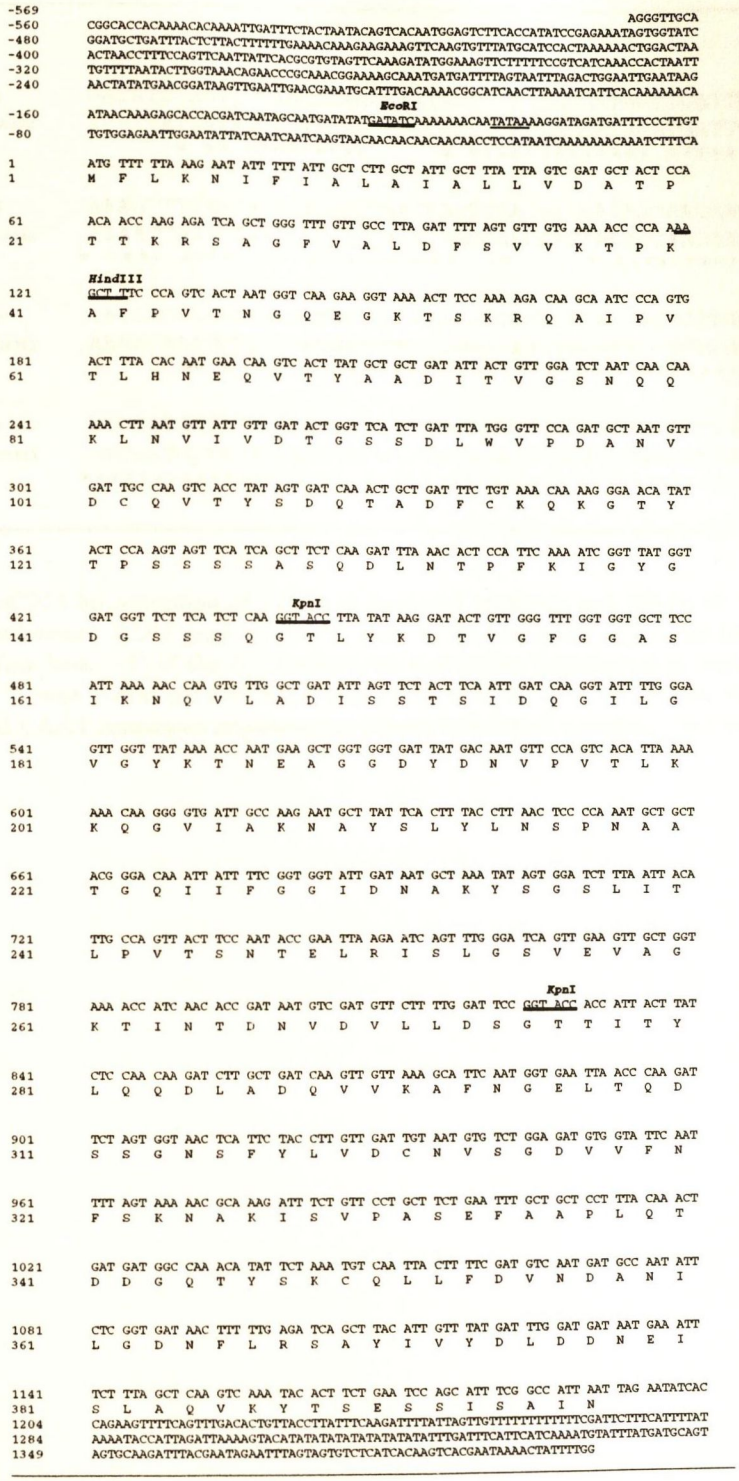
potential ATG translation start codon (Fig. 5.2). The sequence of this putative ORF was compared with sequences in the GenBank database using the BLAST family of computer programs (Altschul *et al.*, 1990) and it was found to show greatest sequence identity (89.6 %) with the *C. albicans SAP2* gene. It was deduced that this ORF comprised the *C. dubliniensis SAP2* gene. A total of 569 bp of putative promoter region upstream of the *CdSAP2* ATG start codon was sequenced. This was compared to the *C. albicans* sequence upstream of the *CaSAP2* gene (Fig. 5.3). Only the first 125 bp of the *C. dubliniensis SAP2* sequence immediately 5' upstream of the ATG start site was compared to the 125 bp of putative promoter region upstream of the CaSAP2 gene published in the GenBank database (accession no. M83663). Both putative promoter regions contain potential TATA consensus sequences. The *C. dubliniensis* TATA sequence is located at position –107, whilst that of the *C. albicans* TATA sequence is situated at position –98. Both promoter regions also show potential CAAT boxes located at position –59 for *C. dubliniensis* and –49 for *C. albicans*. The *C. dubliniensis* putative promoter region shows a 62.4 % identity with that of *C. albicans*.

Figure 5.1 Schematic diagram of the recombinant plasmid pCDS1 containing the *C. dubliniensis SAP2* gene [1]



[1] The cloned DNA insert of recombinant plasmid pCDS1 is represented by the large horizontal box and the thin black lines on either side of this represent vector sequences. The hatched area corresponds to the 1996 bp sequenced from pCDS1 containing the *C. dubliniensis CaSAP2* homologue. The thick black arrow above the hatched area represents the position and direction of transcription of the *C. dubliniensis SAP2* gene. The thin horizontal lines in the lower part of the figure represent the cloned DNA inserts from the recombinant clones pCDS2, pCDS3, and pCDS4 which contained overlapping fragments of the putative *CdSAP2* gene cloned from pCDS1. The letters above the large horizontal box represent restriction endonuclease cleavage sites. The abbreviations are as follows: E, *Eco*RI; Ev, *Eco*RV; H, *Hin*dIII; K, *Kpn*I; N, *Not*I and S, *Sac*I.

Figure 5.2 Nucleotide sequence and deduced amino acid sequence of the *C.dubliniensis CdSAP2* gene



[1] Nucleotide sequences are numbered in the 5' to 3' direction from the first base (+1) of the ATG translation start codon. Amino acid residues are numbered from the initial methionine. The putative TATAA box at position –107 is underlined. The

Figure 5.3 Alignment of the putative promoter regions and 5' coding sequence of *CaSAP2* and *CdSAP2* [1]

```
C. albicans       GATATCTAATTTCAAAAAAAAGAATAGTATAAAAGGATAGTTGATTCCTCTTGGTTGTTG      -66
C. dubliniensis   -----ATGATATCAAAAAAA----CAATATAAAAGGATAGATGATTTCCCTTG-TTGTGG      -76
                        * ** *********     * ************** ***** * **** **** *

C. albicans       AAAATTTGAATAATATCAATCAATCAATCAA-----ATAACAACAACCC-----ACTAGA     -16
C. dubliniensis   AGAATTGGAATATTATCAATCAATCAAGTAACAACAACAACAACAACCTCCATAATCAAA     -16
                  * **** ***** ************** **     * **********      * * *

C. albicans       CATCACCATTTATCAATGTTTTTAAAGAATATTTTCATTGCTCTTGCTATTGCTTTATTA     +45
C. dubliniensis   AAAACAAATCTTTCAATGTTTTTAAAGAATATTTTCATTGCTCTTGCTATTGCTTTATTA     +45
                  *     ** * ************************************************

C. albicans       GTCGATGCTACTCCAACAACAACCAAAAGATCAGCTGGTTTCGTTGCTTTAGATT         +100
C. dubliniensis   GTCGATGCTACTCCAACAACAACCAAAAGATCAGCTGGTTTCGTTGCTTTAGATT         +100
                  ************************************************
```

[1] A total of 225 bp consisting of 125 bp of promoter sequence and 100 bp of 5' coding region of the *C. albicans* and *C. dubliniensis SAP2* genes is shown aligned. The nucleotide sequences are numbered in the 5' to 3' direction from the first base (+1) of the ATG translation start codon (highlighted in bold typeface). The sequeces upstream of the ATG start codon are numbered negatively with the first base before the ATG start codon being –1. The TATA and CAAT consensus sequences are shown underlined. Asterisks represent identical nucleotides.

5.3.2 Analysis of the predicted CdSap2 protein

The *CdSAP2* ORF has the capacity to encode a protein of 397 amino acids with a predicted molecular weight of 42.193 kDa and a pI of 4.49. The predicted molecular weight of the *C. dubliniensis* protein is slightly lower than that of *C. albicans* (42.304 kDa). CdSap2, at 397 amino acids is one amino acid shorter than the corresponding CaSap2 protein. There are 28 residue differences between the two predicted proteins: one deletion at Thr 21, and 27 substitutions (Figure 5.3).

Figure 5.4 An alignment of the predicted CaSap2 and CdSap2 amino acid sequences[1]

```
CaSap2      MFLKNIFIALAIALLVDATPTTTKRSAGFVALDFSVVKTPKAFPVTNGQEGKTSKRQAVP   60
CdSap2      MFLKNIFIALAIALLVDATP-TTKRSAGFVALDFSVVKTPKAFPVTNGQEGKTSKRQAIP   59
            ********************.************************************:*

CaSap2      VTLHNEQVTYAADITVGSNNQKLNVIVDTGSSDLWVPDVNVDCQVTYSDQTADFCKQKGT   120
CdSap2      VTLHNEQVTYAADITVGSNQQKLNVIVDTGSSDLWVPDANVDCQVTYSDQTADFCKQKGT   119
            ********************:******************.**********************

CaSap2      YDPSGSSASQDLNTPFKIGYGDGSSSQGTLYKDTVGFGGVSIKNQVLADVDSTSIDQGIL   180
CdSap2      YTPSSSSASQDLNTPFKIGYGDGSSSQGTLYKDTVGFGGASIKNQVLADISSTSIDQGIL   179
            * **.**********************************.*********:.*********

CaSap2      GVGYKTNEAGGSYDNVPVTLKKQGVIAKNAYSLYLNSPDAATGQIIFGGVDNAKYSGSLI   240
CdSap2      GVGYKTNEAGGDYDNVPVTLKKQGVIAKNAYSLYLNSPNAATGQIIFGGIDNAKYSGSLI   239
            ***********.**************************:**********:**********

CaSap2      ALPVTSDRELRISLGSVEVSGKTINTDNVDVLLDSGTTITYLQQDLADQIIKAFNGKLTQ   300
CdSap2      TLPVTSNTELRISLGSVEVAGKTINTDNVDVLLDSGTTITYLQQDLADQVVKAFNGELTQ   299
            :*****: ***********:*******************************::*****:***

CaSap2      DSNGNSFYEVDCNLSGDVVFNFSKNAKISVPASEFAASLQGDDGQPYDKCQLLFDVNDAN   360
CdSap2      DSSGNSFYLVDCNVSGDVVFNFSKNAKISVPASEFAAPLQTDDGQTYSKCQLLFDVNDAN   359
            **.***** ****:************************.** ****.*.************

CaSap2      ILGDNFLRSAYIVYDLDDNEISLAQVKYTSASSISALT                         398
CdSap2      ILGDNFLRSAYIVYDLDDNEISLAQVKYTSESSISAIN                         397
            ****************************** *****:.
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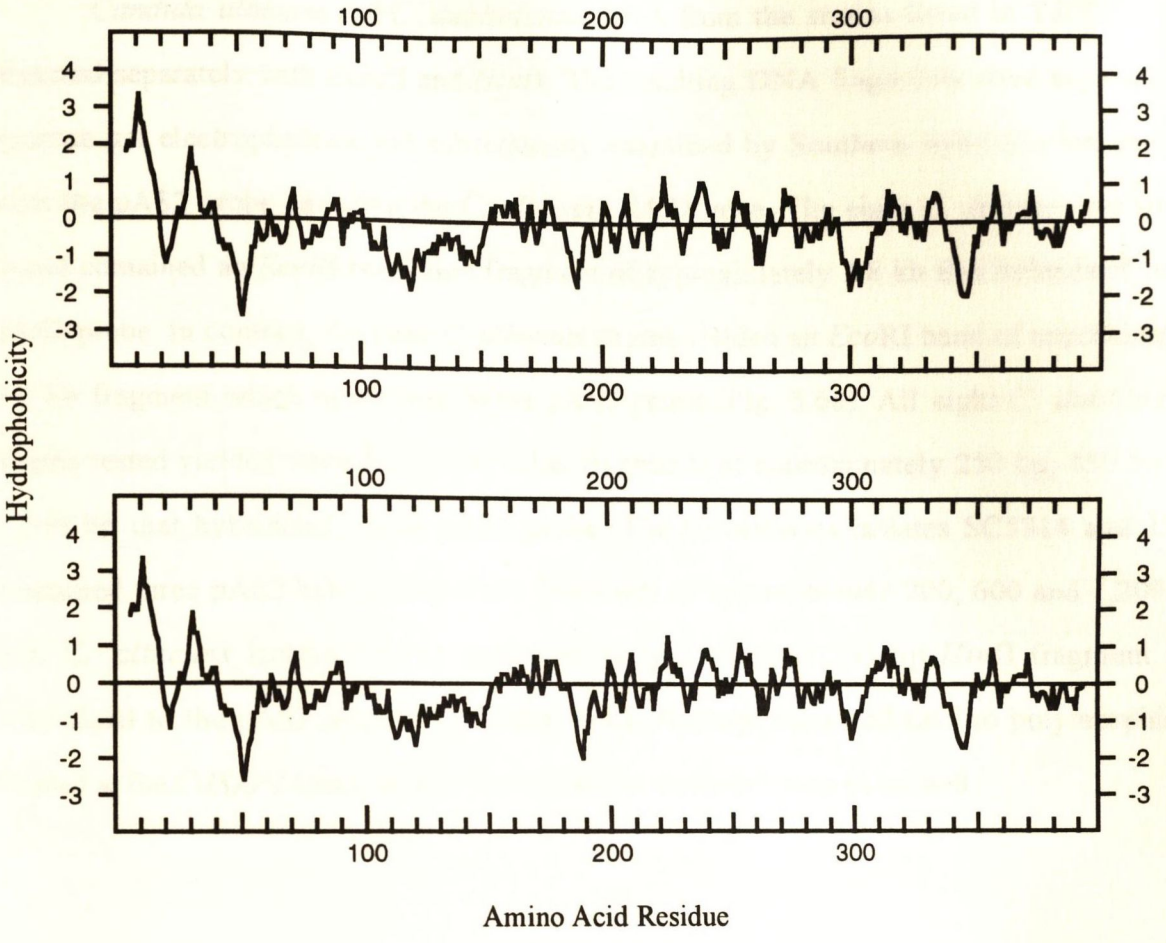
[1] The sequence of the predicted *C. albicans* mature protein starts at residue number 57 (Gln, underlined in bold). The propeptide preceding residue 57 (residue 56 in *C. dubliniensis*) includes a typical signal sequence consisting of residues 6 to 16 (bold typeface) and the Lys-Arg recognition signal for a Kex2-like proteinase adjacent to residue 57 (underlined). The two aspartic acid residues known to be at the active site of this class of proteinases are located at positions 88 and 274 (87 and 273 in CdSap2) and are doubly underlined.

The predicted *C. dubliniensis* Sap2 protein contains a putative prepropeptide N-terminal sequence consisting of a putative signal sequence and the putative propetide sequence. The C. dubliniensis Sap prepropeptide is 55 amino acids in length and is one amino acid shorter than that of the *C. albicans* Sap2 protein. This is due to a deletion of a threonine residue corresponding to residue 21 in the *C. albicans* sequence (Fig. 5.4). The lysine at residue 36 of the prepropeptide, which is invariant in the aspartic proteinase family in general, is also conserved in both species. The *C. dubliniensis* propeptide is preceded by a putative signal sequence IFIALAIALL (von Heijne, 1990) and is identical to that of *C. albicans*. Proteins entering the secretory pathway possess a signal peptide which initiates transport of the protein into the endoplasmic reticulum. The mature Sap2 protein of *C. albicans* has been shown to start at residue 57 which is the amino acid glutamine. This corresponds to a glutamine residue at postion 56 in the predicted *C. dubliniensis* Sap2 protein. In both proteins this residue is preceeded by a putative propeptide processing site ending in the dipeptide Lys-Arg. These sites have been shown to be involved in the propeptide cleavage of Saps 1, 2 and 3 (Morrison *et al.*, 1993; White *et al.*, 1993). Cleavage of propeptides on the carboxyl side of this dipeptide is brought about by enzymes called propeptide convertases (Seidah *et al.*, 1994). In *S. cerevisiae* the enzyme Kex2 performs this function and it is involved in the activation of the secreted α-mating factor Julius *et al.*, 1984). The *C. albicans KEX2* homologue has been shown to affect *C. albicans* proteinase secretion (Newport & Agabian, 1997). Togni and colleagues have shown that this peptidase cleavage site is necessary for the efficient processing of mature proteinase in *C. tropicalis* and that the removal of the propeptide is a prerequisite for the secretion of the mature enzyme (Togni *et al.*, 1996). Other than the deletion of the threonine residue at position 21 in the *C. albicans* sequence, the predicted propeptide sequence in *C. dubliniensis* is identical to that of *C. albicans*.

The sequence of the predicted mature Sap2 proteins from *C. dubliniensis* and *C. albicans* are highly similar. The two putative catalytic aspartic acid residues of CdSap2 are conserved, the first aspartic acid residue is contained in a DTG motif and the second in a DSG motif. This is analagous to the Sap2 protein of *C. albicans*. A hydropathy plot generated by

the method of Kyte & Doolittle (1982) indicates that the predicted CdSap2 protein has a very similar hydrophobicity profile to the corresponding *C. albicans* protein (Fig. 5.4). The predicted CdSap2 protein probably assumes a similar tertiary structure to that observed in *C. albicans*, with the enzyme assuming a bi-lobed structure, and each domain providing one catalytic aspartic residue (Cutfield *et al.*, 1995; Abad-Zapapero *et al.*, 1996).

Figure 5.5 Hydrophobicity profiles of the predicted CaSap2 and CdSap2 proteins [1]

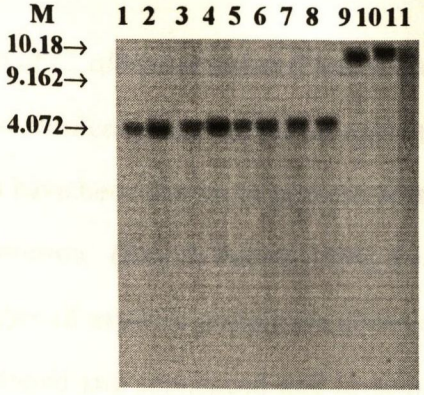


[1] Hydrophobicity profiles of *C. albicans* (top) and *C. dubliniensis* (bottom) Sap2 proteins were generated by the method of Kyte & Doolittle (1982). Regions of the protein with positive hydrophobicity values (i.e. those above the central line, at 0) represent hydrophobic domains.

5.3.3 Polymorphisms at the *C. dubliniensis SAP2* locus

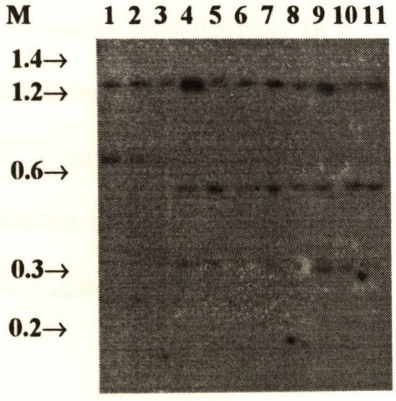
Candida albicans and *C. dubliniensis* DNA from the strains listed in Table 5.2 was digested separately with *EcoR*I and *Hin*fI. The resulting DNA fragments were separated by agarose gel electrophoresis and subsequently examined by Southern hybridisation analysis with the pAS2 probe encoding the *C. albicans SAP2* gene. The eight *C. dubliniensis* strains tested contained an *EcoR*I restriction fragment of approximately 3.8 kb that hybridised to the pAS2 probe. In contrast, the three *C. albicans* strains yielded an *Eco*RI band of approximately 9.6 kb fragment which hybridised to the pAS2 probe (Fig. 5.6a). All eight *C. dubliniensis* strains tested yielded three *Hin*fI restriction fragments of approximately 250 bp, 450 bp and 1,200 bp that hybridised to the pAS2 probe. The *C. albicans* isolates SC5314 and 132A contained three pAS2 hybridising *Hin*fI fragments of approximately 200, 600 and 1,200 bp. The *C. albicans* isolate CA411 contained only a single 1,200 bp *Hin*fI fragment that hybridised to the pAS2 probe (Fig. 5.6b). These findings indicated that no polymorphisms existed at the *CdSAP2* locus for the two restriction endonucleases examined.

Figure 5.6a Southern hybridisation of *Eco*RI digested genomic DNA from *C. albicans* and *C. dubliniensis* isolates hybridised to pAS2 probe [1]



[1] DNA in the lanes was from *C. dubliniensis* (lanes 1-8) or *C. albicans* (lanes 9-11) isolates as follows: lane M, molecular weight markers in kb; lane 1, CM1; lane 2, CM2; lane 3, CD36; lane 4, CD57; lane 5, $CD57^{R}$; lane 6, CD411; lane 7, CBS 2747; lane 8, CBS 8500, lane 9, SC5314; lane 10, 132A; lane 11, CA411.

Figure 5.6b Southern hybridisation of *Hin*fI digested genomic DNA from C. *albicans* and *C. dubliniensis* isolates hybridised to pAS2 probe [1]



[1] DNA in the lanes was from *C. albicans* (lanes 1-3) or *C. dubliniensis* (lanes 4-11) isolates as follows: lane M, molecular weight markers; lane 1, SC5314; lane 2, 132A; lane 3, CA411; lane 4, CD36; lane 5, CM1; lane 6, CM2; lane 7, CD57; lane 8, CD57R, lane 9, CD411; lane 10, CBS 2747; lane 11, CBS 8500.

5.4 Discussion

The ten *SAP* genes of *C. albicans* isolated and sequenced to date comprise a multigene family and encode putative virulence factors in this opportunistic pathogen. Other members of the genus *Candida* which have been shown to possess proteolytic activity are *C. tropicalis*, *C. parapsilosis* and *C. dubliniensis*, (Ray & Payne, 1990; Fusek *et al.*, 1993; McCullough *et al.*, 1995; this study). A number of aspartic proteinase genes of *C. albicans*, *C. tropicalis* and *C. parapsilosis* have been cloned and sequenced and in some instances the protein structure of the corresponding enzymes have been elucidated by X-ray crystallography. *Candida dubliniensis* has been shown to possess homologues of the *C. albicans SAP1-7* genes (Gilfillan *et al.*, 1998) and it has been reported that some isolates of *C. dubliniensis* expressed higher levels of proteinase activity than *C. albicans* when grown in medium containing BSA as the sole nitrogen source (McCullough *et al.*, 1995). The *C. dubliniensis SAP2* gene cloned and sequenced in the present study is the first member of *C. dubliniensis SAP* multigene family to be analysed at the molecular level. Although it is similar to its counterpart in *C. albicans*, *CdSAP2* exhibits a 10.4 % divergence at the nucleotide sequence level. Similar levels of divergence have been reporte for other *C. dubliniensis* genes such as *MDR1 PHR1*and *PHR2* (Table 5.4). However, this is considerably less than the divergence of genes such as *ACT1* and the SSU rRNA genes.

Table 5.4 Percentage divergence of *C. dubliniensis* gene sequences from the corresponding *C. albicans* homologues

Gene	% Divergence	Reference
CdSAP2	10.4	This study
CdMDR1	8.0	Moran *et al.*, 1998
CdPHR1	9.8	Heinz *et al.*, 2000
CdPHR2	8.8	Heinz *et al.*, 2000
CdACT1	2.1	This study and Donnelly *et al.*, 1999
SSU rRNA	1.4	Gilfillan *et al.*, 1998

Housekeeping genes such as *ACT1* and the SSU rRNA genes are subject to considerable functional constraint and are conserved throughout the eukaryotic kingdom. Subsequently a smaller level of nucleotide sequence divergence is observed with these genes from *C. dubliniensis* and *C. albicans*. Presumably, genes such as *SAP2*, *MDR1*, *PHR1* and *PHR2*, whilst exhibiting extensive conserved regions which allow the protein to function, are otherwise subject to less evolutionary constraint than housekeeping genes. The nucleotide sequence of the *C. dubliniensis SAP2* gene was compared to the nucleotide sequences of *C. albicans SAP2*, *C. tropicalis SAPT4* and *C. parapsilosis SAPP2* (*ACPR*) using the CLUSTAL W sequence alignment software package. An evolutionary distance matrix for the group of sequences was generated incorporating corrections for multiple base changes according to the method of Jukes & Cantor (1969; Table 5.5). This matrix shows that the genes from *C. tropicalis* and *C. parapsilosis* are considerably more diverged from *C. albicans* than that of *C. dublinienisis*. This is as expected given the phylogenetic position of each of these species within the genus *Candida*.

Table 5.5 Genetic distance matrix based on comparison of *SAP* gene coding sequences[1]

	C. al.	*C. du.*	*C. tr.*	*C. pa.*
C. albicans	-			
C. dubliniensis	10.4	-		
C. tropicalis	34.2	35.6	-	
C. parapsilosis	43.4	43.3	42.8	-

[1] Values correspond to percentages of difference corrected for multiple base changes by the method of Jukes & Cantor (1969). The *SAP* gene sequences used were as follows: *C. albicans SAP2* (M83663, Wright *et al.*, 1993); *C. dubliniensis SAP2* (this study); *C. tropicalis SAPT4* (L25388, Miyasaki *et al.*, 1994) and *C. parapsilosis SAP1* (*ACPR*; Z11919, de Viragh *et al.*, 1993).

The *CdSAP2* ORF is predicted to encode a protein of 397 amino acids and is 93.9% identical to the corresponding *C. albicans* protein at the amino acid sequence level. Overall, the primary structure of CdSap2 is highly homologous to its *C. albicans* counterpart with the putative prepropeptide sequence and catalytic residues being conserved. The predicted primary structure and hydrophobicity profile of CdSap2 indicates that it probably has an identical tertiary structure to that of *C. albicans* and therefore belongs to the *Candida* subclass of aspartic proteinases. There are substantial differences between the putative promoter regions of both genes (62.4 % identity), although there are conserved elements such as the TATA and CAAT boxes present in both regions. The differences present in the upstream regions of the CdSAP2 and CaSAP2 may have important implications in the regulation of expression of these genes in the two species. However, this is very speculative as only 125 bp of promoter region were compared and eukaryotic promoters may span thousands of base pairs upstream of the coding region.

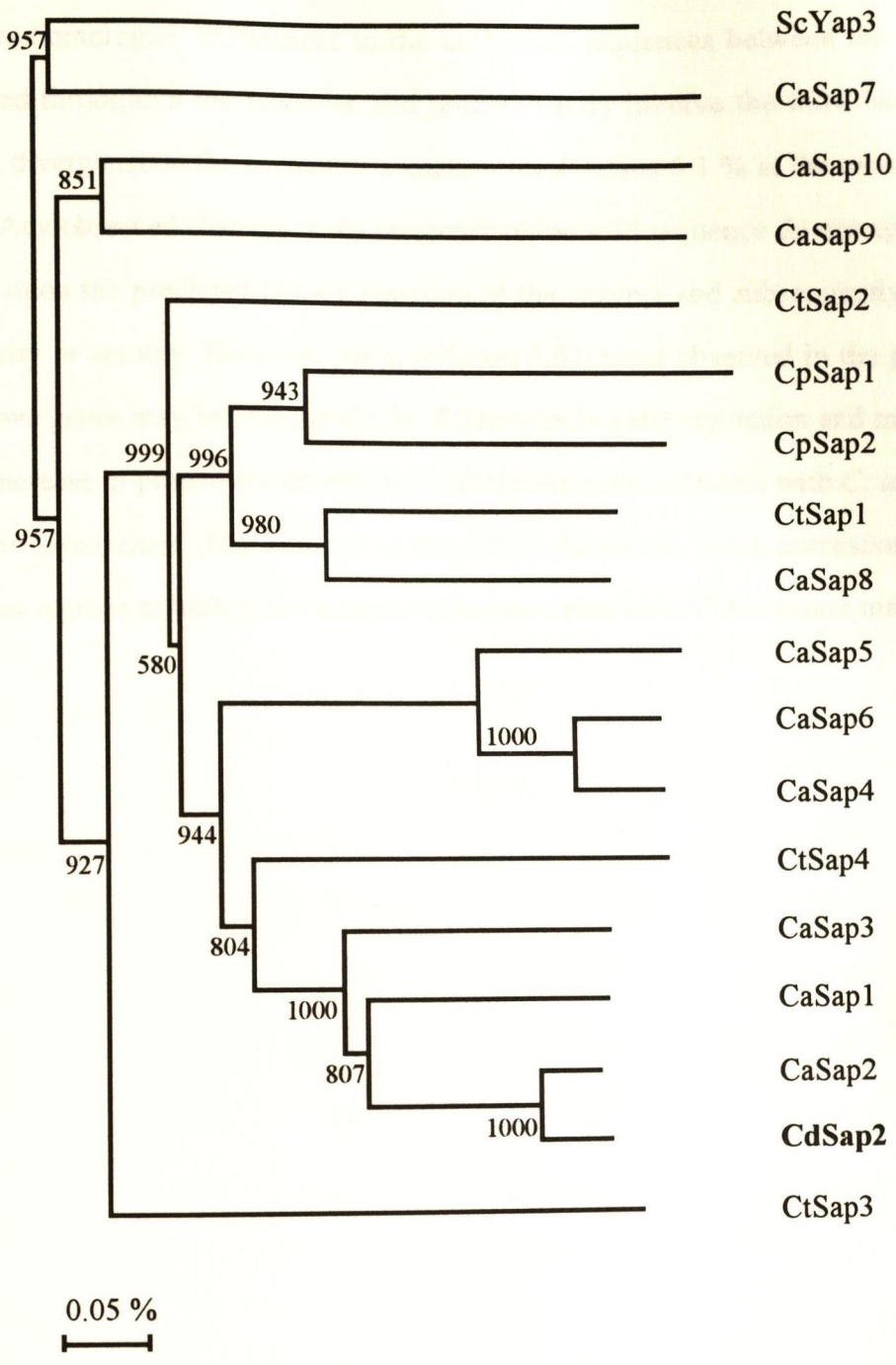
The predicted *C. dubliniensis* Sap2 protein sequence was compared to the predicted protein sequences of the currently known *Candida* Saps using the CLUSTAL W sequence alignment software package. An evolutionary distance matrix for the group of sequences was generated incorporating corrections for multiple base changes according to the method of Jukes & Cantor (1969; Table 5.6). Using this matrix a dendrogram was constructed using the neighbour-joining method of Saitou & Nei (1987). Figure 5.6 shows the dendrogram depicting the relationships that exist between the predicted CdSap2 and the other currently known *Candida* secreted aspartic proteinases, and includes the GPI-anchored proteinase, Yap3, of *S. cerevisiae* as an outlier. The predicted CdSAP2 protein is most closely related to CaSap2 proteinase and falls into the CaSap1-3 grouping. This grouping also contains the *C. tropicalis* Sap4 protein.

Table 5.6 Genetic distance matrix based on comparison of *Candida* SAP protein sequences [1]

	CdSAP2	CaSAP2	CaSAP1	CaSAP3	CtSAP4	CaSAP4	CaSAP6	CaSAP5	CaSAP8	CtSAP1	CpSAP2	CpSAP1	CtSAP2	CtSAP3	CaSaP9	CaSAP10	ScYAP3	CaSAP7
CdSAP2	-																	
CaSAP2	7.1	-																
CaSAP1	25.8	25.6	-															
CaSAP3	28.6	27.0	29.5	-														
CtSAP4	43.4	42.1	42.5	40.6	-													
CaSAP4	43.3	42.0	40.4	43.8	48.7	-												
CaSAP6	42.5	41.8	40.7	44.4	49.2	9.4	-											
CaSAP5	44.8	44.8	42.2	47.7	51.3	22.3	19.6	-										
CaSAP8	45.3	44.1	45.2	43.6	50.8	49.9	50.3	50.5	-									
CtSAP1	41.8	40.4	45.1	41.4	48.3	51.6	51.1	51.6	31.0	-								
ACPR	48.7	48.3	48.3	47.6	54.3	53.8	55.2	54.1	41.7	45.2	-							
ACPL	53.7	53.1	51.5	53.0	55.7	56.1	56.1	58.0	47.9	49.4	42.6	-						
CtSAP2	50.1	51.0	51.8	53.0	54.7	54.7	54.2	54.9	50.4	54.1	53.0	56.7	-					
CtSAP3	56.3	57.0	57.7	57.8	56.5	58.1	57.3	58.9	55.1	56.2	59.6	63.6	60.2	-				
CaSAP9	64.9	64.9	64.6	66.0	64.2	62.7	63.3	65.2	67.7	67.6	66.7	70.6	67.2	64.7	-			
CaSAP10	65.0	66.1	63.1	65.9	63.9	65.0	66.1	65.5	66.5	65.8	64.6	69.3	67.8	63.9	63.4	-		
ScYAP3	66.6	67.9	66.2	65.5	67.1	66.9	66.5	65.7	64.6	64.8	64.2	68.3	64.2	66.8	65.2	71.5	-	
CaSAP7	68.4	65.4	68.4	68.7	67.5	70.0	70.0	70.5	69.3	68.5	70.3	69.1	71.2	68.1	69.7	72.3	66.9	-

[1] Values correspond to percentages of difference corrected for multiple base changes by the method of Jukes & Cantor (1969). The *SAP* gene sequences used were the *C. dubliniensis SAP2* (this study) and the currently known *SAP* genes from *C. albicans*, *C. parapsilosis* and *C. tropicalis* detailed in Table 5.1. The *S. cerevisiae YAP3* proteinase was included as an outlier.

Figure 5.7 Dendrogram depicting relationships between *Candida* Saps [1]



[1] CdSap2, CaSap1-10, CpSap1-2 and CtSap2-4 denote the secreted aspartic proteinases from *C. dubliniensis*, *C. albicans*, *C. parapsilosis* and *C. tropicalis* respectively. ScYap3 is a GPI-anchored proteinase of *S. cerevisiae*. The branch lengths are proportional to the similarity between amino acid sequences and the scale bar represents a 0.05 % difference in amino acid sequence. The numbers at each node were generated by bootstrap analysis, and represent the number of times the arrangement occurred in 1000 randomly generated trees.

In conclusion the *C. dubliniensis SAP2* gene is very similar in sequence to its *C. albicans* homologue. Differences in the nucleotide sequences between the two proteins are dispersed throughout the sequence and predominantly involve the third base of the codons (10.4% divergence at the nucleotide sequence level versus 6.1 % at the amino acid sequence level). Any observed changes in the predicted amino acid sequence do not appear to have any impact upon the predicted tertiary structure of the enzyme and subsequently on its substrate specificity or activity. However, the significant differences observed in the promoter regions of the two genes may be responsible for differences in gene regulation and may be implicated in the increase in proteinase activity by *C. dubliniensis* as compared with *C. albicans* observed by some researchers (McCullough *et al.*, 1995). However, more extensive analysis of the promoter regions of both genes is required before deductions of this nature may be made.

Chapter 6
Expression of *SAP2* and Proteinase Production by *Candida dubliniensis*

6.1 Introduction

Candida albicans is a human commensal organism, which, under certain conditions, can cause superficial infections of mucosal epithelia. However, in recent years the incidence of life threatening disseminated infection caused by this species has increased significantly as the numbers of immunocompromised patients increases. Although the immune status of the host is of vital importance in the transition from commensal to pathogen, the organism itself contributes to the initiation and progression of disease. Unlike some bacterial pathogens which have well documented single dominant virulence factors, the pathogenicity of *C. albicans* is believed to involve a number of factors, the most widely studied being adhesion to host surfaces, transition from yeast to hyphal morphologies, phenotypic switching and the production of hydrolytic enzymes such as aspartic proteinases (Cutler, 1991).

6.1.1 Secreted aspartic proteinases as virulence factors in *C. albicans*

There is much evidence from both *in vivo* and *in vitro* studies to indicate that the *SAP* genes and their protein products (SAPs) play a role in adhesion, colonisation and invasion by *C. albicans*. *Candida albicans* exhibits increased adherence to cells in various tissue models compared to other *Candida* species. Adherence to epithelial cells may be adversely affected by pepstatin A, an inhibitor of aspartic proteinases, suggesting a role for the SAPs in adherence. In instances where pepstatin A did not prevent adherence it reduced cavitation by the yeast cells (Ray & Payne, 1988; Borg & Rüchel, 1988; Ollert *et al.*, 1993; Klotz *et al.*, 1994; Watts *et al.*, 1998). The adherence of *Candida* species to the mucin layer and their subsequent degradation of mucin are essential if they are to traverse the layer and bind to the epithelium (Colina *et al.*, 1996; De Repentigny *et al.*, 2000). The adherence of *Candida* species to small intestinal mucin is thought to be mediated by SAP2. The ability to bind to mucin by *Candida* species is correlated with the hierarchy of virulence of *Candida* species, with *C. dubliniensis*, *C. tropicalis* and *C. albicans* being more highly adherent than other *Candida* species (De Repentigny *et al.*, 2000). The HIV protease inhibitor drugs, which have been shown to inhibit

SAPs 1-3, also inhibit the binding of *C. albicans* to epithelial cells (Borg-von Zepelin *et al.*, 1999; Gruber *et al.*, 1999a and 1999b; Monod *et al.*, 1999; Korting *et al.*, 1999).

SAP antigens have been found during yeast attachment to epithelial cells *in vitro* and *in vivo* in the vaginal secretions of *Candida* vaginitis patients, in oral mucosal candidosis, and in skin lesions resulting from systemic candidosis (Borg & Rüchel, 1988; De Bernardis *et al.*, 1990; Rüchel, 1993). SAP1-3 antigens have been detected in lesions from patients with oral candidosis, with the majority of antigens being secreted by those *C. albicans* cells adhering directly to the epithelial surface (Schaller *et al.*, 1999a). Antibodies to SAP antigens have been detected in human sera with high titres present in individuals presenting with invasive disease (MacDonald & Odds, 1980; Ray & Payne, 1987; Rüchel *et al.*, 1988). Furthermore, SAP2 antibodies have been found to have a protective effect in a rat model of vaginitis (De Bernardis *et al.*, 1997).

Further evidence that the SAPs are *Candida* virulence factors comes from studies on purified SAP proteins that show they have a broad substrate specificity, and are capable of hydrolysing a number of human proteins, including immune system proteins. Proteins that can act as substrates for SAP enzymes include albumin, collagen, IgA, keratin, haemoglobin, and mucin (Remold *et al.*, 1968; Rüchel *et al.*, 1981 and 1983; Negi *et al.*, 1984; Kaminishi *et al.*, 1986 and 1988; Colina *et al.*, 1996; De Repetigny *et al.*, 2000). *Candida* proteinases may also affect the antimicrobial properties of human serum. The enzymes are capable of degrading the Fc portion of IgG, C3 complement component, α-2 macroglobulin and α−1 proteinase inhibitor. The action of the *Candida* proteinase inhibits opsonisation and inhibits the alternative pathway of complement activation (Kaminishi *et al.*, 1995). *Candida* proteinase has also been shown to limit the activation of the kallikrein-kinin system by Hageman factor (Kaminishi *et al.*, 1990). Aspartic proteinase is capable of degrading Interleukin-1β precursor, resulting in the activation of the proinflammatory cytokine IL-1β. Thus SAPs may be involved in the progression of superficial candidosis to chronic inflammation (Beausejour *et al.*, 1998). Saliva contains several antimicrobial agents, including the enzyme lysozyme. Saliva has been shown to have an candidacidal effect (Tobgi *et al.*, 1987; Samaranayake *et al.*, 1994; Wu *et*

al., 1999). It appears to exert some of its anticandidal activity by inhibiting the secretion of the SAP enzymes and the ability of *C. albicans* to grow in human saliva is related to the degree of expression of *SAP*s (Wu *et al.*, 1999; Wu & Samaranayake, 1999).

Studies on vaginitis patients and rat vaginitis models have been particularly useful for demonstrating the role of SAPs as virulence factors in mucosal infection. Vaginal fluids from women with *C. albicans* vaginitis had higher levels of aspartic proteinase activity than women who were carriers (De Bernardis *et al.*, 1990). *Candida albicans* strains isolated from HIV-infected women presenting with vaginitis produced significantly more proteinase activity than those vaginitis patients who were non-HIV infected and those who did not have vaginitis but were positive for *C. albicans*. Rat models of vaginitis have shown that SAP is actively secreted by *C. albicans* in this particular model and treatment with pepstatin A was found to have a protective effect. The ability of *C. albicans* to cause infection in this model would appear to be related to the organism's ability to produce aspartic proteinase. (Agatensi *et al.*, 1991; De Bernardis *et al.*, 1995, 1996, 1997 1999a and 1999b; Stringaro *et al.*, 1997). Furthermore, individual *SAP1*, *2* and *3* mutants were significantly less virulent than the parental strain in the rat model, with the *SAP2* mutant being almost avirulent. Reintroduction of the *SAP2* gene into the *SAP2* mutant restored its virulence (De Bernardis *et al.*, 1999a).

The role of the *SAP* genes and their protein products as virulence factors has also been investigated in murine models of superficial and disseminated candidosis. Pepstatin A had a protective effect in both the rat model of vaginitis and in a murine model of disseminated candidosis (De Bernardis *et al.*, 1997; Fallon *et al.*, 1997). It has been suggested that SAPs are involved in dissemination across the epithelial barrier as the protective effect of pepstatin A in the mouse model of disseminated candidosis was only apparent when the mice where challenged intranasally (Fallon *et al.*, 1997). Studies on various *SAP* mutants reveal that they contribute to the ability of *C. albicans* to establish infection and cause tissue damage. *Candida albicans* derivatives harbouring mutations in the *SAP1*, *2* or *3* genes showed an attenuated virulence in guinea pig and mouse models suggesting that the individual proteins SAPs 1, 2 and 3 are *C. albicans* virulence in these particular models (Hube *et al.*, 1997). SAP2 has been

found to contribute to damage in human endothelial cells and to stimulate them to express E-selectin (Ibrahim *et al.*, 1998). The *SAP1* gene has been shown to be opaque-phase specific in the switching strain WO-1. Misexpression of the *SAP1* gene in the white phase conferred an increased virulence to this phenotype in a cutaneous mouse model (Kvaal *et al.*, 1999). This increased level of virulence was similar to that exhibited by opaque phase cells which express this gene naturally. The white cells, which misexpressed the SAP1 gene, also demonstrated the opaque cell characteristics of increased adhesion and capacity to cavitate the skin (Kvaal *et al.*, 1999). A study using a *C. albicans SAP4-6* triple mutant revealed that this subfamily of SAP proteins or individual members are involved in the progression of systemic disease in guinea pig and mouse models of disseminated infection (Sanglard *et al.*, 1997). Studies using this triple mutant reveal that SAPs 4-6 or individual enzymes of this group contribute to tissue damage in a mouse model of peritonitis (Kretschmar *et al.*, 1999), and they have been implicated in the ability of *C. albicans* to survive phagocytosis by murine peritoneal macrophages (Borg-von Zepelin *et al.*, 1998).

6.1.2 Induction of *SAP* expression in *C. albicans*

Proteolytic activity may be induced in *C. albicans* by growth under restrictive culture conditions that provide nitrogen solely in the form of medium- to high-molecular weight proteins. Proteins such as BSA, haemoglobin, keratin, casein, mucin, and collagen have all been used to induce proteinase production in *C. albicans*, although not all proteins induce proteinase production equally well. Conversely, compounds that are known to inhibit proteinase secretion include low molecular weight compounds such as glycine, glutamic acid, urea, ammonium tartrate, ammonium sulphate, ammonium acetate and ammonium chloride. In general, peptides of 8 or more residues induce proteinase production while those of 7 or less do not. The maximal proteinase induction occurs at an acid pH (3.5 - 4.0) and is inhibited by growth at or around a neutral pH (Remold *et al.*, 1968; Germaine & Tellefson, 1981; Rüchel *et al.*, 1983; Negi *et al.*, 1984; Kaminishi *et al.*, 1986 and 1988; Crandal & Edwards 1987; Ray

& Payne, 1990; Banerjee *et al.*, 1991; Homma *et al.*, 1991; Lerner & Goldman, 1993; Hube *et al.*, 1994; Colina *et al.*, 1996).

Initial experiments showed that *SAP2* was the predominant gene expressed by *C. albicans* yeast cells in BSA medium and it was expressed in the early to mid-log phase only (Wright *et al.*, 1992; Hube *et al.*, 1994; Colina *et al.*, 1996). The *SAP1* and *SAP3* genes appear to be involved in phenotypic switching as shown by studies with the *C. albicans* strain WO-1, which switches between opaque and white phenotypes. The opaque form expresses *SAPs 1-3* and the white form expresses *SAP2* only (Morrow *et al.*, 1992; White *et al.*, 1993; Hube *et al.*, 1994). Although the expression of *SAP2* in opaque cells was affected by known repressors of proteinase activity, such as amino acids, pepstatin A and stationary phase, the expression of *SAP1* and *SAP3* did not appear to be affected (Hube *et al.*, 1994). The closely related *SAP4-6* genes are expressed in serum induction and during pH/temperature shift induction suggesting that the expression of these genes is associated with morphological changes (Hube *et al.*, 1994; White & Agabian, 1995). The expression of *SAP8* appears to be affected by temperature as it was found to be expressed by the *C. albicans* strain DSM 6659 at 25 °C during early exponential growth. This gene was also expressed at 37 °C, although at a lower level than at 25 °C (Monod *et al.*, 1998). The expression of *SAP9* was detected in stationary phase cells, and after expression of *SAP8* had decreased (Morrison *et al.*, 1993; Monod *et al.*, 1998). The protein products of *SAP1, 2* and *3* have been detected in the supernatants of *C. albicans* cultures following growth in complex protein containing media (White *et al.*, 1993; White & Agabian, 1995; Colina *et al.*, 1996; Smolenski *et al.*, 1997).

6.1.3 *In vivo* and *in vitro* expression of *C. albicans SAPs*

Further evidence of the differential regulation and role of the different *SAP* genes was provided by *in vitro* expression studies using various animal and tissue models. Using reconstituted human endothelial cells as an *in vitro* model of oral candidosis Schaller and colleagues demonstrated different patterns of *SAP* expression during the course of infection (Schaller *et al.*, 1998, 1999b and 2000). During the first stages of infection (up to 48 h) *SAP1*

and *SAP3* were detected, followed by *SAP6*. The later stages of infection (after 60 h) were associated with *SAP2* and *SAP8* expression. The expression of these genes was correlated with tissue damage to the culture cells. The early and late pattern of *SAP* expression was also observed *in vivo* in two patients, one of who was in the early stages of infection, and the other who had had oral candidosis for at least one year (Schaller *et al.*, 1998, 1999b, and 2000). Another study investigated the expression of *SAP* genes in salivary samples from patients who were *C. albicans* oral carriers or who had oral candidosis (Naglik *et al.*, 1999). The pattern of expression of *SAP* genes *in vivo* was found to be associated with whether the individual was a carrier of *C. albicans* or symptomatic for oral candidosis. *SAP1* and *3* appeared to be positively correlated with oral candidosis but not with carriage of the organism. *SAP2* was to be expressed in all symptomatic cases and in most carriers. However, the expression of this gene did not occur without the expression of the *SAP4-6* family suggesting that *SAP4-6* may play a role in the regulation of *SAP2*. *SAP7*, the expression of which has not been detected *in vitro*, was found to be expressed in some asymptomatic and in some symptomatic individuals, although the expression of this gene appears to be associated with infection as opposed to carriage (Naglik *et al.*, 1999).

Using an *in vivo* expression technology, Staib *et al.* (1999 and 2000) investigated the roles of *SAP1-6* in a murine model of oesophageal mucosal infection, where *C. albicans* invades the epithelium but does not disseminate. *SAP5* and *SAP6* were strongly expressed and associated with heavy mycelial growth, whereas *SAP1-4* exhibited a low-level of induction. In a murine model of peritonitis *SAP5* was initially expressed, but was not associated with mycelial growth. Invasion of the liver correlated with mycelial growth and expression of *SAP5* and *SAP6*. *SAP5* expression appeared to be maintained after dissemination, and *SAP6* was associated with hyphal growth. *SAP2* was induced during spread to deep organs and appeared to correlate with tissue destruction.

In summary expression of each member of the *SAP* multigene family has been detected *in vivo*. Activity of SAP enzymes is associated with adherence of *C. albicans* to epithelium and with invasion and dissemination of the organism. Members of the family are regulated

differentially *in vitro* and *in vivo* and individual members may have specific roles to play from the initial stages of adherence and colonisation to the progression of systemic disease.

The purpose of this project was to characterise genetic differences between *C. dubliniensis* and *C. albicans* using housekeeping genes and genes encoding putative virulence factors. The *SAP2* gene was selected as a gene encoding a putative virulence factor, and although there was significant nucleotide sequence divergence between the *C. dubliniensis* and *C. albicans* genes as shown in the previous chapter, it is unlikely that the three dimensional structure of the *C. dubliniensis* protein is significantly different from that of *C. albicans*. The aim of this section of the present study was to investigate the expression of the *C. dubliniensis SAP2* gene and the proteinase activity of *C. dubliniensis* in BSA containing media and to compare this with the expression of the *C. albicans SAP2* gene.

6.2 Materials and Methods

6.2.1 *Candida* strains and isolates

The *Candida* strains and isolates used in this part of the present study are listed in Table 5.1 and are described in section 5.2.1. The identity of each strain or isolate was confirmed by the methods described in Chapter 2. The organisms were routinely maintained on PDA agar.

6.2.2 Culture media and growth conditions

Overnight cultures: A single colony from a 48 h yeast culture grown on PDA agar at 37 °C was inoculated into 20 ml of YPD broth at 37 °C for 18 h in a Gallenkamp (Model G25) orbital incubator set at 200 r.p.m. Cell counts of each 18 h culture were performed in a haemocytometer as follows: a 1:10 dilution of each 18 h culture was prepared in sterile distilled water. A small aliquot of this dilution was loaded into the haemocytometer counting chamber and allowed to settle for 5 min. The cells in 5 squares were counted. The cell count was calculated then from this number in c.f.u./ml. This procedure was performed three times and an average cell count was calculated.

***SAP2* inducing media:** In order to analyse phenotypic and genotypic expression of the *SAP2* gene in *C. albicans* and *C. dubliniensis* the yeasts were grown in the *SAP2* induction medium YCB/BSA. YCB/BSA medium consisted of 1.17 % (w/v) Yeast Carbon Base (prepared as a 10x solution in distilled water and filter sterilised; Difco Laboratories), 0.5 % (w/v) BSA (prepared as a 5 % solution in distilled water and filter sterilised; Sigma) and 2 % (w/v) glucose. This induction media was used to grow cells for growth curve experiments, preparation of crude enzyme extracts and extraction of total RNA. In each case, prewarmed YCB/BSA medium was inoculated with cells of the overnight cultures described above to yield a final concentration of 2 x 10^6 c.f.u. per ml and incubated at 30 °C in an orbital shaker set at 200 r.p.m for 4 d.

6.2.3 Growth rate determination

The doubling times of the *C. dubliniensis* and *C. albicans* isolates listed in Table 6.1 in YCB/BSA medium were determined. Initially, a small aliquot of the standardised 18 h overnight cultures was inoculated into 100 ml YCB/BSA medium in a 250 ml Erlenmyer flask to yield a culture density of 2 x 10^6 cells/ml. These cultures were incubated in a Gallenkamp orbital incubator at 200 r.p.m. at 30 °C for 4 d. At intervals, 0.1 ml aliquots were removed from each flask in order to determine the OD_{600} of each culture. Sterile YCB/BSA medium was used as a reference blank. Samples were appropriately diluted in sterile saline so that spectrophotometric readings were not > 1.0. Five millilitre aliquots were also taken at hourly intervals for pH measurement. The cells were removed by filtration and the pH of the supernatant measured. Cultures were sampled hourly throughout the first 7 h of exponential phase and thereafter at 24-hour intervals. Experiments were performed on three separate occasions.

6.2.4 Preparation of samples for RNA extraction, proteinase activity and total protein estimation

For each isolate 300 ml of prewarmed YCB/BSA media in a 1 l Erlenmyer flask was inoculated with a small aliquot of the overnight culture to yield a final concentration of 2 x 10^6 cells/ml. All cultures were incubated at 30 °C for 4 d in an orbital shaker at 200 r.p.m. Aliquots of each batch culture were harvested at 2, 3, 4, 5, 6, 7, 24, 48, 72, and 96 h. The cells of each aliquot were harvested by centrifugation at 3000 r.p.m. in an Eppendorf micro-centrifuge (model 5804C) for 5 min. A 1.5 ml volume of the supernatant from aliquots harvested at 3, 6, 24, 48, 72 and 96 h was collected on ice, the pH adjusted to 7.0 with 1 M NaOH to prevent autodegradation, and then stored at –70 °C following filter sterilisation. These sterilised supernatants were the crude enzyme extracts for measurement of proteinase activity and total protein estimation. The cell pellets from aliquots harvested at 2, 3, 4, 5, 6, 7, and 24 h were used to prepare total cellular RNA as described below. In addition, 100 µl samples of each culture were taken at each time interval and the cell count determined using a haemocytometer and by plating on PDA. Each batch culture was grown on three separate occasions and samples prepared accordingly.

6.2.5 Northern analyses

Total RNA extraction from *Candida* isolates. Total RNA prepared from the *C. dubliniensis* strains grown in YCB/BSA medium using the method described in section 2.5.2 yielded low quantities of degraded RNA. Therefore, total RNA from YCB/BSA cultures of *C. dubliniensis* and *C. albicans* isolates was extracted using the RNeasy Mini Kit system (Qiagen). This procedure involves the mechanical lysis of yeast cells using a bead mill (FastPrep FP120, BIO 101 Savant, Ananchem, London, UK) in the presence of a denaturing guanidine isothiocyanate-containing lysis buffer which inactivates RNases. A Rneasy® spin column containing a silcia-gel-based membrane binds total RNA which is then eluted in a small volume of water. The kit was used according to the manufacturer's instructions with the following exceptions: Qiagen recommend using not more than 10^7 cells for each extraction. This number of cells was contained in less than 10 ml of YCB/BSA culture for each strain at all times. When no more than 10^7 cells of each YCB/BSA culture was used to prepare RNA with the RNeasy Mini Kit little or no RNA was obtained. Therefore the cells contained in 50 ml of culture were used for RNA extraction at 2 and 3 h incubation, 30 ml of culture at 4 and 5 h incubation, 20 ml of culture at 6 and 7 h incubation and 10 ml of culture at 24 h incubation. Using smaller volumes of *C. dubliniensis* culture for RNA extraction resulted in little or no yield of RNA. The Qiagen lysis buffer requires the addition of 100 µl of β-mercaptoethanol per 10 ml of lysis buffer before use; this was increased to 200 µl of β-mercaptoethanol. Purified RNA eluted from the RNeasy spin columns with RNase free water was found to degrade after 24 h storage at -70 °C. To limit this degradation, the RNA was eluted from the RNeasy spin columns using formamide.

PCR amplification of specific *C. dubliniensis* and *C. albicans* sequences to generate probes for Northern analysis. The primer pair S2F/S2R (Table 6.1) was used to amplify a 889 bp DNA fragment from *CaSAP2* and *CdSAP2* using *Taq* DNA polymerase and the proof-reading polymerase *Pwo* (Expand high-fidelity PCR system, Roche). The amplimers obtained using *C. dubliniensis* and *C. albicans* genomic DNA were purified and cloned separately into pBluescript, as described in section 2.4, to yield recombinant plasmids pCdS2

and pCaS2, respectively. The cloned DNA inserts contained within these plasmids were sequenced as described in section 2.6.

The primer pair TEF3F/TEF3R (Table 6.1) was designed using the sequence of the *C. albicans TEF3* gene (accession no. Z12822) and used to amplify a DNA fragment of 762 bp from both *C. albicans* and *C. dubliniensis* genomic DNA. The amplimers obtained from *C. albicans* and *C. dubliniensis* genomic DNA were purified and cloned separately into pBluescript, as described previously in section 2.4, to yield recombinant plasmids CaT3 and CdT3, respectively. The cloned DNA inserts contained within these plasmids were sequenced as described in section 2.6.

The cloned DNA inserts from recombinant plasmids pCdS2, pCaS2, pCdT3 and pCaT3 were excised and gel purified as described in section 2.4. The purified fragments were radioactively labelled with [α-^{32}P]dATP as described in section 2.2.5 for use in Northern hybridisation experiments.

RNA electrophoresis and hybridisation were carried out as described in sections 2.5.3 and 2.5.4.

Table 6.1 Primers used to amplify *C. dubliniensis* and *C. albicans* sequences [1]

Primer	*Sequence*	Nucleotide co-ordinates	Sequence Reference
SF	5'-CGGAATTCCCAGTGACTTTACACAATGA-3'	744 - 763	This study
SR	5'-CGGAATTCACATCGAAAAGTAATTGACATT-3'	1612 - 1633	This study
TEF3F	5'-CGGAATTCCGATTGGTCCAAATGGTGCTGG-3	2113 - 2132	Di Domenico *et al.*, 1992
TEF3R	5'-CGGAATTCCGATCTTGTTACCCATAGCATCG-3'	3012 - 3032	Di Domenico *et al.*, 1992

[1] Primers are complementary to the *C. dubliniensis SAP2* gene sequence (this study) and the *C. albicans TEF3* gene (Di Domenico *et al.*, 1992). Nucleotide co-ordinates shown are numbered in the 5' to 3' direction with the first base of the translation start codon being +1.

6.2.6 Measurement of proteinase enzyme activity

The proteinase activity of the crude enzyme filtrates prepared from culture supernatants collected following 3, 6, 24, 48, 72 and 96 h growth as described above was determined spectrophotometrically by a BSA degradation assay as follows: each assay was set up in triplicate and consisted of 500 µl of 2 % (w/v) BSA, 100 µl of 50 mM sodium citrate pH 3.2, and 200 µl of the crude enzyme preparation. After 1 h incubation with shaking at 37 °C the reaction was stopped by the addition of 200 µl of 20 % (v/v) TCA. Sample blanks consisted of identical ingredients with the TCA being added prior to the addition of crude enzyme preparation. Following a 30 min incubation on ice, samples and blanks were centrifuged for 15 min at 4 °C in a refrigerated Eppendorf centrifuge (Biofuge fresco, Heraeus Instruments, Hanau, Germany). The amount of degraded protein in the clear supernatant was measured by mixing 150 µl of the supernatant with 150 µl of Coomassie® Plus Protein Assay Reagent Kit (Pierce, Rockford, Illinois, USA) in a microtitre plate as per the Micro Protocol (microtitre plate version) described by the manufacturer. The absorbance at 595_{nm} was measured using an automated plate reader (Spectra I; SLT-Labinstruments, Salzburg, Austria). Enzyme activity was measured as the increase in A_{595} between the samples and the blanks. One activity unit was defined as an increase of 0.100/60 min/ml at 595_{nm} (Ollert *et al.*, 1995).

6.2.7 Total protein estimation

The total protein concentration in mg/ml of each crude enzyme preparation was measured with Coomassie® Plus Protein Assay Reagent Kit (Pierce) using the Micro Protocol (microtitre plate version) and comparing against a standard curve as described by the manufacturer. The standard curve was prepared using an albumin standard concentrate and prepared as described in the Coomassie® Plus Protein Assay Reagent Kit booklet. Each crude enzyme sample was appropriately diluted in phosphate buffered saline so that A_{595} readings fell within the absorbance range of the albumin standards. Each dilution was assayed in triplicate.

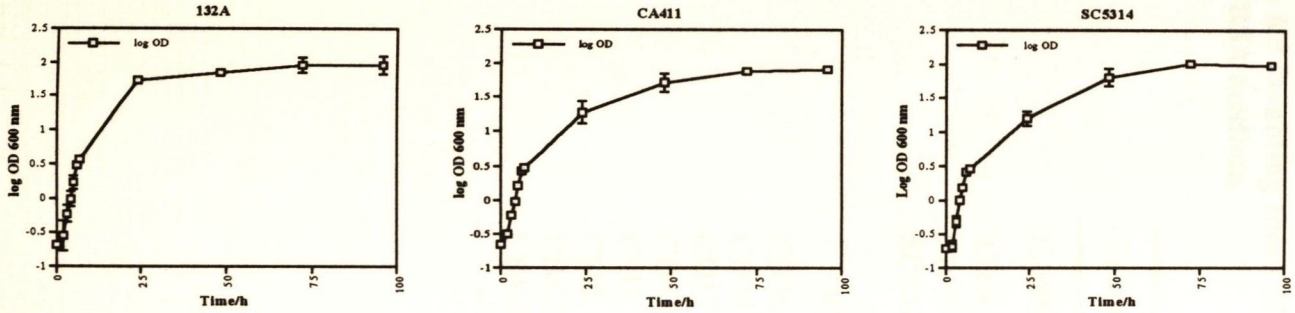
6.3 Results

6.3.1 Growth of *C. dubliniensis* in YCB/BSA medium

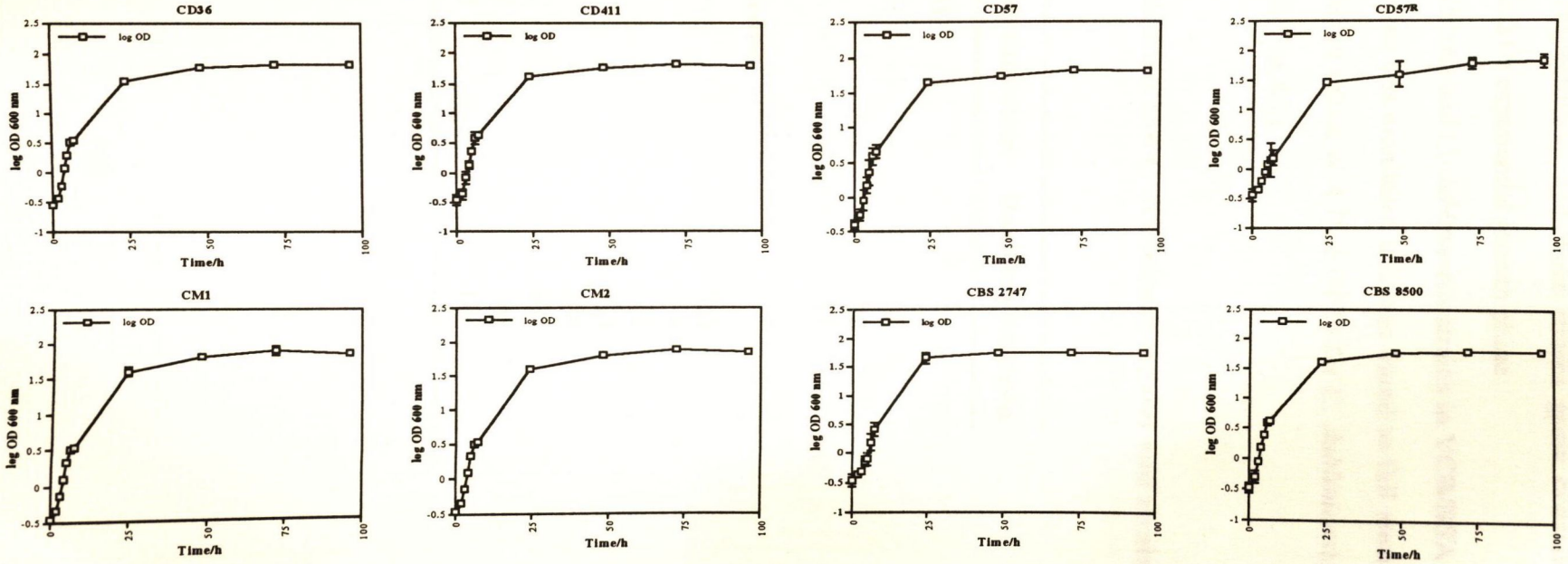
The expression of the *C. albicans SAP2* gene is induced by growth in the defined BSA containing medium YCB/BSA. The ability of *C. albicans* to express this gene in this medium allows it to degrade the BSA protein in the medium to provide it with the amino acids necessary for growth (Wright *et al.*, 1993; Hube *et al.*, 1994). The ability of *C. dubliniensis* to grow in this medium was investigated here in comparison with *C. albicans*. During the first 4 h of exponential growth (24 h) in YCB/BSA medium all the *C. dubliniensis* strains and isolates tested (Table 5.1) grew in the pseudohyphal form. *Candida dubliniensis* CD57^R and CBS 2747 grew in the pseudohypal form throughout the exponential phase of growth. By 24 h all of the *C. dubliniensis* strains and isolates exhibited yeast morphology. With the exception of *C. dubliniensis* isolates CM2 and CBS 2747 the *C. dubliniensis* isolates formed large oval shaped yeast cells. Isolates CM2 and CBS 2747 exhibited unusually shaped, small, elongated, cylindrical cells. This shape has also been observed with these isolates in cultures grown in YPD broth. The cells of all the *C. dubliniensis* strains tested were much smaller when grown in YCB/BSA than when grown in rich medium such as YPD broth. In contrast, all three *C. albicans* strains tested tended to form both yeast and pseudohyphal cells during the first 1 - 2 h of growth and thereafter reverted to the yeast form. Like *C. dubliniensis*, the *C. albicans* strains formed smaller yeast cells in YCB/BSA than when grown in YPD. The growth curves of the *C. dubliniensis* and *C. albicans* organisms listed in Table 5.1 in YCB/BSA medium are shown in Fig. 6.1 and the respective doubling times are shown in Table 6.2. The doubling times for both species in YCB/BSA were longer than the corresponding times in a rich medium such as YPD. The doubling time of *C. albicans* strains in YPD medium is on average 60 – 70 min and that for *C. dubliniensis* strains is approximately 80 – 90 min (data not shown). With the exception of *C. dubliniensis* CD57^R and CBS 2747, the doubling time in YCB/BSA medium for *C. dubliniensis* is on average only slightly longer than that for *C. albicans*. With the co-isolated strains (recovered from the same clinical specimen) CA411 and CD411, the *C.*

Figure 6.1 *In vitro* logarithmic growth curves of *C. albicans* and *C. dubliniensis* strains in YCB/BSA medium [1]

C. albicans



C. dubliniensis



[1] The *C. albicans* and *C. dubliniensis* isolates and strains are listed in Table 6.1. Aliquots of culture of each strain or isolate were withdrawn over 4 d and the OD_{600} of each sample determined. The OD_{600} values in the graphs represent the averages obtained from experiments repeated on 3 different occassions. The error bars are shown.

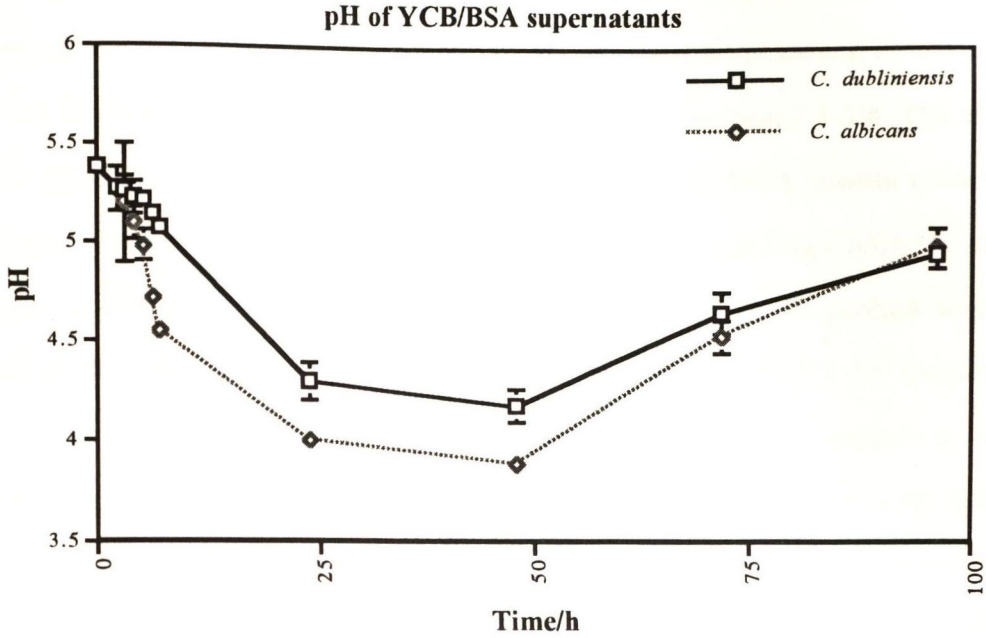
dubliniensis isolate had a doubling time in YCB/BSA (103 min) almost identical to that of the *C. albicans* isolate (104 min). *Candida dubliniensis* CD57^R and CBS 2747 had considerably longer doubling times in YCB/BSA medium than the other *C. dubliniensis* strains tested (12-22 min and 37-47 min longer, respectively), and these strains grew consistently in the pseudohyphal phase throughout the 24 h exponential growth phase.

During growth of the *C. albicans* and *C. dubliniensis* strains in YCB/BSA medium the pH of the supernatants of all cultures was monitored and was found to fall steadily from an initial average value of 5.4 to average values of 4.18 ± 0.089 for *C. dubliniensis* strains and 3.89 ± 0.050 for *C. albicans* strains (Fig. 6.2).

Table 6.2 Doubling times of *C. albicans* and *C. dubliniensis* isolates and strains grown in YCB/BSA medium

Candida strain/isolate	Doubling time/min
C. albicans	
132A	98
CA411	104
SC5314	93
C. dubliniensis	
CD36	103
CD411	103
CD57	111
CD57^R	123
CM1	109
CM2	101
CBS 2747	148
CBS 8500	105

Figure 6.2 pH curve of *C. albicans* and *C. dubliniensis* YCB/BSA cultures [1]



[1] The pH values of each curve represent the averages obtained for either *C. albicans* (calculated from the values obtained for each of the three strains from three separate experiments listed in Table 6.1) or *C. dubliniensis* (calculated from the values obtained for each of the eight strains of *C. dubliniensis* from three separate experiments listed in Table 6.1), with the error bars shown.

6.3.2 *In vitro* expression of *SAP2* in *C. dubliniensis*

In order to determine if the *in vitro* expression in YCB/BSA medium of the *C. dubliniensis SAP2* gene was similar to that exhibited by the *C. albicans SAP2* gene, Northern blot analysis of total cellular RNA extracted from the *C. dubliniensis* and *C. albicans* isolates and strains listed in Table 6.1 was performed as described in section 2.5.3/4. The expression of *CdSAP2* and *CaSAP2* genes was analysed using the purified DNA inserts contained within recombinant clones pCdS2 (encoding *CdSAP2*) and pCaS2 (encoding *CaSAP2*), respectively, as probes. In positive control experiments, *C. albicans* RNA was probed with a 762 bp fragment of the gene encoding translation elongation factor 3 (*TEF3*; Di Domenico *et al.*, 1992). Expression of the *TEF3* gene was used as a positive control because it is expressed under most growth conditions and yields a strong signal in Northern hybridisation experiments. The 762 bp fragment amplified from *CaTEF3* was not used for hybridisation to *C. dubliniensis* RNA as the level of sequence homology of *CaTEF3* to the *C. dubliniensis CaTEF3* homologue was unknown. Therefore, the TEF3F/R primer pair which was originally designed to amplify 762 bp of the *CaTEF3* gene was used to amplify a similarly sized fragment from *C. dubliniensis* genomic DNA. This fragment was cloned into pBluescript to yield the recombinant plasmid pCdT3. The cloned DNA insert from pCdT3 was sequenced fully in both directions and an alignment with the corresponding DNA amplimer from the *C. albicans TEF3* gene showed that the two nucleotide sequences are 95 % identical. It was deduced, therefore, that the cloned DNA insert contained within recombinant plasmid pCdT3 was from the putative *C. dubliniensis TEF3* gene (Fig. 6.3). The DNA insert contained within pCdT3 was excised and purified and used as a probe for *C. dubliniensis* RNA in positive control experiments.

Figure 6.3 Alignment of the 762 bp DNA amplimers from *CaTEF3* and the *C. dubliniensis CaTEF3* homologue [1]

```
C. albicans     ATTGGTCCAAATGGTGCTGGTAAATCCACCTTAATTAACGTTTTGACTGGTGAATTATTA
C. dubliniensis ATTGGTCCAAATGGTGCTGGTAAATCCACTTTAATCAATGTTTTAACTGGTGAATTATTG
                ****************************** ***** ** ***** **************

C. albicans     CCAACCACTGGTGAAGTTTACGTCCACGAAAATTGTCGTATTGCTTACATTAAACAACAT
C. dubliniensis CCAACCACTGGTGAAGTTTACGTCCACGAAAATTGTCGTATTGCTTACATTAAACAACAT
                ************************************************************

C. albicans     GCTTTTGCTCATATTGATAACCATTTGGACAAAACTCCATCTGAATATATTCAATGGAGA
C. dubliniensis GCTTTCGCTCATATTGATAACCATTTAGACAAAACTCCATCTGAATATATTCAATGGAGA
                ***** ******************** **********************************

C. albicans     TTCCAAACTGGTGAAGATAGAGAAACCATGGATAGAGCTTCTAGACAAATCAATGAAGAA
C. dubliniensis TTCCAAACTGGTGAAGATAGAGAAACCATGGATAGAGCTTCTAGACAAATCAATGAAGAA
                ************************************************************

C. albicans     GATGAACAAAACATGAACAAGATTTTCAAAATTGAAGGTACTCCAAGAAGAATTGCTGGC
C. dubliniensis GATGAACAGAACATGAACAAGATCTTTAAAGTTGAAGGTACTCCAAGAAGAATTGCTGGT
                ******** ************** ** *** ****************************

C. albicans     ATTCACGCCAGAAGAAAGTTCAAGAACTCTTATGAATATGAAATTTCTTGGATGTTGGGT
C. dubliniensis ATTCACGCCAGAAGAAAGTTCAAGAACTCTTATGAATATGAAATTTCCTGGATGTTGGGT
                ********************************************** ************

C. albicans     GAAAACATTGGTATGAAGAATGAAAGATGGGTACCAATGATGTCTGTTGACAACACTTGG
C. dubliniensis GAAAACGTTGGTATGAAGAATGAAAGATGGGTACCAATGATGTCTGTTGACAACACTTGG
                ****** ************************************************************

C. albicans     TTGCCAAGAGGTGAATTGATGGAAACTCACGCCAAGTTGGTTGCTGAAGTTGATATGAAA
C. dubliniensis TTACCAAGAGGTGAATTGATGGAAACTCACGCCAAATTGGTTGCTGAAGTTGATATGAAA
                ** ******************************** ************************

C. albicans     GAAGCTTTGGCTTCTGGTCAATTCAGACCATTAACCAGAAAAGAAATTGAAGAACATTGT
C. dubliniensis GAAGCTTTGGCTTCTGGTCAATTCAGACCATTAACCAGAAAGGAAATTGAAGAACATTGT
                ****************************************** ******************

C. albicans     GCTATGTTGGGTTTGGATGCCGAATTGGTTTCTCACTCTAGAATTAGAGGTTTATCTGGT
C. dubliniensis GCTATGTTGGGTTTGGATGCTGAATTAGTCTCCCATTCCAGAATCAGAGGTTTATCTGGT
                ********************* ***** ** ** ** ** ***** ***************

C. albicans     GGTCAAAAAGTTAAATTGGTCTTGGCTGCTTGTACTTGGCAAAGACCTCATTTGATTGTT
C. dubliniensis GGTCAAAGAGTCAAATTGGTCTTGGCTGCTTGTACTTGGCAAAGACCTCATTTGATTGTT
                ******* *** ************************************************

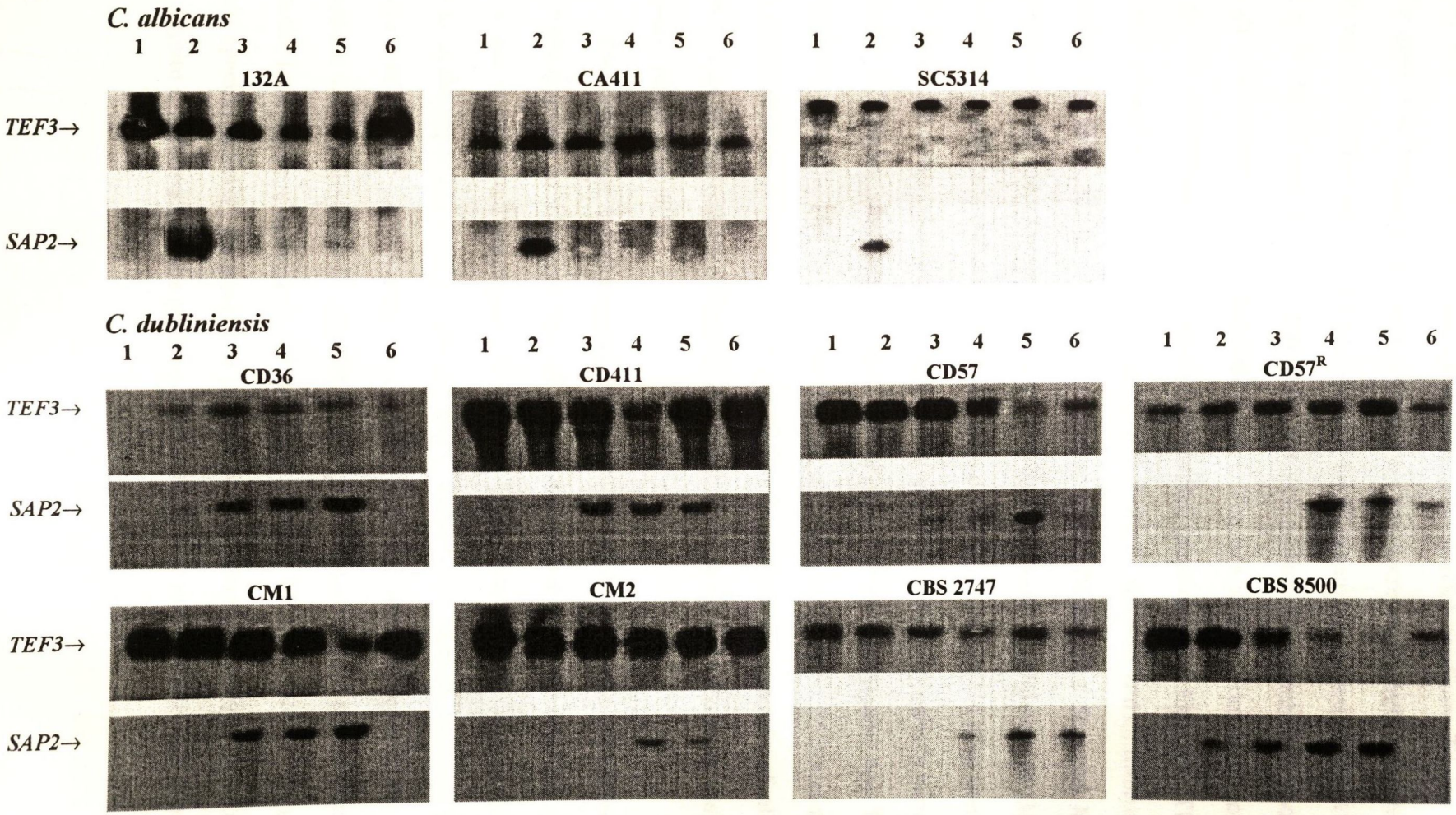
C. albicans     TTGGATGAACCAACCAATTATTTGGATAGAGATTCTTTGGGTGCTTTGTCTAAAGCTTTG
C. dubliniensis TTGGATGAACCAACCAATTATTTGGATAGAGGCTCTTTGGGTGCTTTATCTAAGGCTTTG
                ******************************  ************** ***** ******

C. albicans     AAAGCTTTCGAAGGTGGTATTGTTATCATTACTCACTCTGCT
C. dubliniensis AAAGCTTTCGAAGGGGGTATTGTTATCATTACTCACTCTGCT
                ************** ***************************
```

[1] Sequence alignment of the 762 bp DNA inserts contained within recombinant clones pCaT3 and pCdT3. Asterisks denote identical nucleotides and dashes denote dissimilar nucleotides.

In Northern hybridisation experiments a *SAP2* transcript of approximately 1.3 kb was produced by both *C. albicans* and *C. dubliniensis* strains during the early exponential phase (1-7 h) of growth in YCB/BSA medium (Fig. 6.4). No expression by either *C. albicans* or *C. dubliniensis* strains was detectable after 7 h growth (data not shown). However, expression of a *TEF3* transcript was detected at all stages for all *C. albicans* and *C. dubliniensis* isolates and strains tested. Expression of the *C. albicans SAP2* messenger RNA (mRNA) was detected in total RNA isolated from 3 h cultures for the 3 strains tested. No expression of this transcript was detected following 4, 5, 6, and 7 h incubation. In contrast, the expression of the *C. dubliniensis SAP2* gene varied from strain to strain. *Candida dubliniensis* CD411, CD57, and CM1 expressed *SAP2* mRNA following 4, 5, and 6 h growth in YCB/BSA medium. The signal reached a peak intensity at 6 h and had disappeared by 7 h. *Candida dubliniensis* CD36 and CBS 8500 produced *SAP2* mRNA following 3, 4, 5, and 6 h incubation. The signal also increased in intensity at 6 h but was not detectable at 7 h. The expression of *SAP2* transcripts following 3 h incubation by both strains was weak. *Candida dubliniensis* CD57^{R} and CBS 2747 exhibited similar patterns of *SAP2* expression, with a transcript detected at 5, 6 and 7 hours. Both strains appeared to produce the strongest signal at 5 h and thereafter the signal declined. *Candida dubliniensis* CM2 expressed *SAP2* mRNA following 5 and 6 h of growth, with the strongest expression at 5 h (Fig. 6.4).

Figure 6.4 Expression of the *SAP2* gene by *C. albicans* and *C. dubliniensis* detected by Northern hybridisation [1]



[1] The *C. albicans* and *C. dubliniensis* strains and isolates are listed in Table 6.1. For analysis of *CaSAP2* and *CdSAP2* expression the cloned amplimers from pCaS2 and pCdS2, respectively, were used as probes. The cloned amplimers from pCaT3 and pCdT3 were used to analyse *CaTEF3* and *CdTEF3* expression, respectively. Expression of *TEF3* was used as a postive control. Lanes 1-6 contain RNA prepared at 2, 3, 4, 5, 6, and 7 h, respectively.

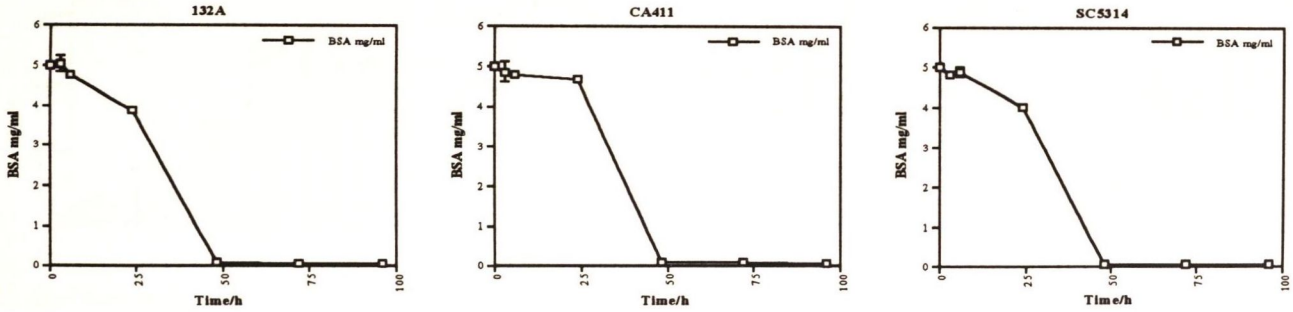
6.3.3 Proteinase activity of *C. albicans* and *C. dubliniensis* YCB/BSA culture supernatants

The *SAP2* gene was expressed by the *C. albicans* and *C. dubliniensis* isolates and strains tested during early exponential growth in YCB/BSA medium. To investigate if the *SAP2* gene was translated into active proteinase and secreted by both species a proteinase assay was performed on culture supernatants from all organisms grown in YCB/BSA medium at 3, 6, 24, 48, 72 and 96 h. Simultaneously, the total protein concentration of each supernatant was also determined using the Bradford method. The rate of BSA breakdown over four days the culture supernatants of each strain is presented graphically in Fig. 6.5. The proteinase enzymes present in the culture supernatants of both the *C. albicans* and *C. dubliniensis* strains degraded the BSA in the medium at similar rates. During the first 24 h of growth, corresponding to the exponential phase, there was a slow breakdown of BSA, with *C. dubliniensis* crude enzyme preparation degrading slightly more protein than that *C. albicans*. At 24 hours the mean BSA concentration in mg/ml in the *C. albicans* culture supernatants was 4.212 ± 0.432 mg/ml while that of the *C. dubliniensis* culture supernatants was 3.767 ± 0.135 mg/ml. This was followed by rapid breakdown of BSA over the next 24 hours to an average BSA concentration of 0.064 ± 0.02 mg/ml for the culture supernatants of all strains and isolates, with no appreciable difference between the two species. At this point the cells were in stationary phase (Fig. 6.5).

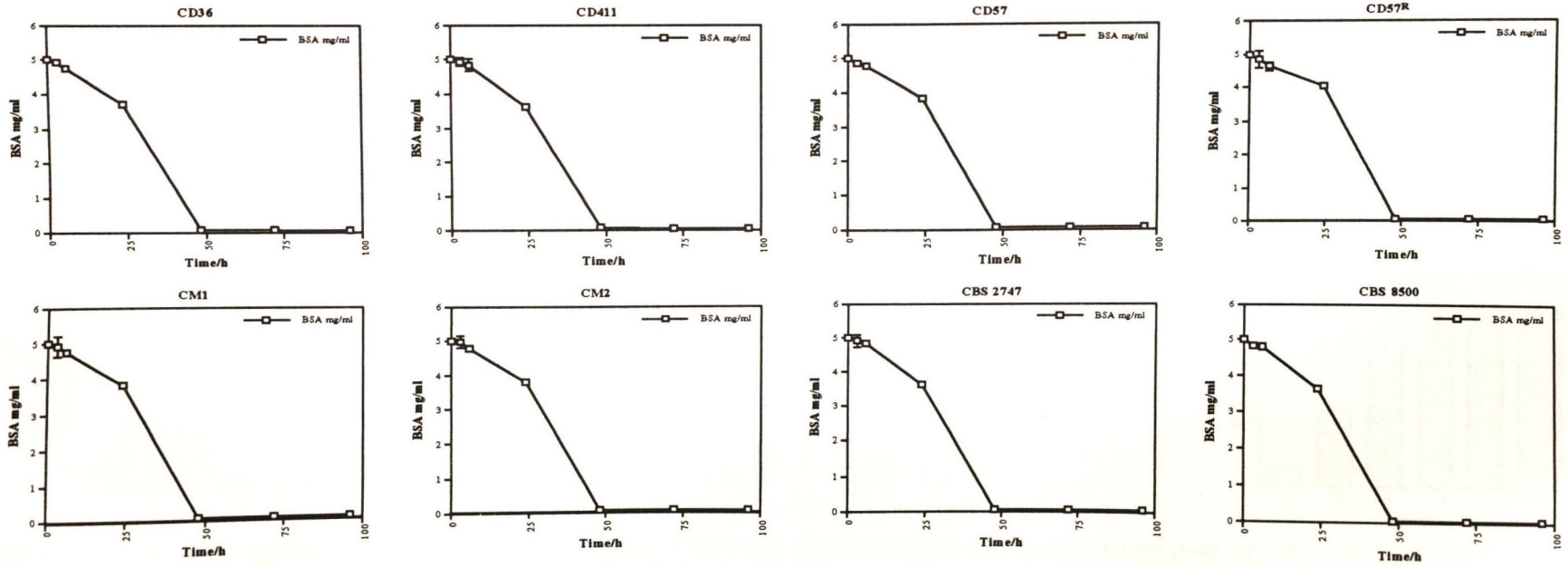
The proteinase activity in the crude enzyme preparations was calculated as the total enzymatic activity in units per litre of culture supernatant (U/l) at each time period for all strains and is presented graphically in Fig. 6.6. These data showed that the total proteinase activity in the supernatants of *C. albicans* and *C. dubliniensis* cultures increased until the peak activity was reached 48-72 h after inoculation. There was little difference in the level of total proteinase activity between *C. albicans* and *C. dubliniensis* culture supernatants. However, when the total enzyme activity in U/l was expressed per 10^8 c.f.u. (U/l/10^8 c.f.u.; referred to here as specific activity) the pattern of enzyme activity was different for the two species (Fig. 6.7). Figure 6.7 shows that the peak specific activity was reached after 6 h growth for all *C.*

Figure 6.5 Graphical representation of the breakdown of BSA in YCB/BSA medium by *C. albicans* and *C. dubliniensis* proteinase over 4 d [1]

C. albicans



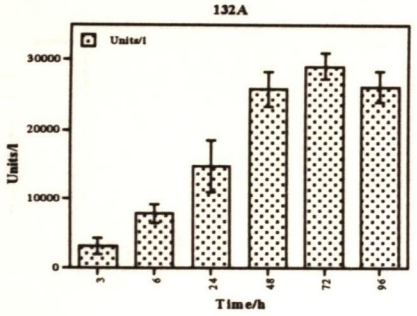
C. dubliniensis

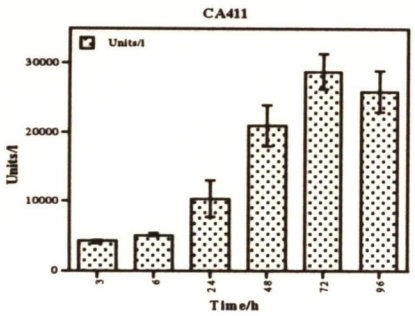


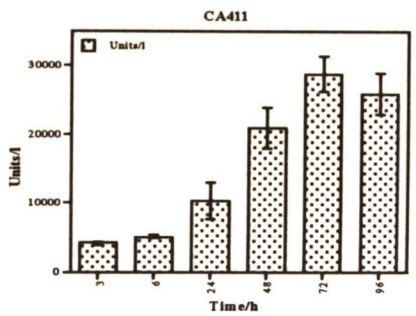
[1] The *C. albicans* and *C. dubliniensis* strains and isolates are listed in Table 6.1. The values BSA concentrations in mg/ml represent the average values calculated from the results obtained from 3 different experiments. The error bars are shown.

Figure 6.6 Total proteinase enzyme activity of *C. albicans* and *C. dubliniensis* YCB/BSA culture supernatants over 4 d [1]

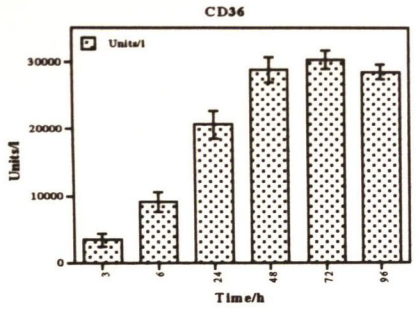
C. albicans

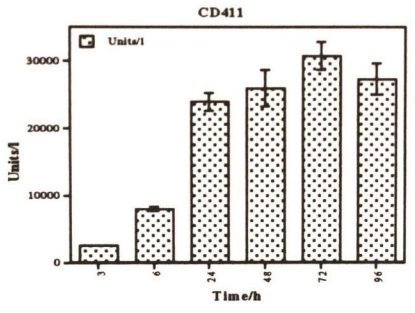


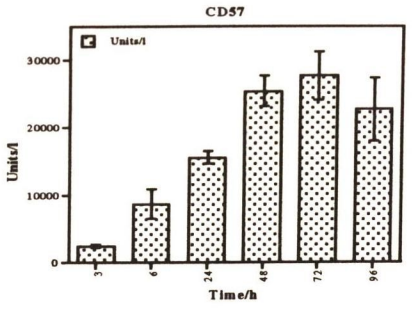


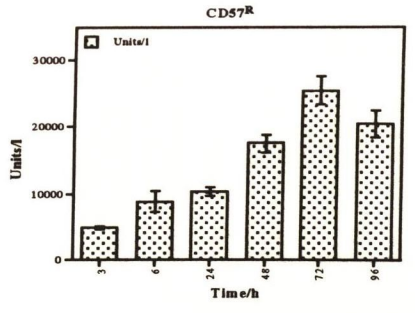


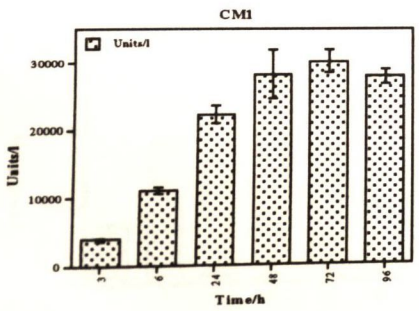
C. dubliniensis

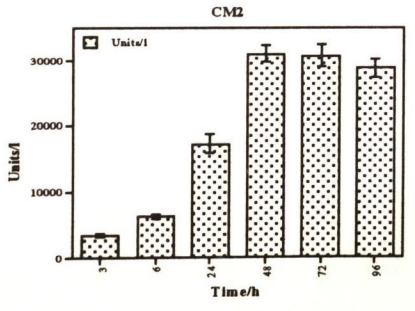


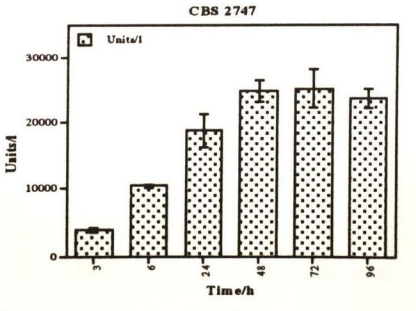


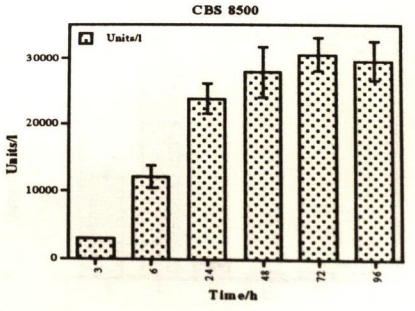








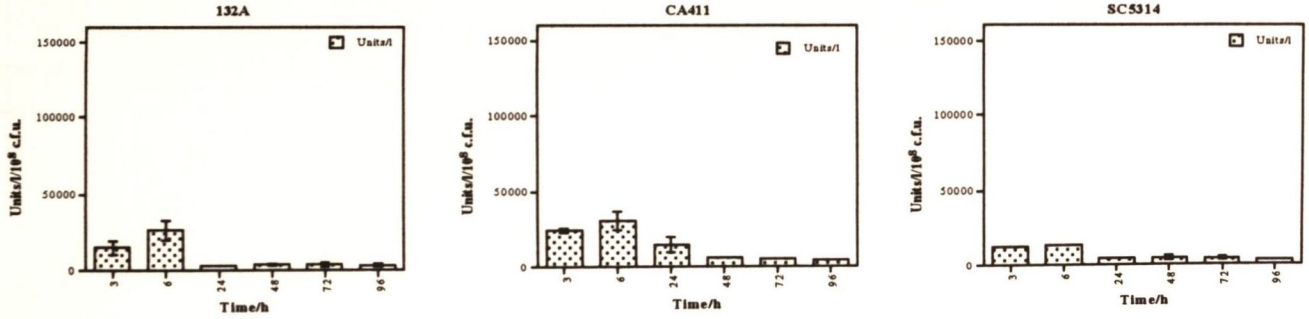




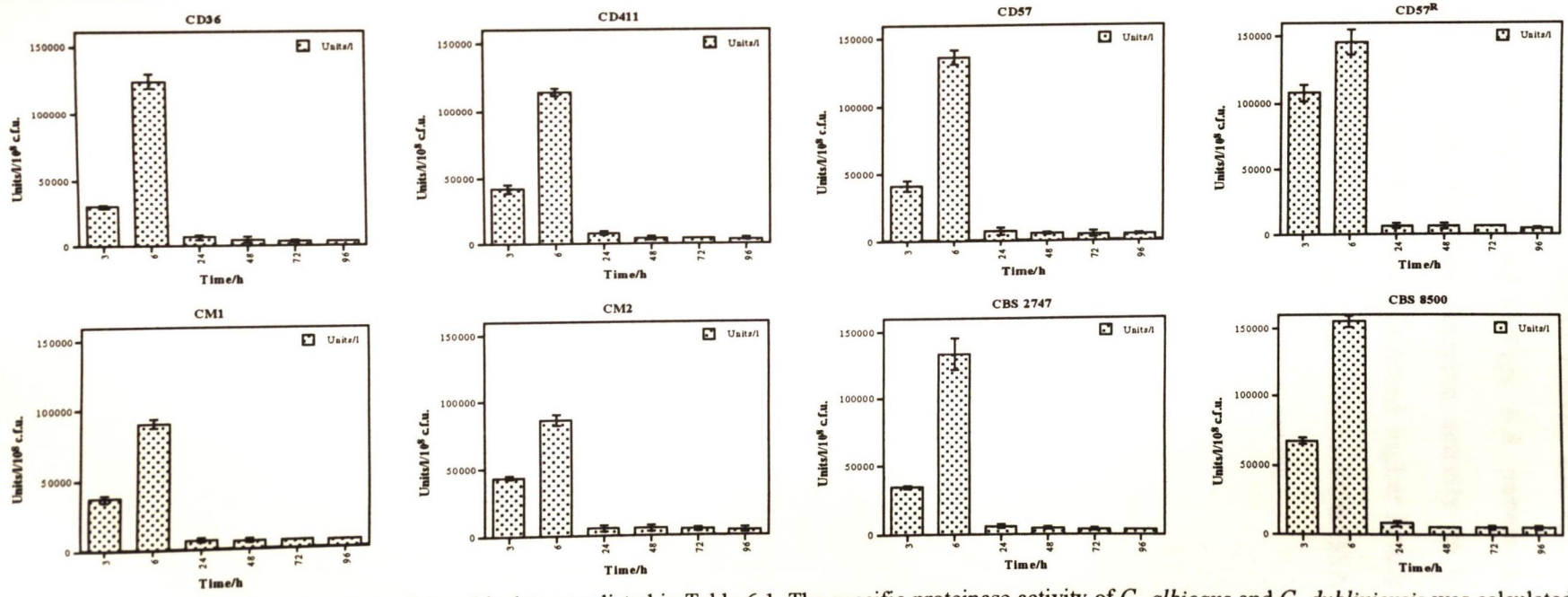
[1] The *C. albicans* and *C. dubliniensis* strains and isolates are listed in Table 6.1. The total enzyme activity at each time point for each strain represents the average of 3 experiments and is measure in units per litre. Error bars are shown.

Figure 6.7 Specific proteinase activity of *C. albicans* and *C. dubliniensis* YCB/BSA culture supernatants over 4 days [1]

C. albicans



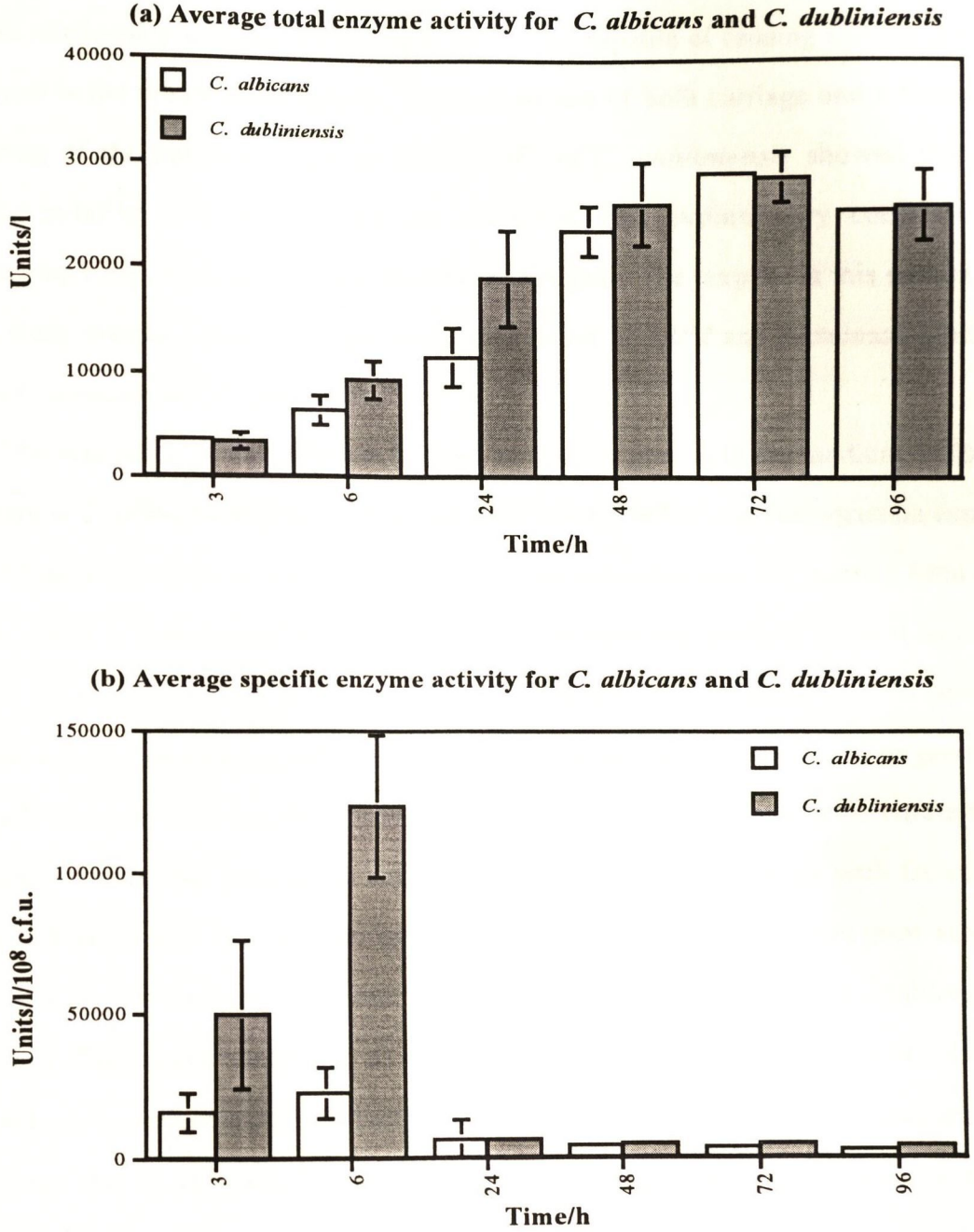
C. dubliniensis



[1] The *C. albicans* and *C. dubliniensis* strains and isolates are listed in Table 6.1. The specific proteinase activity of *C. albicans* and *C. dubliniensis* was calculated at each time point by dividing the total enzyme activity by the number of c.f.u. at that particular time point and is expressed as units per litre per 10^8 c.f.u.

albicans and *C. dubliniensis* cultures, after which the level of specific activity rapidly declined. Notably, the *C. dubliniensis* strains produced a higher level of specific activity than the *C. albicans* strains during the first 6 hours of exponential growth. A comparison of the average *C. albicans* and average *C. dubliniensis* total enzyme activity and specific enzyme activity at 3, 6, 24, 48, 72, and 96 hours is presented in Figs. 6.8. panels (a) and (b), respectively. These data show that although the total enzyme activity in the culture supernatants of both species was similar, *C. dubliniensis* produced higher levels of active secreted proteinase per 10^8 c.f.u. during the first 6 hours of growth in YCB/BSA medium. Thereafter, the average specific activity of both species fell to similar levels.

Figure 6.8 Graphical representation of (a) the average total enzyme activity and (b) specific enzyme activity in *C. albicans* and *C. dubliniensis* YCB/BSA culture supernatants
[1]



[1] The average total and specific activities for *C. albicans* and *C. dubliniensis* were calculated from the individual total and specific activities from the 3 *C. albicans* strains and the 8 *C. dubliniensis* strains, respectively. Error bars are shown.

6.4 Discussion

Candida dubliniensis is phylogenetically very closely related to *C. albicans*. Despite this close relationship and the fact that both species are capable of causing disease in humans *C. albicans* is the predominant species, in the incidence of both carriage and infection. The sequencing of the putative virulence factor *SAP2* of *C. dubliniensis* showed that genes encoding putative virulence factors are subject to less evolutionary constraint than housekeeping genes such as *ACT1* and the SSU rRNA gene. The purpose of this section of the present study was to compare the genotypic expression of *SAP2* and proteinase production between *C. albicans* and *C. dubliniensis*.

The expression of *CdSAP2* was induced *in vitro* by growth in the medium YCB/BSA. The ability of *C. albicans* to grow in this medium is due to the fact that the organism expresses the *SAP2* secreted aspartic proteinase gene. *SAP2* is translated into the inactive form of the enzyme, which is then cleaved and secreted into the surrounding medium where it hydrolyses the BSA to provide amino acids for growth of the organism. *Candida dubliniensis* also possesses a *SAP2* homologue, which exhibits a 10.4 % nucleotide sequence divergence from the *C. albicans* gene (this study, Chapter 5). The results presented here demonstrate that *C. dubliniensis* strains also possess the ability to grow in a minimal medium with the complex protein BSA as the sole source of nitrogen. However, *C. dubliniensis* does not grow as well as *C. albicans* in this medium, as determined by the observed differences in doubling times (Table 6.2). This is not surprising, as the slower growth of *C. dubliniensis* has also been observed in rich medium such YPD broth (data not shown). Kirkpatrick and colleagues (2000) have shown that *C. albicans* has a competitive advantage over *C. dubliniensis* in broth cultures; when standard inoculums of the two species are grown together *C. albicans* outgrows the *C. dubliniensis* and comes to dominate the mixed culture. In the present study, during growth in YCB/BSA the pH of the culture supernatants of both species dropped steadily during the first 48 h of growth, thereafter the pH rose again. This drop in pH has been observed previously for *C. albicans* isolates grown in BSA containing media (Hube *et al.*,

1994; Edison & Manning-Zweerink, 1988). The fall in pH observed for *C. albicans* was greater than that observed for *C. dubliniensis*. Other researchers have observed the greater acidogenic potential of *C. albicans* cultures in human saliva when compared to *C. parapsilosis* and *C. tropicalis* and that this correlates with higher proteinase activity (Wu *et al.*, 1999). However, the difference in acidogenic potential between *C. dubliniensis* and *C. albicans* is slight and may not be significant.

The *SAP2* gene is the predominant gene expressed by *C. albicans* in YCB/BSA medium and it is also expressed by *C. dubliniensis* during growth in this medium (Fig 6.4). Both species express this gene during early exponential growth, although the pattern of expression is somewhat different. *Candida albicans SAP2* transcripts were detected in RNA isolated after 3 h incubation only in YCB/BSA. Other researchers have observed this expression of *SAP2* during early exponential growth, although in some cases the duration of the expression was longer (Wright *et al.*, 1992; Hube *et al.*, 1994; White & Agabian, 1995). Wright *et al.* (1992) found that *C. albicans* ATCC 10261 expressed both *SAP2* and *SAP1* although *SAP2* expression was greater. The expression of *SAP2* transcript was detected at 2 h after the addition of BSA and up to 6 h later. Hube *et al.* (1994) found expression of the *SAP2* gene to occur over 2 hours. White & Agabian (1995) found that *SAP2* was expressed until stationary phase by three *C. albicans* strains including the white phase of strain WO-1. However, opaque cells expressed *SAP2* in the late logarithmic phase only. *Candida dubliniensis* expressed the *SAP2* gene over a longer period of time with expression being detected from 3-7 h after inoculation. Overall the gene was expressed over a period of 3 h, although isolates CD36 and CBS 8500 did express the gene an hour earlier than other *C. dubliniensis* strains. *Candida dubliniensis* CD57^{R} and CBS 2747 expressed the *SAP2* gene later than the other isolates tested, with the first transcripts being detected at 5 h. Interestingly, these two isolates had the longest doubling times in YCB/BSA medium. The fluconazole-resistant *C. dubliniensis* strains CM2 and CD57^{R} have been observed to exhibit a slower growth in other media (G. Moran, Ph.D. Thesis). However, isolate CM2 showed only weak expression of *SAP2* at 5 and 6 hours, but did not have a longer than average doubling time.

Furthermore, the blood isolate CBS 2747, which also exhibited a longer doubling, time (148 m) is a fluconazole-susceptible isolate. Overall, the duration of expression of *SAP2* by *C. dubliniensis* was longer than that by *C. albicans*.

Following expression of the *SAP2* gene in both species, proteinase activity could be detected in the supernatants of all strains tested. Western blotting experiments have shown that the Sap2 protein product is responsible for the proteinase activity observed in culture supernatants of *C. albicans* isolates (White *et al.*, 1993; White & Agabian, 1995; Colina *et al.*, 1996; Smolenski *et al.*, 1997). Therefore, it is possible that the *SAP2* transcript observed in *C. dubliniensis* isolates is translated into an active protein and was responsible for the enzymatic activity observed. However, this has not been confirmed by Western-blotting experiments. The total enzyme activity for all strains of both species reached a peak at 48-72 h, and began to decline after this. This pattern has been observed by Morrison *et al.* (1993) however, the length of time required to reach a peak activity (8 d) was much longer than that observed here. The proteinase activity continued to increase although the level of *SAP2* transcripts was decreasing. This may by due to the fact that the cultures were asynchronous and some cells may have been growing at a slower rate. There is also a possibility of a delay between expression of the *SAP2* gene and the secretion of an active mature Sap2 protein. It is interesting to note that *C. dubliniensis* isolates $CD57^R$ and CBS 2747, both of which have longer doubling times and later expression of *SAP2* mRNA, produced lower peak activities compared to the other strains of *C. dubliniensis* tested. A comparison of the average activities in culture supernatants of *C. albicans* and *C. dubliniensis* reveals that there was little difference in the total enzyme activity between the two species (Fig. 6.8a). However, when the enzyme activity of the *C. dubliniensis* and *C. albicans* culture supernatants is expressed per 10^8 c.f.u. (referred to as specific activity) considerable differences were observed in the specific activity between the two species (Fig 6.8b). Both species showed a peak specific activity at 6 h which had dramatically declined by 24 h. Similarly, this pattern of specific enzyme activity has been observed by other researchers, although the length of time required to reach a peak specific activity varied depending on the different proteinase induction

methods used (Ross *et al.*, 1990; Banerjee *et al.*, 1991; Wu *et al.*, 1999 and 2000). The higher level of specific proteinase activity for *C. dubliniensis* coincides with the finding that the culture supernatants of *C. dubliniensis* strains degraded the BSA in the medium faster than the corresponding culture supernatants of *C. albicans* strains during early exponential growth. The proteinase produced by both species degraded BSA at similar rates after 24 h when there was no significant difference between the specific activities of both species. This pattern appears to reflect the expression of the *SAP2* gene in early exponential growth of both organisms. The average specific activity of the *C. dubliniensis* is considerably higher than that of *C. albicans* and given the prolonged expression of *SAP2* in *C. dubliniensis* it would appear that higher specific activity of the enzyme is associated with the prolonged duration of *SAP2* expression. However, it is important to note that the association of higher specific proteinase activity with prolonged *SAP2* expression is speculative. Obviously other factors may be involved such as the possible expression of other *SAP* genes in this medium by *C. dubliniensis*, and that the duration of *SAP2* expression in *C. albicans* found here may not be representative of the species in general. However, the results of the present study supports the earlier work of McCullough *et al.* (1995) and Lischewski *et al.* (1999). They found that isolates of *C. dubliniensis*, had a significantly higher level of extracellular proteinase activity compared to *C. albicans*.

In conclusion, analysis of the genotypic expression of *SAP2* and proteinase production by *C. dubliniensis* has highlighted further differences between this organism and its close relative, *C. albicans*. Despite the fact that these two species are phenotypically very similar, there are considerable differences at the genetic level and indeed striking differences at the phenotypic level also. Furthermore, given the role of SAP2 in the role of adherence and colonisation of *C. albicans* to mucosal epithelium, the apparent elevated level of extracellular proteinase activity of *C. dubliniensis* warrants further investigation, to further elucidate its role in the pathogenicity of *C. dubliniensis*.

Chapter 7
General Discussion

7.1 General Discussion

Candida dubliniensis was first described as a new *Candida* species closely related to *C. albicans* in July 1995 (Sullivan *et al.*, 1995). In the past 5 years this species has been isolated by laboratories around the world where it is primarily associated with the oral cavities of HIV-infected individuals and AIDS patients (Boerlin *et al.*, 1995; McCullough *et al.*, 1995; Boucher *et al.*, 1996; Hannula *et al.*, 1997; Pujol *et al.*, 1997; Sullivan *et al.*, 1997; Bikandi *et al.*, 1998; Elie *et al.*, 1998; Kirpatrick *et al.*, 1998; Odds *et al.*, 1998; Rodero *et al.*, 1998; Salkin *et al.*, 1998; Velegraki *et al.*, 1998; Jabra-Rizk *et al.*, 1999; Joly *et al.*, 1999; McCullough *et al.*, 1999; Meis *et al.*, 1999; Polacheck *et al.*, 2000). The organism has been recovered from other immunocompromised groups and it has also been associated with insulin dependant diabetes mellitus and with the wearing of dentures (Coleman *et al.*, 1997b; Spencer *et al.*, 1999; Velgraki *et al.*, 1999; Brown *et al.*, 2000; Sano *et al.*, 2000; Willis *et al.*, 2000). Although *C. dubliniensis* has been found as an oral carriage organism it has also been implicated as a causative agent of oral candidosis (Coleman *et al.*, 1997; Velegraki *et al.*, 1999). *Candida dubliniensis* has been shown to develop resistance to the commonly used antifungal drug fluconazole upon exposure both *in vivo* and *in vitro* (Moran *et al.*, 1998; Ruhnke *et al.*, 2000). Although it is most frequently isolated from the oral cavities of HIV-infected populations the organism has been isolated from vaginal, urine, faecal, lung, sputum, wound and blood specimens in HIV-negative individuals (Sullivan *et al.*, 1995; Moran *et al.*, 1997; Odds *et al.*, 1998; Pinjon *et al.*, 1998; Meis *et al.*, 1999; Brandt *et al.*, 2000; Polacheck *et al.*, 2000; Kamei *et al.*, 2000). Despite its recent designation as a new species and its association with HIV-infected individuals, *C. dubliniensis* isolates misidentified as *C. albicans* or *C. stellatoidea* and isolated in 1952 and 1957 have been discovered in culture collections (Sullivan *et al.*, 1995; Meis *et al.*, 1999). The relatively low incidence (3-11.8 %) of recovery of oral *C. dubliniensis* from HIV-negative healthy individuals suggests that an effective, fully functional immune system suppresses the growth of *C. dubliniensis* in the oral cavity.

The aim of the first section of the present study was to confirm the phylogenetic position of *C. dubliniensis* relative to other *Candida* species within the genus *Candida* using

non-ribosomal sequence data. Originally, *C. dubliniensis* was described as a new species of *Candida* based upon analysis of the V3 variable region of the large subunit rRNA gene using a variety of *C. dubliniensis* isolates from diverse geographical locations (Sullivan *et al.*, 1995 and 1997). This was supported by the analysis of the entire coding sequence of the small subunit rRNA gene (Gilfillan *et al.*, 1998) and by analysis of the D1/D2 variable regions of the large subunit rRNA (Kurtzman *et al.*, 1997). The confirmation of the phylogenetic position of *C. dubliniensis* using a non-ribosomal sequence was critical as *Candida* taxonomy is inherently problematic and many species within the genus have been found to be synonyms of other established *Candida* species (Odds, 1988; Wickes *et al.*, 1992; Sullivan *et al.*, 1996). The majority of phylogenetic analyses are based upon rRNA data, although other genes have been used (Fitch & Margoliash, 1967; Woese *et al.*, 1987 and 1990; Gogarten *et al.*, 1989; Iwabe *et al.*, 1989; Pühler *et al.*, 1989; Hennessey *et al.*, 1993; Brown & Doolittle, 1997; Doolittle, 1999). Many phylogenetic trees have been constructed using actin sequences, mainly to confirm the evolutionary relationships inferred using rRNA data (Hightower & Meagher, 1986; Hennessy *et al.*, Fletcher *et al.*, 1994; Wery *et al.*, 1996). In general, the use of actin to infer phylogenetic relationships is particularly useful for the fungi as they have single copies of the gene, unlike higher eukaryotes, which may have many different isoforms of actin (Cox *et al.*, 1995).

As the phylogenetic position of *C. dubliniensis* had been previously established using rRNA data it was decided to confirm this using the actin (*ACT1*) gene. Analysis of the *ACT1* gene of *C. dubliniensis* revealed that it exhibited a sequence identity of 90.6 % with the *ACT1* gene of *C. albicans*. However, a comparison of the spliced coding sequences of both genes showed that the exons are 97.9 % identical at the nucleotide sequence level (Table 3.5). This nucleotide sequence identity is comparable to that exhibited by the V3 variable regions of the large subunit rRNA gene (97.52-97.75 %; Sullivan *et al.*, 1995 and 1997) and that of the small subunit rRNA genes (98.6 %; Gilfillan *et al.*, 1998) of both species. A phylogenetic tree constructed from the *ACT1* coding sequences of *C. dubliniensis* and a variety of other yeast species showed that *C. dubliniensis* was grouped separately from *C. albicans* and other yeast

species in 100 % of trees generated and it is most closely related to *C. albicans* (Fig. 3.8b). This data provided unequivocal confirmation of the phylogenetic position of *C. dubliniensis* as a discrete taxon within the genus *Candida* and it is the first phylogenetic analysis of this species based on non-ribosomal sequences. This confirmation is important given the many anomalies associated with *Candida* taxonomy (Odds, 1988; Sullivan *et al.*, 1996).

The fungal actin genes are unusual in that they are interrupted by introns (Gallwitz & Sures, 1980; Fidel *et al.*, 1988; Wildeman *et al.*, 1988; Deshler *et al.*, 1989; Losberger & Ernst, 1989; Fletcher *et al.*, 1994; Cox *et al.*, 1995; Matheucci *et al.*, 1995; Wery *et al.*, 1996). Analysis of the *ACT1* gene of *C. dubliniensis* revealed the presence of a group IV intron located at the 5' end of the gene interrupting the fourth codon. This intron location is conserved amongst the fungi. The *Candida ACT1*-associated introns all possessed intron consensus elements, nameingly the 5' and 3' consensus sequences and the branchpoint sequence (Mount *et al.*, 1982; Langford *et al.*, 1984; Leer *et al.*, 1984; Molenaar *et al.*, 1984; Teem *et al.*, 1984). An analysis of the intron sequences from *C. albicans* and *C. dubliniensis* revealed that they exhibit considerable sequence divergence (16.6 %; Table 3.4). However, this was less than the intron sequence divergence observed between *C. albicans* and *C. tropicalis* (43.4 %; Table 3.4), indicating that *C. dubliniensis* is more closely related to *C. albicans* than *C. tropicalis*. Analysis of the *C. albicans* and *C. stellatoidea ACT1*-associated intron sequences also revealed that they are so closely related as to be considered a single species (Kamiyama *et al.*, 1989; Sullivan *et al.*, 1995; Boucher *et al.*, 1996). Despite the observed significant divergence in the *ACT1*-associated intron sequences of *C. albicans* and *C. dubliniensis* an analysis of these introns from geographically and epidemiologically unrelated *C. dubliniensis* isolates revealed that the intron sequences were very highly conserved (Fig 3.5). A similar level of intraspecies sequence conservation was found with the *C. albicans* and *C. stellatoidea* isolates examined (Figs. 3.6 and 3.7). Similar intraspecies conservation has been observed by others in the group I self-splicing intron in the large subunit rRNA gene of these three species (Boucher *et al.*, 1996). The divergence observed between the *C. dubliniensis* and *C. albicans ACT1* associated introns is considerably larger than the

divergence observed between the corresponding *ACT1* coding sequences. This is not surprising as there are considerable evolutionary restraints place upon the coding sequence. Since the intron is spliced out from the *ACT1* mRNA it is not subject to these same restraints and it is possible that mutations may accumulate without any deleterious affect on the actin protein. However, despite the considerable sequence divergence observed between these two introns, these two sequences are most closely related to each other when compared with introns from other yeast species (Table 3.4 and Fig. 3.8a).

The *ACT1* sequence data provides an interesting insight into the time frame over which *C. dubliniensis* evolved as a species. The nucleotide and amino acid sequence data indicate that *C. dubliniensis* diverged from *C. albicans* over a time frame of 30,000 to 241,000 years ago (Table 3.6). Given that the fungi diverged from either the plant or animal group about 1275 million years ago, the evolution of *C. dubliniensis* occurred relatively recently. This recent evolution reflects the close relationship that exists between *C. dubliniensis* and *C. albicans*.

The development of a rapid and definitive test for the identification of *C. dubliniensis* has proved to be problematic. The "gold standard" methods are the molecular methods such as nucleotide sequencing of rRNA subunit gene fragments, DNA fingerprinting, RAPD and RFLP analysis which are capable of detecting the considerable genetic differences between these two species (Boerlin *et al.*, 1995; McCullough *et al.*, 1995; Sullivan *et al.*, 1995; Coleman *et al.*, 1997; Schoofs *et al.*, 1997; Sullivan *et al.*, 1997; Kirkpatrick *et al.*, 1998; Odds *et al.*, 1998; Joly *et al.*, 1999; McCullough *et al.*, 1999). Although a number of phenotypic tests have been described many of these have been proven to be unreliable (see Chapter 1).

One of the aims of this project was to develop a rapid and reliable test for the definitive identification of *C. dubliniensis* using the PCR technique. The PCR technique has the advantage that it is applicable to the detection of genetic differences and it is also rapid and relatively inexpensive. The low level of intraspecies sequence variation in the *C. dubliniensis* *ACT1*-associated intron, and the extent of divergence from the *C. albicans* *ACT1*-associated intron facilitated the design of oligonucleotide primers capable of readily discriminating

between isolates of both species using PCR. The inclusion of a rapid method of template preparation from colonies on CHROMagar plates allowed the identification of a suspect *C. dubliniensis* colony in as little as 4 h. The *C. dubliniensis*-specific primers were extensively evaluated in a blind trial with 196 isolates of 11 yeast species (Table 4.2) and was found to be 100 % specific. This method is simple, rapid and reliable and has been used successfully in several recent studies to identify *C. dubliniensis* isolates, including isolates originally misidentified as *C. albicans* (Pincus *et al.*, 1999; Polacheck *et al.*, 2000; Al Mosaid *et al.*, 2000)

The PCR identification systems described elsewhere for discriminating between *C. dubliniensis* and *C. albicans* isolates (Elie *et al.*, 1998; Mannarelli & Kurtzman; 1998; Kurzai *et al.*, 1999; Martin *et al.*, 2000; Tamura *et al.*, 2000) have not been thoroughly evaluated, as only a small number of *C. dubliniensis* isolates were examined in each case. Furthermore, the methods of Elie *et al.* (1998) and Martin *et al.* (2000), PCR ELISA and PCR Line Probe assay, respectively, are not likely to be available in routine diagnostic laboratories. In contrast, the PCR identification technique for *C. dubliniensis* developed in the present study is based upon well-characterised genetic differences, and has been thoroughly evaluated using a large number of isolates and in several studies (Donnelly *et al.*, 1999; Pincus *et al.*, 1999; Polacheck *et al.*, 2000; Al Mosaid *et al.*, 2000). This method when used in conjunction with primary screening on CHROMagar Candida is faster and cheaper than the more routinely used primary isolation on CHROMagar medium followed by identification with commercially available yeast identification systems based on substrate assimilation profiles.

Since 1995 much of the literature pertaining to *C. dubliniensis* has been concerned with development of rapid and dependable identification methods and with the epidemiology of this species. To date, there has been very little research into the virulence of this organism (Gilfillan *et al.*, 1998; Hazen & Masuoka, 2000; Peltroche-Llacsahuanga, 2000). The final aim of this project was to characterise differences between *C. dubliniensis* and *C. albicans* using a gene encoding a putative virulence factor. The *C. dubliniensis SAP2* gene was chosen as there is much evidence to implicate the *SAP2* gene as a virulence factor in *C. albicans* and there is a

substantial body of research on its role in disease both *in vivo* and *in vitro* (discussed in chapter 6). *Candida dubliniensis* has been shown to possess homologues of the *C. albicans SAP1-7* genes (Gilfillan *et al.*, 1998). Furthermore, there have been reports that it produces significantly more aspartic proteinase activity than *C. albicans* (McCullough *et al.*, 1995; Lischewski *et al.*, 1999).

The cloning and sequencing of the *C. dubliniensis SAP2* gene presented here represents the first member of this multigene family to be sequenced in *C. dubliniensis*. The *C. dubliniensis SAP2* gene exhibits a 10.4 % nucleotide sequence divergence from its *C. albicans* homologue (Table 5.5). A comparison of the various *Candida SAP2* gene coding sequences shows that the *SAP2* gene of *C. dubliniensis* is most closely related to *C. albicans*, followed by the *SAP* genes of *C. tropicalis* (34.2 % divergence) and *C. parapsilosis* (43.4 % divergence; Table 5.5). The sequence of the *SAP2* gene provides further insight into the relationships of *C. dubliniensis* with other members of the genus *Candida*. As expected, the *SAP* sequence analysis for *C. albicans*, *C. dubliniensis*, *C. tropicalis* and *C. parapsilosis* showed that *C. dubliniensis* is most closely related to *C. albicans*, followed by *C. tropicalis* and *C. parapsilosis*.

The level of sequence divergence observed between the *C. dubliniensis SAP2* gene and its *C. albicans* homologue is similar to that observed in other genes from the two species including genes such as *MDR1* (8.0 %), *PHR1* (9.8 %) and *PHR2* (8.8 %) (Table 5.4). Genes such as *ACT1* and the small subunit rRNA (SSU rRNA) gene show a much more conserved nucleotide sequence. However, these latter genes are evolutionarily conserved throughout the eukaryotic kingdom due to the fundamental functions they perform within the cell. It is interesting to note that genes such as *CdSAP2*, *CdMDR1*, *CdPHR1* and *CdPHR2*, which may be more affected by environmental selective pressures than *ACT1* or SSU rRNA, all exhibit similar sequence divergence levels from their corresponding *C. albicans* homologues.

The *C. dubliniensis* Sap2 predicted protein is 93.9 % identical to CaSAP2. However, the differences observed in the amino acid sequence do not appear to have any impact upon

the predicted tertiary structure of the protein, as residues important for three-dimensional structure, activation of the zymogen and catalytic activity are conserved.

Although the nucleotide sequence and predicted protein sequence of the *C. dubliniensis SAP2* gene were conserved relative to the *C. albicans SAP2* gene significant differences were observed in the genotypic expression of the two genes *in vitro*. It should be noted that although the phenotypic expression of *SAP2* in *C. dubliniensis* was not confirmed by Western blotting experiments, it was assumed that the *SAP2* transcript expressed by *C. dubliniensis* gave rise to an active secreted protein. The expression of the *SAP2* gene was induced in *C. albicans* and *C. dubliniensis* by growth in the induction medium YCB/BSA. The expression of the *SAP2* gene was detected later in *C. dubliniensis* than in *C. albicans*, however, the duration of the *SAP2* transcript was considerably longer in *C. dubliniensis* than in *C. albicans* (Fig. 6.4). This later expression of *SAP2* in *C. dubliniensis* may be a reflection of the slower growth rate of this species in YCB/BSA medium. Coinciding with this expression of *SAP2* transcripts in *C. albicans* and *C. dubliniensis* was the phenotypic expression of proteinase enzyme in the culture supernatants of both species. Although the total amount of enzyme units was similar for all *C. albicans* and *C. dubliniensis* isolates and strains tested (Fig 6.6), significant differences were observed in the specific enzyme activity (total activity per 10^8 c.f.u.; Fig 6.7). Both species secreted active proteinase from 3 h post inoculation, with a peak in specific activity at 6 hours observed in all strains, which rapidly declined by 24 hours. However, the average specific activity of *C. dubliniensis* was much higher than that of *C. albicans* (Fig 6.8). This higher level of proteinase activity in *C. dubliniensis* culture supernatants was also reflected by the faster rate of breakdown of BSA in the medium in the first 24 h by this species (Fig 6.5).

In summary the prolonged expression of *SAP2* transcript in *C. dubliniensis* may be associated with a higher production of secreted aspartic proteinase. However, *C. dubliniensis* strains did not show a faster doubling time in YCB/BSA medium. This is not surprising as *C. dubliniensis* strains, in general, have slower doubling times than *C. albicans* strains in other media such as YPD. Undoubtedly, the rate of growth of *C. albicans* in YCB/BSA is

contingent upon factors other than the production of aspartic proteinase. The *C. dubliniensis* strains CD57^R and CBS 2747 exhibited longer doubling times and also later expression of *SAP2* transcript. It is possible that their later *SAP2* expression may result in a slower growth. However, it should be not that CD57^R exhibits longer doubling times in YPD than its fluconazole-sensitive parent CD57 (Moran *et al.*, 1997 and 1998).

It is interesting to speculate why *C. dubliniensis* produces a higher level of aspartic proteinase than *C. albicans*. It is possibly an adaptation to living in the oral cavity, enabling it to better withstand the competitive pressures from *C. albicans* as this enzyme has a possible role in adhesion to the mucosal epithelium. Increased expression of SAP2 by *C. dubliniensis* could be involved in the increased adherence of this species to epithelial cells that has been observed by some researchers (McCullough *et al.*, 1995; Gilfillan *et al.*, 1995). One could also speculate that the increased level of aspartic proteinase observed in the early exponential phase is necessary to fulfill particular metabolic requirements of *C. dubliniensis*. However, the elevated expression and secretion of aspartic proteinase in *C. dubliniensis* needs to be investigated *in vivo* before any inferences can be made as to the role this enzyme plays in *C. dubliniensis*.

7.2 Future perspectives

The work presented in this study provides further evidence about the genetic differences between *C. dubliniensis* and *C. albicans*. The *ACT1* sequence analysis has provided confirmation of the phylogenetic position of *C. dubliniensis* within the genus and the *ACT1*-associated intron sequence has provided the basis for a rapid and reliable method of differentiating this novel species from *C. albicans*. A reliable method for the definitive identification of *C. dubliniensis* is essential if the epidemiology of this organism is to be elucidated. The analysis of the expression of *SAP2* and proteinase production in *C. dubliniensis* indicates there are significant differences in the expression of this putative virulence factor gene by *C. dubliniensis* and *C. albicans*. A more detailed study of the *in vitro*

and *in vivo* expression and activity of the *SAP* multigene family in *C. dubliniensis* should be carried out. Furthermore, the expression of this family in *C. dubliniensis* should be investigated in an *in vivo* model of oral candidosis, given this organism's apparent adaptation to the oral cavity of HIV-infected individuals and AIDS patients. Further investigations may provide valuable insights concerning why *C. dubliniensis* has only recently emerged as a human pathogen and its role in oral disease in HIV-infected individuals and AIDS patients.

References

Abad-Zapatero, C., Goldman, R., Muchmore, S. W., Hutchins, C., Stewart, K., Navaza, J., Payne, C. D., Ray, T. L. (1996). Structure of a secreted aspartic protease from *Candida albicans* complexed with a potent inhibitor: implications for the design of antifungal agents. *Protein Sci* **5**, 640-652.

Abad-Zapatero, C., Goldman, R., Muchmore, S. W., Hutchins, C., Oie, T., Stewart, K., Cutfield, S. M., Cutfield, J. F., Foundling S. I., Ray, T. L. (1998). Structure of secreted aspartic proteinases from *Candida*. Implication for the design of antifungal agents. *Adv Exp Med Biol* **436**, 297-313.

Agatensi, L., Franchi, F., Mondello, F., Bevilacqua, R. L., Ceddia, T., De Bernardis, F., Cassone, A. (1991). Vaginopathic and proteolytic *Candida* species in outpatients attending a gynaecology clinic. *J Clin Pathol* **44**, 826-830.

Albertson, G. D., Niimi, M., Cannon, D., Jenkinson, H. F. (1996). Multiple efflux mechanisms are involved in *Candida albicans* fluconazole resistance. *Anitmicrob Agents Chemother* **40**, 2835-2841.

Al-Mosaid, A., Sullivan, D., Salkin, I. F., Shanley, D., Coleman, D. C. (2000). Differentiation of *Candida dubliniensis* from *Candida albicans* on Staib agar and caffeic acid-ferric citrate agar. *J Clin Microbiol* (in press).

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403-410.

Anthony, R. M., Midgley, J., Sweet, S. P., Howell, S. A. (1995). Multiple strains of *Candida albicans* in the oral cavity of HIV positive and HIV negative patients. *Microbial Ecol Health Dis* **8**, 23-30.

Antley, P. P., & Hazen, K. C. (1988). Role of yeast cell growth temperature on *Candida albicans* virulence in mice. *Infect Immun* **56**, 2884-2890.

Asakura, K., Iwaguchi, S.-I., Homma, M. (1991). Electrophoretic karyotypes of clinically isolated yeasts of *Candida albicans* and *C. glabrata*. *J Gen Microbiol* **137**, 2531-2538.

Bagavathi, S. & Malathi, R. (1996). Introns and protein revolution – an analysis of the exon/intron organisation of actin genes. *FEBS Lett* **392**, 63-65.

Banerjee, A., Ganesan, K., Datta, A. (1991). Induction of secretory acid proteinase in *Candida albicans*. *J Gen Microbiol* **137**, 2455-2461.

Barns, S. M., Lane, D. J., Sogin, M. L., Bibeau, C., Weisburg, W. G. (1991). Evolutionary relationships among pathogenic *Candida* species and relatives. *J Bacteriol* **173**, 2250-2255.

Beauséjour, A., Grenier, D., Goulet, U.-P. Deslauriers, N. (1998). Proteolytic activation of the interleukin-1β precursor by *Candida albicans*. *Infect Immun* **66**, 676-681.

Beck-Sague, C. & Jarvis, W. R. (1993). Secular trends in the epidemiology of nosocomial fungal infections in the United States, 1980-1990: National Nosocomial Infections Surveillance System. *J Infect Dis* **167**, 1247-1251.

Bikandi, J., San Millán, R., Moragues, M. D., Cebas, G., Clarke, M., Coleman, D. C., Sullivan, D. J., Quindós, G., Pontón, J. (1998). Rapid identification of *Candida dubliniensis* by indirect immunofluorescence based on differential localization of antigens on *C. dubliniensis* blastospores and *Candida albicans* germ tubes. *J Clin Microbiol* **36**, 2428-2433.

Blum, J. S., Fiani, M. L., Stahl, P. D. (1991). In *Aspartic Proteinases and their Inhibitors*, pp 203-220. Edited by V. Kostka, Walter de Gruyter & Co., Hawthorne, NY.

Bodey, G. P. (1988). The emergence of fungi as major pathogens. *J Hosp Infect* **11**, 411-426.

Boerlin, P., Boerlin-Petzold, F., Durussel, C., Addo, M., Pagani, J., Chave, J., Bille, J. (1995). Cluster of oral atypical *Candida albicans* isolates in a group of human immunodeficiency virus-positive drug users. *J Clin Microbiol* **33**, 1129-1135.

Borg, M. & Rüchel, R. (1988). Expression of extracellular acid proteinase by proteolytic *Candida spp.* during experimental infection of oral mucosa. *Infect Immun* **56**, 626-631.

Borg-von Zepelin, M., Beggah, S., Boggian, K., Sanglard, D., Monod, M. (1998). The expression of the secreted aspartyl proteinases Sap4 to Sap6 from *Candida albicans* in murine macrophages. *Mol Microbiol* **28**, 543-554.

Borg-von Zepelin, M., Meyer, I., Thomssen, R., Wurzner, R., Sanglard, D., Telenti, A., Monod, M. (1999). HIV-Protease inhibitors reduce cell adherence of *Candida albicans* strains by inhibition of yeast secreted aspartic proteases. *J Invest Dermatol* **113**, 747-751.

Boschman, C. R., Bodnar, U. R., Tornatore, M. A., Obias, A. A., Noskin, G. A., et al. (1998). Thirteen-year evolution of azole resistance in yeast isolates and prevalence of resistant strains carried by cancer patients at a large medical center. *Antimicrob agents Chemother* **42**, 734-738.

Boucher, H., Mecure, S., Montplaisir, S., Lemay., G. (1996). A novel group I intron in *Candida dubliniensis* is homologous to a *Candida albicans* intron. *Gene* **180**, 189-196.

Brandt, M. E., Harrison, L. H., Pass, M., Sofair, A. N., Huie, S., Li, R. K., Morrison, C. J., Warnock, D. W., Hajjeh, R. A. (2000). *Candida dubliniensis* fungemia: the first four cased in North America. *Emerg Infect Dis* **6**, 46-49.

Brown, J. R. & Doolittle, W. F. (1997). *Archaea* and the prokaryote-to-eukaryote transition. *Microbiol Mol Biol Rev* **61**, 456-502.

Brown, D. M., Jabra-Rizk, M. A., Falkler Jr., W. A., Baqui, A. A., Meiller, T. F. (2000). Identification of *Candida dubliniensis* in a study of HIV-seropositive pediatric dental patients. *Pediatr Dent* **22**, 234-238.

Coleman, D. C., Bennett, D. E., Sullivan, D. J., Gallagher, P. J., Henman, M. C., Shanley, D. B., Russell, R. J. (1993). Oral *Candida* in HIV infection and AIDS: new perspectives/new approaches. *Crit Rev Microbiol* **19**, 61-82.

Coleman, D. C., Bennett, D. E., Gallagher, P. J. et al. (1995). Oral candidiasis and HIV infection; antifungal drug resistance and changes in *Candida* population dynamics. In: Greenspan J. S., Greenspan D., eds. *Oral manifestations of HIV infection.* Quintessence Publishing Co., Inc: Chicago, 112-118.

Coleman, D., Sullivan, D., Harrington, B., Haynes, K., Henman, M., Shanley, D., Bennett, D., Moran, G., McCreary, C., O'Neill, L. (1997a). Molecular and phenotypic analysis of *Candida dubliniensis*: a recently identified species linked with oral candidosis in HIV-infected and AIDS patients. *Oral Dis* **3**, S106-S101.

Coleman, D. C., Sullivan, D. J., Bennett, D. E., Moran, G. P., Barry, H. J., Shanley, D. B. (1997b). Candidiasis: the emergence of a novel species *Candida dubliniensis*. AIDS 11, 557-567.

Coleman, D. C., Rinaldi, M. G., Haynes, K. A., Rex, J. H., Summerbell, R. C., Anaissie, E. J., Li, A., Sullivan, D. J. (1998). Importance of *Candida* species other than *Candida albicans* as opportunistic pathogens. *Med Mycol* **36**, 156-165.

Colina, A. R., Aumont, F., Deslauriers, N., Belhumeur, P., de Repentigny, L. (1996). Evidence for degradation of gastrointestinal mucin by *Candida albicans* secretory aspartyl proteinase. *Infect Immun* **64**, 4514-4519.

Cox, G. M., Rude, T. H., Dykstra, C. C., Perfect, J. R. (1995). The actin gene from *Cryptococcus neoformans*: structure and phylogenetic analysis. *J Med Vet Mycol* **33**, 261-266.

Crandall, M. & Edwards Jr., J. E. (1987). Segregation of proteinase-negative mutants from heterozygous *Candida albicans*. *J Gen Microbiol* **133**, 2817-2824.

Cushion, M., Ruffolo, J., Walzer, P. (1988). Analysis of the developmenal stages of *Pneumocystis carinii in vitro*. *Lab Invest* **58**, 324-331.

Cutfield, S., Marshall, C., Moody, P., Sullivan, P., Cutfield, J. (1993). Crystallization of inhibited aspartic proteinase from *Candida albicans*. *J Mol Biol* **234**, 1266-1269.

Cutfield, S. M., Dodson, E. J., Anderson, B. F., Moody, P. C., Marshall, C. J., Sullivan, P. A., Cutfield J. F. (1995). The crystal structure of a major secreted aspartic proteinase from *Candida albicans* in complexes with two inhibitors. *Structure* **3**, 1261-1271.

Cutler, J. E. (1991). Putative virulence factors of *Candida albicans*. *Annu Rev Microbiol* **45**, 187-218.

Davies, D. R. (1990). The structure and function of the aspartic proteinases. *Annu Rev Biophys Biophys Chem* **19**, 189-215.

De Bernardis, F., Agatensi, L., Ross, I. K., Emerson, G. W., Lorenzini, R., Sullivan, P. A., Cassone, A. (1990). Evidence for a role for secreted aspartate proteinase of *C. albicans* in vulvovaginal candidiasis. *J Infect Dis* **161**, 1276-1283.

De Bernardis, F., Cassone, A., Sturtevant, J., Calderone, R. (1995). Expression of *Candida albicans SAP1* and *SAP2* in experimental vaginitis. *Infect Immun* **63**, 1887-1892.

De Bernardis, F., Chiani, P., Ciccozzi, M., Pellegrini, G., Ceddia, T., D'Offizzi, G., Quinti, I., Sullivan, P. A., Cassone, A. (1996). Elevated aspartic proteinase secretion and experimental pathogenicity of *Candida albicans* isolates from oral cavities of subjects infected with human immunodeficiency virus. *Infect Immun* **64**, 466-471.

De Bernardis, F., Boccanera, M., Adriani, D., Spreghini, E., Santoni, G., Cassone, A. (1997). Proctective role of anitmannan and anit-aspartyl proteinase antibodies in an experimental model of *Candida albicans* vaginitis in rats. *Infect Immun* **65**, 3399-3405.

De Bernardis, F., Arancia, S., Morelli, L., Hube, B., Sanglard, D., Schafer, W., Cassone, A. (1999a). Evidence that members of the secretory aspartyl proteinase gene family, in particular *SAP2*, are virulence factors for *Candida* vaginitis. *J Infect Dis* **179**, 201-208.

De Bernardis, F., Modnello, F., Scaravelli, G., Pachi, A., Girolamo, A., Agatensi, L., Cassone, A. (1999b). High aspartyl proteinase production and vaginitis in human immunodeficiency virus-infected women. *J Clin Microbiol* **37**, 1376-1380.

Denning, D. W. (1995). Can we prevent azole resistance in fungi? *Lancet* **346**, 454-455.

De Repentigny, L., Aumont, F., Bernard, K., Belhumeur, P. (2000). Characterization of binding of *Candida albicans* to small intestinal mucin and its role in adherence to mucosal epithelial cells. *Infect Immun* **68**, 3172-3179.

Deshler, J. O., Larson, G., Rossi, J. J. (1989). *Kluyveromyces lactis* maintains *Saccharomyces cerevisiae* intron-encoded splicing signals. *Mol Cell Biol* **9**, 2208-2213.

De Viragh, P. A., Sanglard, D., Togni, G., Falchetto, R., Monod, M. (1993). Cloning and sequencing of two *Candida parapsilosis* genes encoding acid proteases. *J Gen Microbiol* **139**, 335-342.

Di Domenico, B. J., Lupisella, J., Sandbaken, M., Chakraburtty, K. (1992). Isolation and sequence analysis of the gene encoding translation elongation factor 3 from *Candida albicans*. *Yeast* **8**, 337-352.

Donnelly, S. M., Sullivan, D. J., Shanley, D. B., Coleman, D. C. (1999). Phylogenetic analysis and rapid identfication of *Candida dubliniensis* based on analysis of *ACT1* intron and exon sequences. *Microbiol* **145**, 1871-1882.

Doolittle, W. F. (1999). Phylogenetic classification and the universal tree. *Science* **284**, 2124-2128.

Douglas, J. L., Houston, J. G., McCourtie, J. (1981). Adherence of *Candida albicans* to human buccal epithelial cells after growth on different carbon sources. *FEMS Microbiol Lett* **12**, 241-243.

Dreyer, T., Halkjaer, B., Svenden, I., Otterson, M. (1985). In *Aspartic Proteinases and their Inhibitors*, pp 41-44. Edited by V. Kostka, Walter de Gruyter & Co., Hawthorne, NY.

Dubois, N., Colina, A. R., Aumont, F., Belhumeur, P., de Repentigny, L. (1998). Overexpression of *Candida albicans* secretory aspartyl proteinase 2 and its expression in *Saccharomyces cerevisiae* do not augment virulence in mice. *Microbiology* **144**, 2299-2310.

Edison, A. M. & Manning-Zweerink, M. (1988). Comparison of the extracellualr proteinase activity produced by a low-virulence mutant of *Candida albicans* and its wild-type parent. *Infect Immun* **56**, 1388-1390.

Edman, J. C., Kovacs, J. A., Masur, H., Santi, D. V., Elwood, H. J., Sogin, M. L. (1988). Ribosomal RNA sequence shows *Pneumocystis carinii* to be a member of the fungi. *Nature* **334**, 519-522.

Eisenstein, B. I. (1990). New opportunistic infections – more opportunities. *N Engl J Med* **323**, 1625-1627.

Elie, C. M., Lott, T. J., Reiss, E., Morrison, C. J. (1998). Rapid identification of *Candida* species with species-specific DNA probes. *J Clin Microbiol* **36**, 3260-3265.

Fallon, K., Bausch, K., Noonan, J., Huguenel, E., Tamburini, P. (1997). Role of aspartic proteases in disseminated *Candida albicans* infection in mice. *Infect Immun* **65**, 551-556.

Fell, J. W. (1993). Rapid identification of yeast species using three primers in a polymerase chain reaction. *Mol Mar Biol Biotechnol* **3**, 174-180.

Feng, D.-F., Cho, G. Doolittle, R. F. (1997). Determining divergence times with a protein clock: Update and reevaluation. *Proc Natl Acad Sci USA* **94**, 13028-13033.

Fidel, S., Doonan, J. H., Morris, N. R. (1988). *Aspergillus nidulans* contains a single actin gene which has unique intron locations and encodes a γ-actin. *Gene* **70**, 283-293.

Fisher-Hoch, S. P., Hutwagner, L. (1995). Opportunistic candidiasis: an epidemic of the 1980s. *Clin Infect Dis* **21**, 897-904.

Fitch, W. M., & Margoliash, E. (1967). Construction of phylogenetic trees. *Science* 155, 279-284.

Fletcher, L. D., McDowell, J. M., Tidwell, R. R., Meagher, R. B., Dykstra, C. C. (1994). Structure, expression and phylogenetic analysis of the gene encoding Actin 1 in *Pneumocystis carinii*. *Genetics* **137**, 743-750.

Foltmann, B. (1981). Gastric proteinases – structure, function, evolution and mechanism of action. *Essays Biochem* **17**, 52-84.

Fonzi, W. A. & Irwin, M. M. (1993). Isogenic strain construction and gene mapping in *Candida albicans*. *Genetics* **134**, 717-728.

Fox, J. L (1993). Fungal infection rates are increasing. *ASM News* **10**, 515-518.

Fridkin, S. K. & Jarvis, W. R. (1996). Epidemiology of nosocomial fungal infections. *Clin Microbiol Rev* **9**, 199-511.

Frischauf, A. M., Lehrach, H., Poustka, A., Murray, N. (1983). Lambda replacement vectors carrying polylinker sequences. *J Mol Biol* **170**, 827-842.

Fusek, M., Smith, E. A., Monod, M., Foundling, S. I. (1993). *Candida parapsilosis* expresses and secretes two aspartic proteinases. *FEBS Lett* **327**, 108-112.

Gales, A. C., Pfaller, M. A., Houston, A. K., Joly, S., Sullivan, D. J., Coleman, D. C., Soll, D. R. (1999). Identification of *Candida dubliniensis* based on temperature and utilization of xylose and α-methly-D-glucoside as determined with the API 20C AUX and Vitek YBC systems. *J Clin Microbiol* **37**, 3804-3808.

Gallagher, P. J., Bennett, D. E., Henman, M. C., Russell, R. J., Flint, S. R., Shanley, D. B., Coleman, D. C. (1992). Reduced azole susceptibility of oral isolates of *Candida albicans* from HIV-positive patients and a derivative exhibiting colony morphology variation. *J Gen Microbiol* **138**, 1901-1911.

Gallwitz, D. & Sures, I. (1980). Structure of a split yeast gene: complete nucleotide sequence of the actin gene in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci USA* 77(5), 2546-2550.

Gaudet, J., Julien, J., Lafay, J. F., Brygoo, Y. (1989). Phylogeny of some *Fusarium* species, as determined by large-subunit rRNA sequence comparison. *Mol Biol* **6**, 227-242.

Germaine, G. R. & Tellefson, L. M. (1981). Effect of pH and human saliva on proteinase production by *Candida albicans*. *Infect Immun* **31**, 323-326.

Gilfillan, G. D., Sullivan, D. J., Haynes, K., Parkinson, T., Coleman, D. C., Gow, N. A. R. (1998). *Candida dubliniensis*: phylogeny and putative virulence factors. *Microbiology* **144**, 829-838.

Gogarten, J. P., Kibak, H., Dittrich, P., Taiz, L., Bowman, E. J., Bowman, B. J., Manolson, M. F., Poole, R. J., Date, T., Oshima, T., Konishi, J., Denda, K., Yoshida, M.

(1989). Evolution of the vacuolar H^+-ATPase: implications for the origin of eukaryotes. *Proc Natl Acad Sci USA* **86**, 6661-6665.

Gogarten, J. P., Olendzenski, L., Hilario, E., Simon, C., Holsinger, K. E. (1996). Dating the ancestor of organisms. *Science* **274,** 1450-1751.

Guého, E., Kurtzman, C. P., Peterson, S. W. (1989). Evolutionary affinities of heterobasidiomycetous yeasts estimated from 18S and 25S ribosomal RNA sequence divergence. *Syst Appl Microbiol* **12**, 230-236.

Guého, E., Kurtzman, C. P., Peterson, S. W. (1990). Phylogenetic relationships among species of *Sterigmatomyces* and *Fellomyces* as determined from partial rRNA sequences. *Int J Syst Bacteriol* **40**, 60-65.

Gruber, A., Speth, C., Lukasser-Vogl, E., Zangerle, R., Borg-von Zepelin, M., Dierich, M. P., Wurzner, R. (1999a). Human immunodeficiency virus type 1 protease inhibitor attenuates *Candida albicans* virulence properties *in vitro*. *Immunopharmacology* **41**, 227-234.

Gruber, A., Berlit, J., Speth, C., Lass-Florl, C., Kofler, G., Nagl, M., Borg-von Zepelin, M., Dierich, M. P., Wurzner, R. (1999b). Dissimilar attenuation of *Candida albicans* virulence properties by human immunodeficiency virus type 1 protease inhibitors. *Immunobiology* **201**, 133-144.

Gupta, R. S. (1998). Protein phylogenies and signature sequences: a reappraisal of evolutionary relationships among archaebacteria, eubacteria, and eukaryotes. *Microbiol Mol Biol Rev* **62**, 1435-1491.

Hancock, J. M., & Dover, G. A. (1989). Paradoxes of molecular coevolution in the rDNA multigene family, pp 206-220. In *Evolutionary Biology of Transient Unstable Populations*. Fontdevila, A. (ed). Berlin: Springer-Verlag.

Hannula, J., Saarela, M., Alaluusua, S., Slots, J., Asikainen, S. (1997). Phenotypic and genotypic characterization of oral yeasts from Finland and the United States. *Oral Microbiol Immunol* **12**, 358-365.

Haynes, K. A. & Westerneng, T. J. (1996). Rapid identification of *Candida albicans*, *C. glabrata*, *C. parapsilosis* and *C. krusei* by species-specific PCR of large subunit ribosomal DNA. *J Med Microbiol* **44**, 390-396.

Hazen, K. C. (1995). New and emerging yeast pathogens. *Clin Microbiol Rev* **8**, 162-278.

Hazen, K. C. & Masuoka, J. (2000). Cell surface hydrophobicity (CSH) comparison of *Candida albicans* and *Candida dubliniensis*. XIV Congress of the International Society of Human and Animal Mycoses (ISHAM), Buenos Aires. Abstract pathogenesis P-150, p252.

Heinz, W. J., Kurzai, O., Brakhage, A. A., Fonzi, W. A., Korting H. C., Frosch, M., Muhlschlege, F. A. (2000). Molecular responses to changes in the environmental pH are conserved between the fungal pathogens *Candida dubliniensis* and *C. albicans*. *Int J Med Microbiol* **290**, 231-238.

Hendriks, L., Goris, A., Van De Peer, Y., Neefs, J., Vancanneyt, M., Kersters, K., Hennebert, G. L., De Wachter, R. (1991). Phylogenetic analysis of five medically important *Candida* species deduced on the basis of small ribosomal subunit RNA sequences. *J Gen Microbiol* **137**, 1223-1230.

Hennesey, E. S., Drummond, D. R., Sparrow, J. C. (1993). Molecular genetics of actin function. *Biochem J* **282**, 657-671.

Hightower, R. C. & Meagher, R. B. (1986). The molecular evolution of actin. *Genetics* **114,** 315-332.

Holmber, K. & Meyer, R. D. (1986). Fungal infections in patients with AIDS and AIDS-related complex. *Scand J Infect Dis* **18**, 179-192.

Homma, M., Chibana, H., Tanaka, K. (1993). Induction of extracellualar proteianse in *Candida albicans. J Gen Microbiol* **139**, 1187-1193.

Hori, H. & Osawa, S. (1979). Evolutionary change in 5S RNA secondary structure and a phylogenic tree of 54 5S RNA species. *Proc Natl Acad Sci USA* **76**, 381-385.

Hsu, I., Delbaere, L. T. J., James, M. N. G., Hoffmann, T. (1977). Penicillopepsin from *Penicillium janthinellum* crystal structure at 2.8 Å and sequence homology with porcine pepsin. *Nature* **266**, 140-145.

Hube, B., Turver, C. J., Odds, F. C., Eiffert, H., Boulnois, G. J., Kochel, H., Ruchel, R. (1991). Sequence of the *Candida albicans* gene encoding the secretory aspartate proteinase. *J Med Vet Mycol* **29**, 129-132.

Hube, B., Monod, M., Schofield, D. A., Brown, A. J. P., Gow, N. A. R. (1994). Expression of seven members of the gene family encoding secretory aspartyl proteinases in *Candida albicans. Mol Microbiol* **14**, 87-99.

Hube, B. (1996). *Candida albicans* secreted aspartyl proteinases. *Curr Top Med Mycol* 7, 55-69.

Hube, B., Sanglard, D., Odds, F. C., Hess, D., Monod, M., Schafer, W., Brown, A. J., Gow, N. A. (1997). Disruption of each of the secreted aspartyl proteinase genes *SAP1*, *SAP2* and *SAP3* of *Candida albicans* attenuates virulence. *Infect Immun* **65**, 3529-3538.

Hube, B., Ruchel, R., Monod, M., Sanglard, D., Odds, F. C. (1998). Functional aspects of secreted *Candida* proteinases. *Adv Exp Med Biol* **436**, 339-344.

Ibrahim, A. S., Filler, S. G., Sanglard, D., Edwards, J. E. Jr., Hube, B. (1998). Secreted aspartyl proteinases and interactions of *Candida albicans* with human endothelial cells. *Infect Immun* **66**, 3003-3005.

Iwabe, N., Kuma, K., Hasegawa, M., Osawa, S., Miyata, T. (1989). Evolutionary relationship of archaebacteria, eubacteria, and eukaryotes inferred from phylogenetic trees of duplicated genes. *Proc Natl Acad Sci USA* **86**, 9355-9359.

Jabra-Rizk, M. A., Falkler Jr., W. A., Merz, W. G., Kelley, J. I., Baqui, A. A., Meiller, T. F. (1999a). Coaggregation of *Candida dubliniensis* with *Fusobacterium nucleatum*. *J Clin Microbiol* **37**, 1464-1468.

Jabra-Rizk, M., Baqui, A. A. M. A., Kelley, J. I., Falkler Jr., W. A., Merz, W. G., Meiller, T. F. (1999b). Identification of *Candida dubliniensis* in a prospective study of patients in the United States. *J Clin Microbiol* **37**, 321-326.

Jabra-Rizk, M. A., Falkler, J. R., Merz, W. G., Baqui, A. A. M. A., Kelley, J. I., Meiller, T. F. (2000). Retrospective identification and characterization of *Candida dubliniensis* isolates

among *Candida albicans* clinical laboratory isolates from human immunodeficiency virus (HIV)-infected and non-HIV-infected individuals. *J Clin Microbiol* **38**, 2423-2426.

James, M. N. G. & Sielecki, A. R. (1986). Molecular structure of an aspartic proteinase zymogen, porcine pepsinogen at 1.8 Å resolution. *Nature* **319**, 33-38.

Jarvis, W. R. (1995). Epidemiology of nosocomial fungal infections, with emphasis on *Candida* species. *Clin Infect Dis* **20** 1526-1530.

Johnson, E. M., Warnock, D. W., Luker, J., Porter, S. R., Scully, C. (1995). Emergence of azole drug resistance in *Candida* species from HIV-infected patients receiving prolonged fluconazole therapy for oral candidosis. *Antimicrob Agents Chemother* **35**, 103-114.

Joly, S., Pujol, C., Rysz, M., Vargas, K., Soll, D. R. (1999). Development and characterization of complex DNA fingerprinting probes for the infectious yeast *Candida dubliniensis*. *J Clin Microbiol* **37**, 1035-1044.

Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, vol. 3, pp. 21-132. Edited by H. N. Munro. New York: Academic Press.

Julius, D., Blake, A., Blair, L., Kunisawa, R., Thomer, J. (1984). Isolation of the putative structural gene for the lysine-arginine-cleaving endopeptidase required for processing of yeast prepro-alpha-factor. *Cell* **37**, 129-132.

Kamei, K., McCullough, M. J., Stevens, D. A. (2000). Initial case of *Candida dubliniensis* infection from Asia: non-mucosal infection. *Med Mycol* **38**, 81-83.

Kaminishi, H., Hagihara, Y., Hayashi, S., Cho, T. (1986). Isolation and characterisation of collagenolytic enzyme produced by *Candida albicans*. *Infect Immun* **53**, 312-316.

Kaminishi, H., Hagihara, Y., Tanaka, M., Cho, T. (1988). Degradation of bovine achilles tendon collagen by *Candida albicans* proteinase *J Med Vet Mycol* **26**, 315-318.

Kaminishi, H., Tanaka, M., Cho, T., Maeda, H., Hagihara, Y. (1990). Activation of the plasma kallikrein-kinin system by *Candida albicans* proteinase. *Infect Immun* **58**, 2139-2143.

Kaminishi, H., Miyaguchi, H., Tamaki, T., Suenaga, N., Hisamatsu, M., Mihashi, I., Matsumoto, H., Maeda, H., Magihara, Y. (1995). Degradation of humoral host defense by *Candida albicans* proteinase. *Infect Immun* **63**, 984-988.

Kamiyama, A., Niimi, M., Tokunaga, M., Nakayama, H. (1989). DNA homology between *Candida albicans* strains: evidence to justify the synonymous status of *Candida stellatoidea*. *Mycopathologia* **107**, 3-7.

Kay, J., Valler, M. J., Keilova, H., Kostka, V. (1981) In *Proteinases and their Inhibitors* pp 1-17. Edited by V. Turk, Walter de Gruyter and Co., Hawthorne, NY.

Kirkpatrick, W. R., Revankar, S. G., McAtee, R. K., Lopez-Ribot, J. L., Fothergill, A. W., McCarthy, D. I., Sanche, S. E., Cantu, R. A., Rinaldi, M. G., Patterson, T. F. (1998). Detection of *Candida dubliniensis* in oropharyngeal samples from human immunodeficiency virus-infected patients in North America by primary CHROMagar Candida screening and susceptibility testing of isolates. *J Clin Microbiol* **36**, 3007-3012.

Kirkpatrick, W. R., Lopez-Ribot, J. L., McAtee, R. K., Patterson, T. F. (2000). Growth competition between *Candida dubliniensis* and *Candida albicans* under broth and biofilm growing conditions. *J Clin Microbiol* **38**, 902-904.

Klein, R. S., Harris, C. A., Butkus Small, C. *et al.* (1984). Oral candidiasis in high risk patients as the initial manifestation of the acquired immunodeficiency syndrome. *N Engl J Med* **311**, 354-358.

Klotz, S. A. (1994). Adherence of *Candida albicans* to endothelial cells is inhibited by prostaglandin I_2. *Infect Immun* **62**, 1497-1500.

Koehler, A. P., Chu, K.-C., Houang, E. T. S., Cheng, A. F. B. (1999). Simple, reliable and cost-effective yeast identification scheme for the clinical laboratory. *J Clin Microbiol* **37**, 422-426.

Korn, E. D. (1978). Biochemistry of actomyosin-dependent cell motility (A Review). *Proc Natl Acad Sci USA* **75**, 588-599.

Korting, H. C., Schaller, M., Eder, G., Hamm, G., Bohmer, U., Hube, B. (1999). Effects of the human immunodeficiency virus (HIV) proteinase inhibitors saquinavir and indinavir on *in vitro* activities of secreted aspartyl proteinases of *Candida albicans* isolates from HIV-infected patients. *Antimicrob Agents Chemother* **43**, 2038-2042.

Krainer, A. R. & Maniatis, T. (1988). RNA splicing. In *Transcription and Splicing*, pp 131-206. Edited by B. D. Hames & D. M. Glover. Washington DC: IRL Press.

Kretschmar, M., Hube, B., Bertsch, T., Sanglard, D., Merker, R., Schroder, M., Hof, H., Nichterlein, T. (1999). Germ tubes and proteinase activity contribute to virulence of *Candida albicans* in murine peritonitis. *Infect Immun* **67**, 6637-6642.

Kurzai, O., Heinz, W. J., Sullivan, D. J., Coleman, D. C., Frosch, M., Muhlschlegel, F. A. (1999). Rapid PCR test for discriminating between *Candida albicans* and *Candida dubliniensis* isolates using primers derived from the pH-regulated *PHR1* and *PHR2* genes of *C. albicans*. *J Clin Microbiol* **37**, 1587-1590.

Kurtzman, C. P. (1989). Estimation of phylogenetic distances among ascomycetous yeasts from partial sequencing of ribosomal RNA. *Yeast* **5**, S351-S354.

Kurtzman, C. P. & Robnett, C. J. (1997). Identification of clinically important ascomycetous yeasts based on nucleotide divergence in the 5' end of the large-subunit (26S) ribosomal DNA gene. *J Clin Microbiol* **35**, 1216-1223.

Kurtzman, C. P. (1989). Estimation of phylogenetic distances among ascomycetous yeasts from partial sequencing of ribosomal RNA. *Yeast* **5**, S351-S354.

Kurtzman, C. P. & Robnett, C. J. (1997). Identification of clinically important ascomycetous yeasts based on nucleotide divergence in the 5' end of the large-subunit (26S) ribosomal DNA gene. *J Clin Microbiol* **35**, 1216-1223.

Kvaal, C., Lachke, S. A., Srikantha, T., Daniels, K., McCoy, J., Soll, D. R. (1999). Misexpression of the opaque-phase-specific gene *PEP1* (*SAP1*) in the white phase of *Candida albicans* confers increased virulence in a mouse model of cutaneous infection. *Infect Immun* **67**, 6652-6662.

Kwon-Chung, K. J., Riggsby, W. S., Uphoff, R. A., Hicks, J. B., Whelan, W. L., Reiss, E., Magee, B. B., Wickes, B. L. (1989). Genetic differences between Type I and Type II *Candida stellatoidea*. *Infect Immun* **57**, 527-532.

Kyte, J. & Doolittle, R. F. (1982). A simple method for displaying the hydropathic character of a protein. *J Mol Biol* **157**, 105-132.

Langford, C. J., Klinz, F-J., Donath, C., Galwitz, D. (1984). Point mutations identify the conserved intron-contained TACTAAC box as an essential splicing signal sequence in yeast. *Cell* **36**, 645-653.

Larone, D. H. (1993). Staining methods. In *Medically Improtant Fungi: A Guide to Identification*, pp. 186-192. Edited D. H. Larone. Washington, DC: ASM.

Leer, R. J., van Raamsdonk-Duin, M. M. C., Hagendoorn, M. J. M., Mager, W. H., Planta, R. J. (1984). Structural comparison of yeast ribosomal protein genes. *Nuc Acids Res* **12**, 6685-6700.

Le Guennec, R., Reynes, J., Mallié, M. (1995). Fluconazole- and itraconazole-resistant *Candida albicans* strains from AIDS patients: multilocus enzyme electrophoresis analysis and antifungal susceptibilities. *J Clin Microbiol* **33**, 2732-2737.

Lennox, E. S. (1955). Transduction of linged genetic characters by the host of the bacteriophage p1. *Viriology* **1**, 190-206.

Lerner, G. G. & Goldman, R. C. (1993). Stimuli that induce production of *Candida albicans* extracellular aspartyl proteinase. *J Gen Microbiol* **139**, 1643-1651.

Lischewski, A., Hrmsen, D., Wilms, K., Baier, G., Gunzer, U., Klinker, H., Wilhelm, M., Schwinn, A., Hacker, J. (1999). Molecular epidemiology of *Candida albicans* isolates from AIDS and cancer patients using a novel standardized CARE-2 DNA fingerprinting technique. *Mycoses* **42**, 371-383.

Loomis W. F. & Smith, D. W. (1990). Molecular phylogeny of *Dictyostelium discoideum* by protein sequence comparison. *Proc Natl Acad Sci USA* **87**, 9093-9097.

Losberger, C. & Ernst, J. F. (1989). Sequence of the *Candida albicans* gene encoding actin. *Nucleic Acids Res* **17**, 9488.

Macdonald, F. &Odds, F. C. (1980). Inducible proteinase of *Candida albicans* in diagnostic serology and in the pathogenesis of systemic candidosis. *J Med Microbiol* **13**, 423-435.

Macdonald, F. (1984). Secretion of inducible proteinase by pathogenic *Candida* species. *Sabouraudia* **22**, 79-82.

Magee, B. B., Hube, B., Wright, R. J., Sullivan, P. J., Magee, P. T. (1993). The genes encoding the secreted aspartyl proteinases of *Candida albicans* constitute a family with at least three members. *Infect Immun* **61**, 3240-3243.

Mahrous, M., Lott, T. J., Meyer, A., Sawant, A. D., Ahearn, D. G. (1990). Electrophoretic karyotyping of typical and atypical *Candida albicans*. *J Clin Microbiol* **28**, 876-881.

Mannarelli B. M. & Kurtzman, C. P. (1998). Rapid identification of *Candida albicans* and other human pathogenic yeasts by using short oligonucleotides in a PCR. *J Clin Microbiol* **36**, 1634-1641.

Martin, C., Roberts, D., Van Der Weide, M., Rossau, R., Jannes, G., Smith, T., Maher, M. (2000). Development of a PCR-based line probe assay for identification of fungal pathogens. *J Clin Microbiol* **38**, 3735-3742.

Matheucci Jr., E., Henrique-Silva, F., El-Gogary, S., Rossini, C. H. B., Leite, A., Escobar Vera, J., Carle Urioste, C., Crivellaro, O., El-Dorry, H. (1995). Structure, organization and promoter expression of the actin-encoding gene in *Trichoderma reesei*. *Gene* **161**, 103-106.

McCullough, M. J., Ross, B. C., Dwyer, B. D. et al. (1994). Genotype and phenotype of oral *Candida albicans* from patients infected with the human immunodeficiency virus. *Microbiol* **140**, 1195-1202.

McCullough, M., Ross, B., Reade, P. (1995). Characterization of genetically distinct subgroup of *Candida albicans* strains isolated from oral cavities of patients infected with human immunodeficiency virus. *J Clin Microbiol* **33**, 696-700.

McCullough, M. J., Clemons, K. V., Stevens, D. A. (1999). Molecular and phenotypic characterization of genotypic *Candida albicans* subgroups and comparison with *Candida dubliniensis* and *Candida stellatoidea*. *J Clin Microbiol* **37**, 417-421.

Meis, J. F. G., Ruhnke, M., De Pauw, B. E., Odds, F. C., Siegert, W., Verwiej, P. E. (1999). *Candida dubliniensis* candidemia in patients with chemotherapy-induced neutropenia and bone marrow transplantation. *Emerg Infect Dis* **5**, 150-153.

Mecure, S., Senechal, S., Auger, P. et al (1996). *Candida albicans* serotype analysis by flow cytometry. *J Clin Microbiol* **34**, 2106-2112.

Meiller, T. F., Jabra-Rizk, M. A., Baqui, A. A., Kelley, J. I., Meeks, V. I., Merz, W. G., Falkler, W. A. (1999). Oral *Candida dubliniensis* as a clinically improtant species in HIV-seropositive patients in the United States. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* **88**, 573-580.

Mertins, P. & Gallwitz, D. (1987). A single intronless actin gene in the fission yeast *Schizosaccharomyes pombe*: nucleotide sequence and transcripts formed in homologous and heterologous yeast. *Nuc Acids Res* **15**,7369-7379.

Miyasaki, S. H., White, T. C., Agabian, N. (1994). A fourth secreted aspartyl proteinase gene (*SAP4*) and a CARE2 repetitive element are located upstream of the *SAP1* gene in *Candida albicans*. *J Bacteriol* **176**, 1702-1710.

Molenaar, T., Woudt, L. P., Jansen, A. E. M., Mager, W. H., Planta, R. J. (1984). Structure and organization of two linked ribosomal protein genes in yeast. *Nuc Acids Res* **12**, 7345-7358.

Monod, M., Togni, G., Hube, B., Sanglard, D. (1994). Multiplicity of genes encoding secreted aspartic proteinases in *Candida* species. *Mol Microbiol* **13**, 357-368.

Monod, M., Hube, B., Hess, D., Sanglard, D. (1998). Differential regulation of *SAP8* and *SAP9*, which encode two new members of the secreted aspartic proteinase family in *Candida albicans*. *Microbiology* **114**, 2731-2737.

Monod, M., Borg-von Zepelin, M., Telenti, A., Sanglard, D. (1999). The inhibition of *Candida albicans* secreted aspartic proteases by three different HIV protease inhibitors. *Dermatology* **198**, 412-414.

Moran, G. P., Sullivan, D. J. Henman, M. C., McCreary, C. E., Harrington, B. J., Shanley, D. B., Coleman, D. C. (1997). Antifungal drug susceptibilities of oral *Candida dubliniensis* isolates from human immunodeficiency virus (HIV)-infected and non-HIV-infected subjects and generation of stable fluconazole-resistant derivatives. *Antimicrob Agents Chemother* **41**, 617-623.

Moran, G. P., Sanglard, D., Donnelly, S. M., Shanley, D. B., Sullivan, D. J., Coleman, D. C. (1998). Identification and expression of multidrug transporters responsible for fluconazole resistance in *Candida dubliniensis*. *Antimicrob Agents Chemother* **42**, 1819-1830.

Morrison, C. J., Hurst, S. F., Bragg, S. L., Randall, J. K., Diaz, H., McLauglin, D. W., Reiss, E. (1993). Purification and characterisation of the extracellular aspartyl proteinase of *Candida albicans*: removal of extraneous proteins and cell wall mannoprotein and evidence for lack of glycosylation. *J Gen Microbiol* **139**, 1177-1186.

Morrow, B., Srikantha, T., Soll, D. R. (1992). Transcription of the gene for a pepsinogen, *PEP1*, is regulated by white-opaque switching in *Candia albicans*. *Mol Cell Biol* **12**, 2997-3005.

Naglik, J. R., Newport, G., White, T. C., Fernandes-Naglik, L. L., Greenspan, J. S., Greenspan, D., Sweet, S. P., Challacombe, S. J., Agabian, N. (1999). *In vivo* analysis of secreted aspartyl proteinase expression in human oral candidiasis. *Infect Immun* **67**, 2482-2490.

Negi, M., Tsuboi, R., Matsui, T., Ogawa, H. (1984). Isolation and characterisation of proteinase from *Candida albicans*: substrate specificity. *J Invest Derm* **83**, 32-36.

Nellen, W., Donath, C., Moos, M., Gallwitz, D. (1981). The nucleotide sequences of the actin genes from *Saccharomyces carlsbergensis* and *Saccharomyces cerevisiae* are identical except for their introns. *J Mol App Genetics* **1**, 239-244.

Newport, G. & Agabian, A. (1997). *KEX2* influences *Candida albicans* proteinase secretion and hyphal formation. *J Biol Chem* **272**, 28954-28961.

Ng, R. & Abelson, J. (1980). Isolation and sequence of the gene for actin in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci USA* 77, 3912-3916.

Nguyen, M. H., Peacock, J. E., Morris, A. J. *et al.* (1996). The changing face of candidemia: emergence of non-*Candida albicans* species and antifungal resistance. Am J Med **100**, 617-623.

Odds, F. C. (1988). *Candida and Candidosis*, 2nd edn. London: Ballière Tindall.

Odds, F. C., Schmid, J., Soll, D. R. et al. (1990). Epidemiology of *Candida* infection in AIDS. In: *Mycoses in AIDS Patients*, pp.67-74. H. Vanden Bossche, D. W. R. Mackenzie, G. Cauwenburgh, J. Van Cutsem, E. Drouhet, B. Dupont (Eds.). Plenum Press: New York.

Odds, F. C. & Bernaerts, R. (1994). CHROMagar Candida, a new differential isolation medium for presumptive identification of clinically imortant *Candida* species. *J Clin Microbiol* **32**, 1923-1929.

Odds, F. C., Van Nuffel, L., Dams, G. (1998). Prevalence of *Candida dubliniensis* isolates in a yeast stock collection. *J Clin Microbiol* **36**, 2869-2873.

Ollert, M. W., Söhnchen, R., Korting, H. C., Ollert, U., Bräutigam, S., Bräutigam, W. (1993). Mechanisms of adherence of *Candida albicans* to cultured human epidermal keratinocytes. *Infect Immun* **61**, 4560-4568.

Olsen G. J &Woese, C. R. (1993). Ribosomal RNA: a key to phylogeny. *FASEB J* 7, 113-123.

Park, S., Wong, M., Marras, S. A. E., Cross, E. W., Kiehn, T. E., Chaturvedi, V., Tyagi, S., Perlin, D. S. (2000). Rapid identification of *Candida dubliniensis* using a species-specific molecular beacon. *J Clin Microbiol* **38**, 2829-2836.

Pearl, L. H. & Taylor, W. R. (1987). Sequence specificity of retroviral proteases. *Nature* **328**, 482.

Peltroche-Llacsahuanga, H., Schmidt, S., Seibold, M., Lutticken, R., Haase, G. (2000a). Differentiation between *Candida dubliniensis* and *Candida albicans* by fatty acid methyl ester analysis using gas-liquid chromatography. *J Clin Microbiol* **38**, 3696-3704.

Peltroche-Llacsahuanga, H., Schnitzler, N., Schmidt, S., Tintelnot, K., Lütticken, R., Haase, G. (2000b) Phagocytosis, oxidative burst, and killing of *Candida dubliniensis* and *Candida albicans* by human neutrophils. *FEMS Microbiol Lett* **191**, 151-155.

Perfect, J. R. & Schell, W. A. (1996). The new fungal opportunists are coming. *Clin Infect Dis* **22**, S112-S118.

Peterson, S. W., & Kurtzman, C. P. (1991). Ribosomal RNA sequence divergence among sibling species of yeasts. *Syst Appl Microbiol* **14**, 124-129.

Pfaller, M. A. (1994). Epidemiology and control of fungal infections. *Clin Infect Dis* **19**, S8-S13.

Pfaller, M. A. (1996). Nosocomial candidiasis: emerging species, reservoirs, and modes of transmission. *Clin Infect Dis* **22**, S89-S94.

Pfaller, M. A., Messer, S. A., Gee, S., Joly, S., Pujol, C., Sullivan, D. J., Coleman, D. C., Soll, D. R. (1999). *In vitro* susceptibilities of *Candida dubliniensis* isolates tested against the new triazole and echinocandin antifungal agents. *J Clin Microbiol* **37**, 870-872.

Pierce V. A. & Crawford, D. L. (1997). Phylogenetic analysis of glycolytic enzyme expression. *Science* **276**, 256-259.

Pincus, D. H., Coleman, D. C., Pruitt, W. R., Padhye, A. A., Salkin, I. F., Geimer, M., Bassel, A., Sullivan, D. J., Clarke, M., Hearn, V. (1999). Rapid identification of *Candida dubliniensis* with commercial yeast identification systems. *J Clin Microbiol* **37**, 3533-3539.

Pinjon, E., Sullivan, D., Salkin, I., Shanley, D., Coleman, D. (1998). Simple, inexpensive, reliable method for differentiation of *Candida dubliniensis* from *Candida albicans*. *J Clin Microbiol* **36**, 2093-2095.

Polacheck, I. (1991). The discovery of melanin production in *Cryptococcus neoformans* and its impact on diagnosis and the study of virulence. *Zentralbl Bakteriol* **276**, 120-123.

Polacheck, I., Strahilevitz, J., Sullivan, D., Donnelly, S., Salkin, I. F., Coleman, D. C. (2000). Recovery of *Candida dubliniensis* from non-human immunodeficiency virus-infected patients in Israel. *J Clin Microbiol* **38**, 170-174.

Pollard, T. D. (1990). Actin. *Curr Opin Cell Biol* **2**, 33-40.

Powderly, W. G. (1992). Mucosal candidiasis caused by non-*albicans* species of *Candida* in HIV-positive patients. *AIDS* **6**, 604-605.

Pühler, G., Leffers, H., Gropp, F., Palm, P., Klenk, H., Lottspeich, F., Garrett, R. A., Zillig, W. (1989). Archaebacterial DNA-dependent RNA polymerases testify to the evolution of the eukaryotic nuclear genome. *Proc Natl Acad Sci USA* **86**, 4569-4573.

Pujol, C., Renaud, F., Mallie, M., de Meeus, T., Bastide, J.-M. (1997). Atypical strains of *Candida albicans* recovered from AIDS patients. *J Med Vet Mycol* **35**, 115-121.

Raue, H. A., Klootwijk, J., Musters, W. (1988). Evolutionary conservation of structure and function of high molecular weight ribosomal RNA. *Prog Biophys Molec Biol* **51**, 77-129.

Ray, T. L. & Payne, C. D. (1987) Detection of *Candida* acid proteinase (CAP) antibodies in systemic candidiasis by enzyme immunoassay. *Clin Res* **35**, 711A.

Ray, T. L. & Payne, C. D. (1988). Scanning electron microscopy of epidermal adherence and cavatition in murine candidiasis: a role for *Candida* acid-proteinase. *Infect Immun* **56**, 1942-1949.

Ray, T. L. & Payne, C. D. (1990). Comparative production and rapid purification of *Candida acid* proteinase from protein supplemented cultures. *Infect Immun* **58**, 508-514.

Redding, S.W., Zellars, R. C., Kirkpatrick, W. R., McAtee, R. K., Caceres, M. A., Fothergill, A. W., Lopez-Ribot, J. L., Bailey, C. W., Rinaldi, M. G., Patterson, T. F.

(1999). Epidemiology of oropharyngeal *Candida* colonization and infection in patients receiving radiation for head and neck cancer. *J Clin Microbiol* **37**, 3896-3900.

Reisler, E. (1993). Actin molecular structure and function. *Curr Opin Cell Biol* **5**, 41-47.

Remold. J., Fasolk. H., Staib, F. (1968). Purification and characterization of a proteolytic enzyme from *Candida albicans*. *Biochim Biophys Acta* **167**, 399-406.

Rodero, L., Losso, M., Canteros, C., Hochenfellner, F., Davel, G. (1998). *Candida dubliniensis*: 1st isolation in Argentina. *Rev Argent Microbiol* **30**, 39-41.

Ross, I. K., De Bernardis, F., Emerson, G. W., Cassone, A., Sullivan, P. A. (1990). The secreted aspartate proteinase of *Candida albicans*: physiology of secretion and virulence of a proteinase-deficient mutant. *J Gen Microbiol* **136**, 687-694.

Rüchel, R. (1981). Properties of a purified proteinase from the yeast *Candida albicans*. *Biochem Biophys Acta*. **659**, 99-113.

Rüchel, R., Uhlemann, K., Boning, B. (1983). Secretion of acid proteinases by different species of the genus *Candida*. *Abl Bakt Hyg I Abt Orig A* **255**, 537-548.

Rüchel, R. (1983). On the role of proteinases from *Candida albicans* in the pathogenesis of acronecrosis. *Zent Bakt Mik Hyg A* **255**, 524-536.

Rüchel, R., Boning-Stutzer, B., Mari, A. (1988). A synoptical approach to the diagnosis of candidosis, relying on serological antigen and antibody tests, on culture, and on evaluation of clinical data. *Mycoses*, **31**, 87-106.

Ruhnke, M., Grosch-Worner, I., Lischewski, A., Neubauer, A., Steinmuller, A., Trautmann, M., Morschhaüser, J. (1999). Genotypic relatedness of yeast isolates from women infected with human immunodeficiency virus and their children. *Mycoses* **42**, 385-394.

Ryder, N. S., Wagner, S., Leitner, I. (1998). *In vitro* activities of terbinafine against cutaneous isolates of *Candida albicans* and other pathogenic yeasts. *Antimicrob Agents Chemother* **42**, 1057-1061.

Saitou, N. & Nei, M. (1987). The neighbour joining method for reconstructing phylogneetic trees. *Mol Biol Evol* **4**, 406-425.

Salkin, I. F., Pruitt, W. R., Sullivan, D., Coleman, D., Pincus, D. H. (1998). Distinctive carbohydrate assimilation profiles used to identify the first clinical isolates of *Candida dubliniensis* recovered in the United States. *J Clin Microbiol* **36**, 1467.

Samaranayake, L. P. (1992). Oral mycoses in HIV infection. *Oral Surg Oral Med Oral Pathol* **73**, 171-180.

Samaranayake, Y. H., MacFarlane, T. W., Samaranayake, L. P., Aitchison, T. C. (1994). The *in vitro* proteolytic and saccharolytic activity of *Candida* species in human saliva. *Oral Microbiol Immunol* **9**, 229-235.

Sambrook, J., Fritsch, E. F., Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Laboratory.

Sanglard, D., Togni, G., de Viragh, P. A., Monod, M. (1992). Disruption of the gene encoding the secreted acid protease (*ACP*) in the yeast *Candida tropicalis*. *FEMS Microbiol Lett* **74**, 149-156.

Sanglard, D., Kuchler, K., Ischer, F., Pagani J.-L., Monod, M., Bille, J. (1995). Mechanisms of resistance to azole antifungal agents in *Candida albicans* isoaltes from AIDS patients involve specific multidrug tansporters. *Antimicrob Agents Chemother* **39**, 2378-2386.

Sanglard, D., Hube, B., Monod, M., Odds, F. C., Gow, N. A. (1997). A triple deletion of the secreted aspartyl proteinase genes *SAP4*, *SAP5*, and *SAP6* of *Candida albicans* causes attenuated virulence. *Infect Immun* **65**, 3539-3546.

Sano A., Vilela, M. M., Takahashi, I., Fukushima, K., Takizawa, K., da Silva, M. T., Uno, J., Nishimura, K., Miyaji, M. (2000). Isolation of *Candida dubliniensis* from the oral cavity of an HIV-positive child in Brazil. *Nippon Ishinkin Gakkai Zasshi* **41**, 177-181.

Schaller, M., Schafer, W., Korting, H. C., Hube, B. (1998). Differential expression of secreted aspartyl proteinases in a model of human oral candidosis and in patient samples from the oral cavity. *Mol Microbiol* **29**, 605-615.

Schaller, M., Hube, B., Ollert, M. W., Schafer, W., Borg-von Zepelin, M., Thoma-Greber, E., Korting, H. C. (1999a). *In vivo* expression and localization of *Candida albicans* secreted aspartyl proteinases during oral candidiasis in HIV-infected patients. *J Invest Dermatol* **112**, 383-386.

Schaller, M., Korting, H. C., Schafer, W., Bastert, J., Chen, W., Hube, B. (1999b). Secreted aspartic proteinase (Sap) activity contributes to tissue damage in a model of human oral candidosis. *Mol Microbiol* **1**, 169-180.

Schaller, M., Schackert, C., Korting, H. C., Januschke, E., Hube, B. (2000). Invasion of *Candida albicans* correlates with expression of secreted aspartic proteinases during experimental infection of human epidermis. *J Invest Dermatol* **114**, 712-713.

Scherer, S. & Stevens, D. A. (1988). A *Candida albicans* dispersed, repeated gene family and its epidemiologic applications. *Proc Natl Acad Sci USA* **85**, 1452-1456.

Schmid, J., Odds, F. C., Wiselda, M. J. *et al.* (1992). Genetic similarity and maintenance of *Candida* albicans strains from a group of AIDS patients, demonstrated by DNA fingerprinting. *J Clin Microbiol* **30**, 935-941.

Schoofs, A., Odds, F. C., Colebunders, R., Ieven, M., Goossens, H. (1997). Use of specialised isolation media for recognition and identification of *Candida dubliniensis* isolates from HIV-infected patients. *Eur J Clin Microbiol Infect Dis* **16**, 296-300.

Schwartz, R. M. & Dayhoff, M. O. (1978). Origins of prokaryotes, mitochondria and chloroplasts. *Science* **199**, 395-403.

Selik, R. M., Starcher, E. T., Curran, J. W. (1987). Opportunistic diseases reported in AIDS patients: frequency, associations, and trends. *AIDS* **1**, 175-182.

Shaberg, D. R., Culver, D. H., Gaynes, R. P. (1991). Major trends in the microbial etiology of nosocomial infection. *Am J Med* **91**, S72-S75.

Shah, D. M., Hightower, R. C., Meagher, R. B. (1983). Genes encoding actin in higher plants: intron positions are highly conserved but the coding sequences are not. *J Mol Appl Genetics* **2**, 111-126.

Shewale, J. G., Takahashi, T., & Tang, J. (1985). In *Aspartic proteinases and their Inhibitors*, pp 101-116. Edited by V. Kostka, Walter de Gruyter & Co., hawthorne, NY.

Small, J. V., Rottner, K., Kaverina, I. (1999). Functional design in the actin cytoskeleton. *Curr Opin Cell Biol* 11, 54-60.

Smolenski, G., Sullivan, P. A., Cutfield, S. M., Cutfield, J. F. (1997). Analysis of secreted aspartic proteinases from *Candida albicans*: purification and characterization of individual Sap1, Sap2 and Sap3 isoenzymes. *Microbiology* **143**, 349-356.

Sogin, M. L., Morrison, H. G., Hinkle, G., Silberman, J. D. (1996). Ancestral relationships of the major eukaryotic lineages. *Microbiologia SEM* **12**, 17-28.

Southern, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol Biol* **98**, 503-517.

Spencer, P. M. & Jackson, G. G. (1989). Fungal and mycobacterial infections in patients infected with the human immunodeficiency virus. *Antimicrob Agents Chemother* **23**, 107-125.

Staib, F. (1965). Serum-proteins as nitrogen source for yeast-like fungi. *Sabouraudia* **4**, 187-193.

Staib, F., Seibold, M., Antweiler, E., Fröhlich, B., Weber, S., Blisse, A. (1987). The brown colour effect (BCE) of *Cryptococcus neoformans* in the diagnosis, control and epidemiology of *C. neoformans* infections in AIDS patients. *Zentralbl Bakteriol Microbiol Hyg* **266**, 167-177.

Staib, P. & Morschhäuser, J. (1999). Chlamydospore formation on Staib agar as a species-specific characteristic of *Candida dubliniensis*. *Mycoses* **42**, 521-524.

Staib, P., Kretschmar, M., Nichterlein, T., Kohler, G., Michel, S., Hof, H., Hacker, J., Morschhäuser, J. (1999). Host-induced, stage-specific virulence gene activation in *Candida albicans* during infection. *Mol Microbiol* **32**, 553-546.

Staib, P., Mich, S., Kohler, G., Morschhäuser, J. (2000). A molecular genetic system for the pathogenic yeast *Candida dubliniensis*. *Gene* **242**, 393-398.

Staib, P., Kretschmar, M., Nichterlein, T., Hof, H., Morschhäuser, J. (2000). Differential activation of a *Candida albicans* virulence gene family during infection. *Proc Natl Acad Sci USA* **97**, 6102-6107.

Stringaro, A., Crateri, P., Pellegrini, G., Arancia, G., Casone, A., De Bernardis, F. (1997). Ultrastructural localization of the secretory aspartyl proteinase in *Candida albicans* cell wall *in vitro* and in experimentally infected rat vagina. *Mycopathologia* **137**, 95-105.

Subramanian, E., Swan, I. D. A., Davies, D. R. (1976). *Biochem Biophys Res Commun* **68**, 875-880.

Sullivan, D., Bennett, D., Henman, M., Harwood, P., Flint, S., Mulcahy, F., Shanley, D., Coleman, D. (1993). Oligonucleotide fingerprinting of isolates of *Candida* species other than *C. albicans* and of atypical *Candida* species from human immunodeficiency virus-positive and AIDS patients. *J Clin Microbiol* **31**, 2124-2133.

Sullivan, D. J., Weterneng, T. J., Haynes, K. A., Bennett, D. E., Coleman, D. C. (1995). *Candida dubliniensis* sp. nov.: phenotypic and molecular characterization of a novel species associated with oral candidosis in HIV-infected individuals. *Microbiology* **141**, 1507-1521.

Sullivan D. J., Henman, M. C., Moran, G. P., O'Neill, L. C., Bennett, D. E., Shanley, D. B., Coleman, D. C. (1996). Molecular genetic approaches to identification, epidemiology and taxonomy of non-*albicans Candida* species. *J Med Microbiol* **44**, 399-408.

Sullivan, D., Haynes, K., Bille, J., Boerlin, P., Rodero, L., Lloyd, S., Henman, M., Coleman, D. (1997). Widespread geographic distribution of oral *Candida dubliniensis* strains in human immunodeficiency virus-infected individuals. *J Clin Microbiol* **35**, 960-964.

Sullivan, D. & Coleman, D. (1997). *Candida dubliniensis*: an emerging opportunistic pathogen. *Curr Top Med Mycol* **8**, 15-25.

Sullivan, D. & Coleman, D. (1998). *Candida dubliniensis*: characteristics and identification. *J Clin Microbiol* **36**(2), 329-334.

Sullivan, D. J., Moran, G. P., Donnelly, S., Gee, S., Pinjon, E., McCartan, B., Shanley, D. B., Coleman, D. C. (1999). *Candida dubliniensis*: an update. *Rev Iberoam Micol* **16**, 72-76.

Symersky, J., Monod, M., Foundling, S. I. (1997). High-resolution structure of the extracellular aspartic proteinase from *Candida topicalis* yeast. *Biochemistry* **36**, 12700-12710.

Tamura, M., Watanabe, K., Imai, T., Mikami, Y., Nishimura, K. (2000). New PCR primer pairs specific for *Candida dubliniensis* and detection of the fungi from the *Candida albicans* clinical isolates in Japan. *Clin Lab* **46**, 33-40.

Teem, J. L., Abovich, N., Kaufer, N. F.. Schwindinger, W. F., Warner, J. R., Levy, A., Woolford, J., Leer, R. J., van Raamsdonk-Duin, M. M. C., Mager, W. H., Planta, R. J., Schultz, L., Friesen, J. D., Fried, H., Rosbash, M. (1994). A comparison of yeast ribosomal protein gene DNA sequences. *Nucleic Acids Res* **12**, 8295-8312.

Thompson, J. D., Higgins, D. G., Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673-4680.

Tietz, H.-J., Kussner, A., Thanos, M. et al. (1995). Phenotypic and genotypic characterisation of unusual vaginal isolates of *Candida albicans* from Africa. *J Clin Microbiol* **33**, 2462-2465.

Timmins, E. M., Howell, S. A., Alsberg, B. K., Noble, W. C., Goodacre, R. (1998). Rapid differentiation of closely related *Candida* species and strains by pyrolysis-mass spectrometry and Fourier transform-infrared spectroscopy. *J Clin Microbiol* **36**, 367-374.

Tintelnot, K., Haase, G., Seibold, M., Bergmann, F., Staemmler, M., Franz, T., Naumann, D. (2000). Evaluation of phenotypic markers for selection and identification of *Candida dubliniensis*. *J Clin Microbiol* **38**, 1599-1608.

Tobgi, S. T., Samaranayake, L. P., MacFarlane, T. W. (1987). The *in vitro* susceptibility of *Candida* species to lysozyme. *Oral Microbiol Immunol* **9**, 229-235.

Togni, G., Sanglard, D., Monod, M. (1994). Acid proteinase secreted by *Candida tropicalis*: virulence in mice of a proteinase negative mutant. *J Med Vet Mycol* **32**, 257-265.

Togni, G., Sanglard, D., Quadroni, M., Foundling, S. I., Monod, M. (1996). Acid proteinase secreted by *Candida tropicalis*: functional analysis of preproregion cleavages in C. tropicalis and *Saccharomyces cerevisiae*. *Microbiology* **142**, 493-503.

Van de Peer, Y., Van den Broeck, I., De Rijk, P., De Wachter, R. (1994). Database on the structure of small ribosomal subunit RNA. *Nuc Acids Res* **22**, 3488-3494.

Velegraki, A., Nicolatau, O., Theodoridou, M., Mostrou, G., Legakis, N. J. (1999). Paediatric AIDS-related linear gingival erythema: aform of erythematous candidiasis. *J Oral Pathol Med* **28**, 178-182.

Velegraki, A. & Logotheti, M. (1998). Presumptive identification of an emerging yeast pathogen: *Candida dubliniensis* (sp. nov.) reduces 2,3,5-triphenyltetrazolium chloride. *FEMS Immunol Med Microbiol* **20**, 239-241.

Voight, K., Cigelnik, E., O'Donnell, K. (1999). Phylogeny and PCR identification of clinically important zygomycetes based on nuclear ribosomal-DNA sequence data. *J Clin Microbiol* **37**, 3957-3964

Von Heijne, G. (1990). The signal peptide. *J Membr Biol* **115**, 195-201.

Warnock, D. W. (1992). Azole drug resistance in *Candida* species. *J Med Microbiol* **37**, 225-226.

Watts, H. J., Cheah, F. S., Hube, B., Sanglard, D., Gow, N. A. (1998). Altered adherence in strains of *Candida albicans* harbouring null mutations in secreted aspartic proteinase genes. *FEMS Microbiol Lett* **159**, 129-135.

Welch, M. D., Holtzman, D. A., Drubin, D. G. (1994). The yeast actin cytoskeleton. *Curr Opin Cell Biol* **6**, 110-119.

Wery, J., Dalderup, M. J. M., Ter Linde, J., Boekhouts, T., Van Ooyen, A. J. J. (1996). Structural and phylogenetic analysis of the actin gene from the yeast *Phaffia rhodozyma*. *Yeast* **12**, 641-651.

White, T. C., Miyasaki, S. H., Agabian, N. (1993). Three distinct secreted aspartyl proteinases in *Candida albicans*. *J Bacteriol* **175**, 6126-6133.

White T. C. & Agabian, N. (1995). *Candida albicans* secreted aspartyl proteinases: isoenzyme pattern is determined by cell type, and levels are determined by environmental factors. *J Bacteriol* **177**, 5215-5221.

Wickes, B. L., Hicks, J. B., Merz, W. G., Kwon-Chung, K. J. (1992). The molecular analysis of synonymy among medically important yeasts within the genus *Candida*. *J Gen Microbiol* **138**, 901-907.

Wildeman, A. G. (1988). A putative ancestral actin gene present in a thermophilic eukaryote: novel combination of intron positions. *Nuc Acids Res* **16**, 2553-2564.

Willis, A. M., Coulter, W. A., Sullivan, D. J., Coleman, D. C., Hayes, J. R., Bell, P. M., Lamey, P. J. (2000). Isolation of *C. dubliniensis* from insulin-using diabetes mellitus patients. *J Oral Pathol Med* **29**, 86-90.

Wingard, J. R., Merz, W. G., Rinaldi, M. G., Johnson, T. R., Karp, J. E., Saral, R. (1991). Increase in *Candida krusei* infection among patients with bone marrow transplantation and neutropaenia treated prophylactically with fluconazole. *N Engl J Med* **325**, 1274-1277.

Wingard, J. R., Merz, W. G., Rinaldi, M. G., Johnson, T. R., Karp, J. E., Saral, R. (1993). Association of *Torulopsis glabrata* infections with fluconazole prophylaxis in neutropenic bone marrow transplant patients. *Antimicrob Agents Chemother* **37**, 1847-1879.

Wingard, J. R. (1995). Importance of *Candida* species other than *C. albicans* as pathogens in oncology patients. *Clin Infect Dis* **20**, 115-125.

Wlodawer, A., Miller, M., Jaskolski, M., Sathyanarayana, B. K., Baldwin, E., Weber, I. T., Selk, L. M., clawson, L., Schneider, J., Kent, S. B. H. (1989). Conserved folding in retroviral proteases: crystal structure of a synthetic HIV-1 protease. *Science* **245**, 616-621.

Woese, C. R. (1987). Bacterial Evolution. *Microbiol Rev* **51**, 221-271.

Woese, C. R., Kandler, O., Whellis, M. L. (1990). Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. *Proc Natl Acad Sci USA* **87**, 4576-4579.

Wright, R. J., Carne, A., Hieber, A. D., Lamont, I. L., Emerson, G. W., Sullivan, P. A. (1992). A second gene for a secreted aspartate proteinase in *Candida albicans*. *J Bacteriol* **174**, 7848-7853.

Wu, T. & Samaranayake, L. P. (1999). The expression of secreted aspartyl proteinases of *Candida* species in human whole saliva. *J Med Microbiol* **48**, 711-720.

Wu, T., Samaranayake, L. P., Leung, W. K., Sullivan, P. A. (1999). Inhibition of growth and secreted aspartyl proteinase production in *Candida albicans* by lysozyme. *J Med Microbiol* **48**, 721-730.

Wu, T., Wright, K., Hurst, S. F., Morrison, C. J. (2000). Enhanced extracellular production of aspartyl proteinase, a virulence factor, by *Candida albicans* isolates following growth in subinhibitory concentrations of fluconazole. *Antimicrob Agents Chemother* **44**, 1200-1208.

Yamada, Y., Nagahama, T., Kawasaki, H., Banno, I. (1990a). The phylogenetic relationship of the genera *Phaffia* Miller, Yoneyama et Soneda and *Cryptococcus* Kutzing

emend. Phaff et Spencer (*Cryptococcaceae*) based on the partial sequences of 13S and 26S ribosomal ribonucleic acids. *J Gen Appl Microbiol* **36**, 403-414.

Yamada, Y., Nakagawa, Y., Banno, I. (1990b). The molecular phylogeny of the Q_{10}-equipped species of the heterobasidiomycetous yeast genus *Rhodosporidium* banno based on the partial sequences of 18S and 26S ribosomal ribonucleic acids. *J Gen Appl Microbiol* **36**, 435-444.

Zuckerkandl, E., & Pauling, L. (1965). Molecules as documents of evolutionary history. *J Theor Biol* **8**, 357-366.

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Phylogenetic analysis and rapid identification of *Candida dubliniensis* based on analysis of *ACT1* intron and exon sequences

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The phylogenetic position of *Candida dubliniensis* has previously been established on the basis of the sequence of rRNA genes. In order to confirm the relationship between *C. dubliniensis* and other yeast species, particularly *Candida albicans*, using non-rRNA gene sequences the *ACT1* gene was chosen for analysis. Three overlapping fragments that together span the entire *C. dubliniensis ACT1* gene (*CdACT1*) were amplified from a recombinant phage isolated from a genomic DNA λ library using PCR. These were cloned and used to determine the contiguous sequence of the gene. Analysis of the sequence data revealed the presence of a 1131 bp ORF interrupted by a single 632 bp intron at the 5′ extremity of the gene. Comparison of the *CdACT1* sequence with the *C. albicans* homologue (*CaACT1*) revealed that although the exons are 97·9% identical the introns are only 83·4% identical. Phylogenetic trees generated using *ACT1* exon and intron sequences from a range of yeast species unequivocally confirmed the phylogenetic position of *C. dubliniensis* as a unique taxon within the genus *Candida*. Analysis of the *ACT1*-associated intron sequences from 10 epidemiologically unrelated *C. dubliniensis* isolates from disparate geographical locations showed a very low level of intraspecies sequence variation. In order to develop an accurate and rapid method to identify *C. dubliniensis* from primary isolation plates the significant divergence between the *C. dubliniensis* and *C. albicans ACT1* intron sequences was exploited by designing *C. dubliniensis*-specific PCR primers. Using a rapid boiling method to produce template DNA directly from colonies from primary isolation plates in 10 min, these primers were used in a blind test with 122 isolates of *C. dubliniensis*, 53 isolates of *C. albicans*, 10 isolates of *C. stellatoidea* and representative isolates of other clinically relevant *Candida* and other yeast species. Only the *C. dubliniensis* isolates yielded the *C. dubliniensis*-specific 288 bp amplimer. Use of this technique on colonies suspected to be *C. dubliniensis* allows their correct identification as *C. dubliniensis* in as little as 4 h.

Keywords: *ACT1*, phylogenetics, *Candida dubliniensis*, PCR identification

INTRODUCTION

Candida dubliniensis is a yeast species first described in 1995 (Sullivan *et al.*, 1995). Although *C. dubliniensis* is phenotypically similar to *C. albicans*, the two species differ significantly at the genetic level. In particular, phylogenetic analysis of large- and small-subunit rRNA gene sequences provided the basis for the designation of *C. dubliniensis* as a separate species (Sullivan *et al.*, 1995, 1997; Gilfillan *et al.*, 1998). The first isolates identified as *C. dubliniensis* were recovered from the oral cavities of Irish human immunodeficiency virus (HIV)-infected individuals. However, over the last three

Abbreviations: HIV, human immunodeficiency virus.

The EMBL accession numbers for the nucleotide sequences reported in this paper are AJ236897 (*Candida dubliniensis* CD36), AJ237918 (*Candida tropicalis* NCPF 3111) and AJ237919 (*Candida stellatoidea* ATCC 11006).

years there have been increasing numbers of reports of the recovery of *C. dubliniensis* isolates by laboratories throughout the world, including Europe, North and South America, and Australia (Coleman *et al.*, 1997b; Sullivan *et al.*, 1997; Sullivan & Coleman, 1998; Salkin *et al.*, 1998; Kirkpatrick *et al.*, 1998). Although the majority of these isolates have been primarily associated with oral candidosis in HIV-infected and AIDS patients (Coleman *et al.*, 1997a), *C. dubliniensis* isolates have also been recovered form the oral cavities, gastrointestinal tracts and vaginas of HIV-negative individuals (Moran *et al.*, 1997, 1998; Sullivan & Coleman, 1998; Odds *et al.*, 1998). There have also been reports of *C. dubliniensis* isolates associated with systemic disease (Pinjon *et al.*, 1998; Meis *et al.*, 1999). The majority of clinical isolates of *C. dubliniensis* have been shown to be susceptible to commonly used antifungal drugs, including fluconazole (Moran *et al.*, 1997, 1998; Kirkpatrick *et al.*, 1998; Pfaller *et al.*, 1999). However, fluconazole resistance has been detected in clinical isolates (Moran *et al.*, 1997, 1998; Kirkpatrick *et al.*, 1998; Pfaller *et al.*, 1999) and isolates of *C. dubliniensis* susceptible to fluconazole can be readily induced to produce fluconazole-resistant derivatives following exposure to the drug *in vitro* (Moran *et al.*, 1997, 1998).

The prevalence of *C. dubliniensis* in the oral cavities of HIV-infected individuals and AIDS patients and reports of its association with disease in other body sites warrant in-depth epidemiological analysis. However, these investigations have been hampered by the lack of a simple, reliable method capable of unequivocally differentiating between *C. dubliniensis* and *C. albicans* in the clinical laboratory. Indeed, since *C. dubliniensis* and *C. albicans* share the ability to produce germ tubes and chlamydospores, features previously used for the definitive identification of *C. albicans*, it is likely that many isolates of *C. dubliniensis* have been misidentified as *C. albicans*. Investigations of our own collection of stored oral *Candida* isolates, originally identified as *C. albicans*, have shown that 1·8% of isolates recovered from asymptomatic normal healthy individuals and 16·5% of isolates recovered from HIV-infected individuals were in fact *C. dubliniensis* (Coleman *et al.*, 1997a). In a similar study, Odds *et al.* (1998) have recently shown that approximately 2% of a stored archival culture collection of 2500 yeast isolates, originally identified as *C. albicans*, was *C. dubliniensis*. They found that the prevalence of *C. dubliniensis* was significantly higher among HIV-infected individuals than among HIV-negative individuals (Odds *et al.*, 1998). Although first described in 1995, the earliest known *C. dubliniensis* isolates were recovered in the 1950s, thus predating the HIV pandemic. One of these strains, NCPF 3108, was recovered in the UK in 1957 and was originally deposited in the British National Collection for Pathogenic Fungi as *C. stellatoidea* (Sullivan *et al.*, 1995), while another strain, CBS 2747, which was recovered in the Netherlands in 1952, was originally deposited in the Centraal Bureau fur Schimmelcultures as *C. albicans* (Meis *et al.*, 1999).

A variety of tests have been developed to discriminate between *C. dubliniensis* and *C. albicans* based upon phenotypic characteristics. These include carbohydrate assimilation profiles and colonial coloration on differential media such as CHROMagar Candida and methyl blue-Sabouraud agar (Sullivan *et al.*, 1995, 1997; Coleman *et al.*, 1997a; Schoofs *et al.*, 1997). However, some of these assays have been shown to be unreliable in some instances and should only be used for the presumptive identification of *C. dubliniensis* from clinical specimens (Schoofs *et al.*, 1997; Sullivan & Coleman, 1998; Kirkpatrick *et al.*, 1998). The accuracy of *C. dubliniensis* isolate identification based on carbohydrate assimilation profiles has been improved by the recent inclusion of the assimilation profiles of some *C. dubliniensis* strains in the databases of commercially available yeast identification systems, including the API ID 32C and 20C AUX systems. It has been reported recently that *C. dubliniensis* and *C. albicans* can be distinguished on the basis of differential growth at 45 °C, with isolates of the former species unable to grow at this temperature (Pinjon *et al.*, 1998). However, in a recent study a significant number of *C. albicans* isolates were found to be unable to grow at this temperature (Kirkpatrick *et al.*, 1998). Currently, the most reliable tests available to differentiate between these species are based on molecular techniques such as DNA fingerprinting with repetitive-sequence-containing probes, randomly amplified polymorphic DNA (RAPD) analysis and pulsed-field gel electrophoresis (Sullivan *et al.*, 1995), but these are not suitable for the analysis of large sample numbers in routine diagnostic laboratories. However, since the differences between *C. dubliniensis* and *C. albicans* are most pronounced at the genetic level such differences should provide the basis for a specific and rapid identification test. One molecular technique with the required degree of specificity and ease of use is the polymerase chain reaction (PCR). This technology is increasingly available in diagnostic laboratories and due to its speed, reproducibility and high sample volume throughput is ideally suited for application to large numbers of clinical isolates.

The phylogenetic position of *C. dubliniensis* in relation to other yeast species has been established on the basis of the comparison of small- and large-subunit rRNA gene sequences (Sullivan *et al.*, 1995, 1997; Gilfillan *et al.*, 1998). In the present study we sought to confirm these phylogenetic relationships using sequences of non-rRNA gene origin. It was also hoped that these sequence data would lead to the identification of *C. dubliniensis*-specific nucleotide sequences that could be exploited in the design of a rapid PCR-based identification test. To achieve these goals the *ACT1* gene of *C. dubliniensis* was chosen for analysis. *ACT1* encodes actin, a protein that is abundant in all eukaryotic cells, where it is the major component of cytoplasmic microfilaments. Due to structural constraints the amino acid sequence of actin proteins from different eukaryotic species is highly conserved (Korn *et al.*, 1978; Hightower *et al.*, 1986; Pollard *et al.*, 1990; Hennessey *et al.*, 1993; Welch *et al.*,

1994). Since *C. albicans* and *C. dubliniensis* are very closely related it was anticipated that the *ACT1* genes of these species would be very similar. Results presented in this study for *C. dubliniensis* and in a previous study for *C. albicans* (Losberger & Ernst, 1989) showed that both *ACT1* genes contain a single class IV intron and it was anticipated that these intron sequences would be subject to less evolutionary conservation than the actin-protein-coding exons. Therefore we decided to investigate whether the exons and introns of *C. albicans* and *C. dubliniensis* would be sufficiently divergent to allow an accurate determination of the phylogenetic relationship between the two species and to allow the design of *C. dubliniensis*-specific primers suitable for rapid and specific identification of this species in the clinical laboratory using a rapid template DNA preparation procedure.

METHODS

***Candida* strains and culture media.** All *C. dubliniensis* strains were isolated by this laboratory or received from other laboratories and identified using the molecular and phenotypic methods described by Sullivan *et al.* (1995). All *Candida* strains and isolates were routinely grown on Potato Dextrose Agar (PDA, Oxoid) at pH 5·6 for 48 h at 37 °C. For liquid culture, *Candida* strains and isolates were grown at 37 °C in Yeast Peptone Dextrose Broth (YPD) in an orbital incubator (Gallenkamp) set at 150 r.p.m.

Bacterial strains and culture media. *Escherichia coli* DH5α was used as the host strain for phagemid pBluescript II KS(+/−) (Stratagene) and was maintained on Luria–Bertani (LB) agar, supplemented with 100 μg ampicillin ml^{-1} to maintain plasmids where appropriate. For liquid culture, strains harbouring plasmids were grown at 37 °C in LB broth containing 100 μg ampicillin ml^{-1} in an orbital incubator set at 150 r.p.m. Transformation of *E. coli* DH5α and identification of transformants containing recombinant plasmids were carried out by standard protocols (Sambrook *et al.*, 1989). *E. coli* LE 392 and its P2 phage lysogenic derivative (P2 392) were used for propagating the bacteriophage λ cloning vector EMBL3 and its recombinant derivatives. These strains were grown and maintained on LB agar supplemented with 10 mM $MgSO_4$ and 0·2% (w/v) maltose. Organisms for phage infection were grown in LB broth containing 10 mM $MgSO_4$ and 0·2% (w/v) maltose (Sambrook *et al.*, 1989).

Chemicals, enzymes, radioisotopes and oligonucleotides. Analytical-grade or molecular-biology-grade chemicals were purchased from Sigma-Aldrich, BDH or Boehringer Mannheim. Enzymes were purchased from Promega or Boehringer Mannheim and used according to the manufacturer's instructions. [α-^{32}P]dATP (3000 Ci $mmol^{-1}$; 110 TBq $mmol^{-1}$) was purchased from Amersham. Custom-synthesized oligonucleotides were purchased from Genosys Biotechnologies (Europe).

DNA extraction procedures. Plasmid DNA for restriction endonuclease digestion and Southern hybridization was prepared by the alkaline lysis method described by Sambrook *et al.* (1989). Plasmid DNA for sequencing was prepared using a Quantum Prep Plasmid Miniprep kit (Bio-Rad). Total cellular DNA from *Candida* isolates was prepared as described by Gallagher *et al.* (1992). High-molecular-mass total cellular DNA from *C. dubliniensis* for the construction of a genomic library was isolated by the method described by Bennett *et al.* (1998). *Candida* template DNA for use in PCR experiments with the *C. dubliniensis*-specific oligonucleotide primer pair DUBF/DUBR (Table 1) was prepared as follows. A single colony from a culture grown for 48 h at 37 °C on PDA or CHROMagar Candida medium (CHROMagar Candida, Paris, France) was suspended in 50 μl sterile distilled water. Cell suspensions were boiled for 10 min and the lysed cells subjected to a clearing spin for 5 min at 20000 ***g***. Template DNA contained in 25 μl supernatant was used for PCR amplification.

Table 1. PCR primers used in this study

Primer	Sequence	Nucleotide co-ordinates*	RE site†
C. albicans			
5′F	5′-CGGAATTCCTTAGAAACATTATCTCGAT-3′	−49 to −30	*Eco*RI
5′R	5′-GCTCTAGAGAGAAATATTATGTCGACAA-3′	126 to 145	*Xba*I
ACTF	5′-CGGAATTCAATGGACGGTGGTATGTT-3′	−1 to 17	*Eco*RI
ACTR	5′-CGGAATTCAATGGATGGACCAGATTCGTCG-3′	1746 to 1767	*Eco*RI
3′F	5′-CGGAATTCTAAGATTATTGCTCCACCAG-3′	1641 to 1660	*Eco*RI
3′R	5′-GCTCTAGACCAGATTTCCAGAATTTCAC-3′	1792 to 1811	*Xba*I
INTF	5′-CGGAATTCAATGGACGGTGGTATGGT-3′	−1 to 17	*Eco*RI
INTR	5′-CGGAATTCGAGCGTCGTCACCGGC-3′	724 to 739	*Eco*RI
C. dubliniensis			
DUBF	5′-GTATTTGTCGTTCCCCTTTC-3′	251 to 270	
DUBR	5′-GTGTTGTGTGCACTAACGTC-3′	519 to 538	

*Primers were complementary to *ACT1* gene sequences as follows: *CaACT1*, accession no. X16377 (Losberger & Ernst, 1989); and *CdACT1*, accession no. AJ236897 (this study). Nucleotide co-ordinates shown are numbered in the 5′ to 3′ direction with the first base of the translation start codon being +1.

†Restriction endonuclease recognition sequence included within the primer sequence (underlined).

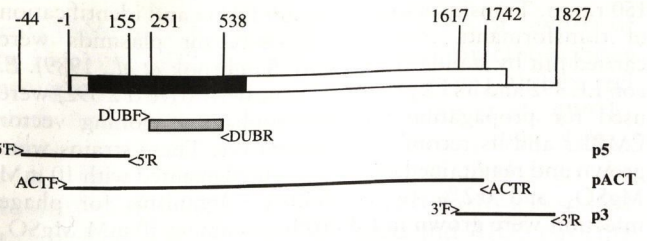

Fig. 1. Schematic diagram of the *C. dubliniensis ACT1* gene (*CdACT1*), which is represented by the large horizontal box. The black area corresponds to the position of the intron at the 5′ end of the gene. The thin horizontal lines in the lower part of the figure represent sequences amplified from the *CdACT1*-encoding recombinant phage λCDACT1 using the primer pairs 5′F/5′R, ACTF/ACTR and 3′F/3′R (Table 1). The names of the recombinant plasmids obtained when these amplimers were cloned in pBluescript are shown on the right. The location of sequences amplified with the *C. dubliniensis*-specific primer pair DUBF/DUBR is indicated by the shaded box. The nucleotide co-ordinates of the sequences contained in each amplimer relative to *CdACT1* sequences are shown at the top (numbering the *CdACT1* ATG start codon +1).

```
Ca    GTATGTTTTAATTTAGCTTCAATTCTAATTGATTGATTAATCAGTTGATTGGTTTCAATA
Cd    GTATGTTTATATTTAACTTAGATT-TAATTGATTGATTAATCAGTTGGATGATTTCATTT
      ********  ***** ***  *** ********************  ** ***** *

Ca    TGACAAA-------TGGGTAG-----GGTGGGAAAACTTCAT-TTTCAATTCAGATCAAA
Cd    CGATAGAGTGTTGTTGTTTAGATCTGGGTGGGAAAAGAACCCATTTCCATTCAGATCAAG
       ** * *       **  ***      **********   *   **** ***********

Ca    CTTTTTTGTTGTCGACATAATATTTCTCGTTTGGGATGTTACTGTCACATTAATAATACA
Cd    -TTTTTTGTTGTCGACATAATATTTCTCGTTTGGGATGTTACTGTCACATTA-----ACA
      ************************************************     ***

Ca    CACACATCAGCTTATAATTTTGAAAGTAATTTATCAGATATGTTGTGACGATCAATGGAA
Cd    CACA----AGCTTATAATTTTGAA-GTGGTACATCAGG--AGTT-TGACTACCATTGGAT
      ****    ********** ** *  *****    *** **** * ** ****

Ca    ATGGCTAACTTCAATGTATCTGTTCTTCCCCTTTTTCAAAGTTCACGTTT-*-TTTGATT
Cd    GTGTTCCAATTTAGTGTATTTGTCGTTCCCCTTTC---AATTTCGTGTTTAAGTTTAATT
       **    * ** * ***** ***  *********    ** ***  ****   *** **

Ca    GATTGATTGATTGATCTGTCGGCAGTGGTTTCAAAACCATTCGGTGAGTAATCCTATCAA
Cd    GATTGATTGATTGATCTGTCGGCAATGGTTTCAAA-CCATTCGGTGAATAATATCATTGA
      ************************ ********** ********** ****   **  *

Ca    TCAATGTTACGACAAAAGGCTCAATATTCAAAATTGCAATGTTTTATGTTTTCCTACGTG
Cd    TCAA--TTAAAAAACAAGGTTTAATACTTCAA-TGACAATGTTTAATGTTTTTCAACAAG
      ****  ***  * * **** * **** *  ** *  ******** ******* * **  *

Ca    TACTTGTGCAAGGCAATTGATTCAACATTGCTTTTGGTGTTTGACGAGTTTCTAGTTTGG
Cd    CGTTTGTGCAAATCAATTGATTCATGATTGCCTTTGATGTT-GACGAGTTTCCAATTTCG
         ********  **********  ***** **** **** ********** * *** *

Ca    ACTTGTGTTGTTATCTGGACTATA-CAGATTTCCCGGCTCACTATGAATTTTTTTTTTCG
Cd    AGTTCTG--GTTATCTGACCTATAACAGATTTCC-GGTTCATTGTAAAT-----TTTTCG
      * ** **  ********  ***** ********* ** *** * * ***      ******

Ca    ACGCTCAGTGCACACAACTATAAACAACACAAACACAAACACAGCAAGAAAAAAAAAAAC
Cd    ACG-TTAGTGCACACAAC--------ACACAA---CAAAAACAGCAACAAAAAAAA----
      *** * ***********          ******   **** ******* ********

Ca    GAACATTGAATTGAAACCAAGCCAACTGAAA-AATTCC------TTATTTAAATGACTGT
Cd    -AATATTGTATTGAAACCAA--CAACTGCAACAAGTCCCCTTTTTTTTTTTTAATGACTGT
       ** ***** **********  ****** ** ** ***       ** *** *********

Ca    CATACTAACCCATTTTT-ATAG
Cd    CGTACTAACCCATTTTTTATAG
      * *************** ****
```

Fig. 2. Comparison of nucleotide sequences of the *C. albicans* and *C. dubliniensis ACT1*-associated introns. The *C. albicans* sequence was from strain ATCC 10123 (GenBank accession no. X16377; Losberger & Ernst, 1989) and the *C. dubliniensis* sequence from strain CD36 (EMBL accession no. AJ236897; this study). The 5′ intron consensus sequence (GTATG), the 3′ intron consensus sequence (YAG) and the branchpoint sequence (TACTAAC) are in bold type. Asterisks indicate identical nucleotides and dashes indicate deletions. Sequences of the *C. dubliniensis*-specific primers DUBF/DUBR (Table 1) are underlined.

Large-scale *E. coli* phage lysates were prepared according to the plate method of Sambrook *et al.* (1989) and recombinant phage DNA was purified from phage preparations using a Wizard Lambda Preps kit (Promega).

Construction of a *C. dubliniensis* CD36 genomic DNA library. The DNA library was constructed by ligating *Sau*3A-generated partial digestion products of *C. dubliniensis* CD36 chromosomal DNA >10 kb in size with *Bam*HI-generated pre-prepared λ bacteriophage replacement vector EMBL3 arms (Promega) followed by packaging *in vitro* into pre-prepared phage heads and tails (Promega) according to the manufacturer's instructions. Previous studies have shown that DNA fragments ranging in size from 9 to 23 kb can be cloned into the EMBL3 vector (Frischauf *et al.*, 1983). The packaged recombinant phage particles were propagated on the *E. coli* lysogenic strain P2 392. A recombinant library containing 2·0 $\times 10^5$ p.f.u. was obtained.

Recombinant phages were propagated on *E. coli* LE 392 to yield ~ 1000 p.f.u. per 90 mm Petri plate and were transferred from the plaques onto nitrocellulose membrane filters (Schleicher & Schuell) by overlaying the plaques with the filters, which were then screened by plaque hybridization (Sambrook *et al.*, 1989) using α-^{32}P-labelled DNA probes.

DNA hybridization. Probe DNA used in screening the *C. dubliniensis* genomic DNA library and in Southern hybridization experiments was labelled with [α-^{32}P]dATP (3000 Ci mmol^{-1}; 110 TBq mmol^{-1}) by random priming with a random hexanucleotide primer labelling kit (Prime-a-Gene, Promega). All hybridizations were performed under conditions of high stringency (65 °C), as described by Sambrook *et al.* (1989).

PCR isolation of *ACT1*-associated introns. Amplification of *ACT1*-associated intron sequences from *Candida* strains was performed in 100 µl reaction mixtures containing 100 pmol each of the forward and reverse primers, INTF/INTR (Table 1), 250 µM deoxynucleotide triphosphates, 2·0 mM $MgSO_4$, 20 mM Tris/HCl (pH 8·8 at 25 °C), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0·1% (v/v) Triton X-100, 1 U $Vent_R$ DNA polymerase (New England Biolabs) and 500 ng template DNA. PCR reactions were performed in a DNA Thermal Cycler (Perkin Elmer Cetus) with 30 cycles of 1 min at 94 °C, 1 min at 52 °C and 1 min at 72 °C, followed by 72 °C for 10 min. The amplimers were purified using the Wizard PCR Preps DNA Purification system (Promega) and cloned into pBluescript using standard procedures (Sambrook *et al.*, 1989).

DNA sequencing. This was performed by the dideoxy chain-terminating method of Sanger *et al.* (1977) using an automated Applied Biosystems 370A DNA sequencer and dye-labelled terminators (ABI Prism Dye Terminator Cycle Sequencing Ready Reaction kit, Applied Biosystems). Searches of the GenBank database for nucleotide sequence similarities were performed using the BLAST family of computer programs (Altschul *et al.*, 1990). Nucleotide sequence alignments were carried out using the CLUSTAL W sequence analysis program (Thompson *et al.*, 1994).

PCR identification of *C. dubliniensis*. PCR identification of *C. dubliniensis* using the *C. dubliniensis*-specific primer pair DUBF/DUBR (Table 1) was carried out in a 50 µl final volume containing 10 pmol each of the forward and reverse primers, 2·5 mM $MgCl_2$, 10 mM Tris/HCl (pH 9·0 at 25 °C), 10 mM KCl, 0·1% (v/v) Triton X-100, 2·5 U *Taq* DNA polymerase

(Promega) and 25 µl template-DNA-containing cell supernatant (prepared as described above). Each reaction mixture also contained 10 pmol each of the universal fungal primers RNAF/RNAR (Fell, 1993), which amplify approximately 610 bp from all fungal large-subunit rRNA genes and were used as an internal positive control. Cycling conditions consisted of 6 min at 95 °C followed by 30 cycles of 30 s at 94 °C, 30 s at 58 °C, 30 s at 72 °C, followed by 72 °C for 10 min. Amplification products were separated by electrophoresis through 2·0% (w/v) agarose gels containing 0·5 µg ethidium bromide ml^{-1} and were visualized on a UV transilluminator.

RESULTS

Isolation and sequence analysis of the *C. dubliniensis ACT1* gene

A *C. dubliniensis* CD36 genomic library, constructed in bacteriophage λ EMBL3, was screened by plaque hybridization with a radioactively labelled probe consisting of the entire *C. albicans ACT1* (*CaACT1*) gene cloned into pBR322 (p1002, a gift from B. Magee, University of Minnesota). Approximately 10000 recombinant plaques were screened, from which 10 p1002-reactive plaques were detected. The plaque with the strongest signal was selected and subjected to single-plaque purification. DNA was extracted from the recombinant phage, termed λCDACT1, and restriction endonuclease digestion analysis and Southern hybridization analysis showed that λCDACT1 contained a DNA insert of approximately 15 kb. Attempts to subclone smaller hybridizing fragments from the cloned insert DNA of λCDACT1 into the vector phagemid pBluescript, to facilitate the sequencing of the *C. dubliniensis ACT1* (*CdACT1*) gene, failed. A number of p1002-reactive recombinant plasmids were obtained. However, upon further investigation these were shown to contain both *ACT1* and EMBL3 vector homologous sequences, and no recombinant plasmids harbouring only *ACT1*-homologous DNA were obtained. It was concluded that the *ACT1*-homologous insert DNA from λCDACT1 was

Table 2. Yeast species and strains used in the phylogenetic analysis of *C. dubliniensis*

Yeast strain*	*ACT1* intron sequence†	Reference
C. albicans		
132A	This study	Gallagher *et al.* (1992)
179B	This study	Gallagher *et al.* (1992)
ATCC 10123	X16377	Losberger & Ernst (1989)
C. dubliniensis		
CD36 (Ireland)	AJ236897; this study	Sullivan *et al.* (1995)
CD91 (Ireland)	This study	This study
CD70 (UK)	This study	Sullivan *et al.* (1997)
NCPF 3108 (UK)	This study	Sullivan *et al.* (1995)
CD93 (Finland)	This study	This study
94191 (Spain)	This study	Pinjon *et al.* (1998)
P2 (Switzerland)	This study	Boerlin *et al.* (1995)
CD71 (Argentina)	This study	Sullivan *et al.* (1997)
CM2 (Australia)	This study	Sullivan *et al.* (1995)
CD92 (Canada)	This study	This study
C. glabrata		
ATCC 90876	AF069746	Unpublished data submitted to GenBank
C. stellatoidea		
ATCC 11006	AJ237919; this study	Kwon-Chung *et al.* (1989)
303530	This study	bioMérieux‡
303531	This study	bioMérieux‡
C. tropicalis		
NCPF 3111	AJ237918; this study	NCPF catalogue
K. lactis		
J7	M25826	Deshler *et al.* (1989)
S. cerevisiae		
A364A	L00026	Gallwitz & Sures (1980)

*Abbreviations: ATCC, American Type Culture Collection, (Manassas, VA, USA); NCPF, National Collection of Pathogenic Fungi, Bristol, UK. The country of origin of the *C. dubliniensis* isolates is shown in parentheses.

†Accession numbers are for the EMBL/GenBank nucleotide sequence databases.

‡From the culture collection of bioMérieux, St Louis, MO, USA.

unstable when cloned in pBluescript. Therefore it was decided to amplify *CdACT1* from the recombinant phage by PCR using a mixture containing *Taq* polymerase and the proof-reading polymerase *Pwo* (Expand high-fidelity PCR system, Boehringer) and three primer sets homologous to regions of the *CaACT1* gene and flanking sequences, including 5′F/5′R, ACTF/ACTR and 3′F/3′R (Table 1, Fig. 1). The three amplimers, containing overlapping sequences, obtained following PCR with these primers were cloned into pBluescript to yield recombinant plasmids p5, pACT and p3, respectively (Fig. 1). The insert DNA cloned in p5 and p3 was sequenced fully in both directions using universal primers, while the insert DNA cloned in pACT was sequenced fully by primer walking. These three overlapping sequences yielded a contiguous sequence of 1827 bp revealing an ORF of 1131 bp interrupted by a single 632 bp intron at the 5′ end (Fig. 1). The overall nucleotide sequence identity between this ORF (*CdACT1*) and the *CaACT1* gene was 90·6%. This divergence is mainly due to differences between the intron sequences, which are 83·4% identical (Fig. 2), while the spliced coding sequences, which are identical in length in both species, are 97·9% identical. The differences between the exon sequences correspond to a total of 24 base changes. However, only one of these base substitutions [A → G, at position 660, numbering the sequences in the 5′–3′ direction from the first base (+1) of the translation start codon of *CdACT1*], results in a change in the predicted amino acid sequence, a conservative substitution from isoleucine to valine. At 632 bp the *CdACT1* intron is 25 bp shorter than the corresponding sequence in *CaACT1*; however, it is situated in exactly the same position at the 5′ end of the gene and is recognizable by the presence of yeast intron consensus sequences (Fig. 2). These are the 5′ consensus sequence GTATG, the 3′ consensus sequence YAG, and the branchpoint sequence TACTAAC located near the 3′ end of the intron which is essential for efficient splicing (Mount, 1982; Langford *et al.*, 1984; Leer *et al.*, 1984; Molenaar *et al.*, 1984; Teem *et al.*, 1984).

In order to determine the level of intraspecies intron sequence conservation in epidemiologically unrelated isolates from geographically divergent parts of the world the introns of nine additional *C. dubliniensis* isolates, two additional *C. albicans* isolates (132A and 179B) and three *C. stellatoidea* isolates (Table 2) were amplified using the primer set INTF/INTR, which were complementary to *CaACT1* sequences flanking the intron (Table 1). The intron sequences of the 10 *C. dubliniensis* isolates tested, including CD36 (Table 2), were found to be very highly conserved, with only one or two base changes found within each isolate. Similar intraspecies sequence conservation was observed with the *C. albicans* and *C. stellatoidea* strains studied.

Phylogenetic analysis based on *ACT1* sequences

The *ACT1* gene has been used extensively to infer interspecies relationships across broad evolutionary distances (Zakut *et al.*, 1982; Mertins & Gallwitz, 1987; Wildeman *et al.*, 1988; Fletcher *et al.*, 1994; Cox *et al.*, 1995; Wery *et al.*, 1996). This part of the study was undertaken to confirm the phylogenetic position of *C. dubliniensis* in relation to other yeast species using *ACT1* sequences. This is the first time that the phylogeny of *C. dubliniensis* has been investigated using non-rRNA gene sequences. Since the *ACT1* gene of many yeast species contains highly conserved (i.e. exon) and less well-conserved (i.e. intron) sequences, these regions were compared separately. Firstly, the *ACT1* spliced coding sequences of *C. albicans*, *C. dubliniensis*, *C. glabrata*, *Kluyveromyces lactis* and *Saccharomyces cerevisiae*, obtained in this study or from the databases (Table 3), were compared using the CLUSTAL W sequence alignment software package. Secondly, the *ACT1*-associated intron sequences from selected strains of *C. albicans*, *C. dubliniensis*, *C. stellatoidea*, *C. tropicalis*, *C. glabrata* and *K. lactis* (Table 4) were obtained either from GenBank or following amplification using the INTF/INTR primer set (Table 1) and also compared using CLUSTAL W. An evolutionary distance matrix for each group of sequences was generated incorporating corrections for multiple base changes according to the method of Jukes & Cantor (1969) (Tables 3 and 4). These data indicated that the *C. dubliniensis* coding and intron sequences differ from the corresponding *C. albicans* sequences by 2·1% and 16·6%, respectively. Evolutionary trees constructed using the neighbour-joining method of Saitou & Nei (1987) based on these data are shown in Fig. 3. These trees and the bootstrap values determined for each node unequivocally confirmed the unique species designation of *C. dubliniensis* and its phylogenetic position in relation to the other yeast species, including *C. albicans*. In addition, these data also confirm that *C. albicans* and *C. stellatoidea* are

Table 3. Genetic distance matrix based on comparison of *ACT1* gene coding sequences

Values correspond to percentages of difference corrected for multiple base changes by the method of Jukes & Cantor (1969). The *ACT1* gene coding sequences used were as follows: *C. albicans* ATCC 10123 (X16377; Losberger & Ernst, 1989); *C. dubliniensis* CD36 (AJ236897; this study); *S. cerevisiae* A364A (L00026; Gallwitz & Sures, 1980); *C. glabrata* NCPF 90876 (AF069746; O. Kurzai and others, unpublished data) and *K. lactis* J7 (M25826; Deshler *et al.*, 1989). Neither the *C. tropicalis* nor the *C. stellatoidea* *ACT1* coding sequences are currently available in the databases, so they could not be compared with the sequences of the other yeast species used to construct the matrix.

	C. al.	*C. du.*	*S. ce.*	*C. gl.*	*K. la.*
C. albicans	–				
C. dubliniensis	2·1	–			
S. cerevisiae	12·9	12·6	–		
C. glabrata	13·2	12·3	9·8	–	
K. lactis	13·2	12·6	10·1	7·8	–

Table 4. Genetic distance matrix based on comparison of *ACT1*-associated intron sequences

Values correspond to percentages of difference corrected for multiple base changes by the method of Jukes & Cantor (1969). The intron sequences used were as follows: *C. albicans* ATCC 10123 (X16377; Losberger & Ernst, 1989); *C. stellatoidea* ATCC 11006 (AJ237919, this study); *C. dubliniensis* CD36 (AJ236897; this study); *C. tropicalis* NCPF 3111 (AJ237918, this study); *C. glabrata* NCPF 90876 (AF069746; unpublished data submitted to GenBank) and *K. lactis* J7 (M25826; Deshler *et al.*, 1989). The sequence of the *S. cerevisiae ACT1*-associated intron (L00026; Gallwitz & Sures, 1980) was not included in the construction of the matrix because it was only 308 bp in length, significantly shorter than the intron sequences of the other yeasts studied, and so valid genetic distance determinations with this sequence and the others used to construct the matrix could not be made.

	C. al.	*C. st.*	*C. du.*	*C. tr*	*C. gl.*	*K. la.*
C. albicans	–					
C. stellatoidea	0·2	–				
C. dubliniensis	16·6	16·6	–			
C. tropicalis	43·4	43·5	47·1	–		
C. glabrata	54·8	55·0	57·1	54·0	–	
K. lactis	58·1	58·3	54·7	61·4	63·1	–

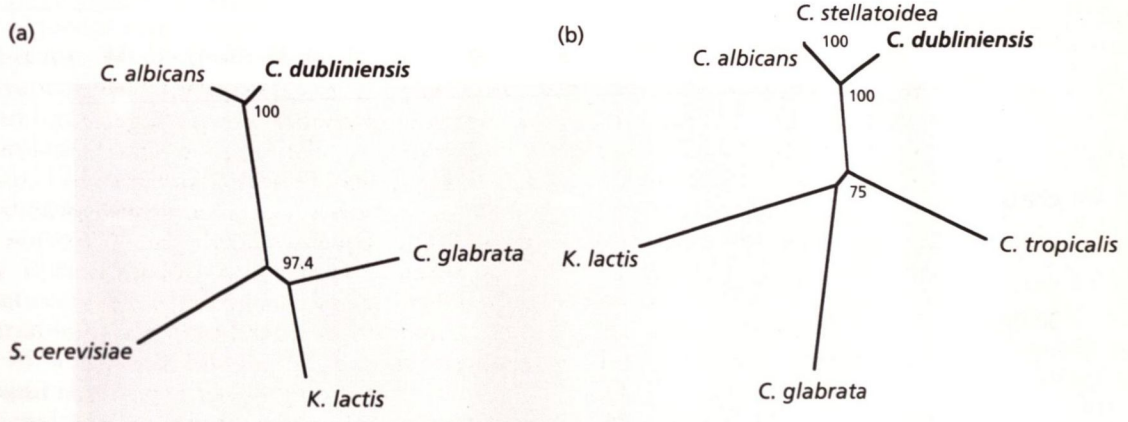

Fig. 3. Unrooted phylogenetic neighbour-joining trees generated from the alignment of the *ACT1*-exon (a) and -intron (b) sequences of *C. dubliniensis* and other yeast species. Numbers at the nodes were generated by bootstrap analysis (Felsenstein, 1985) and represent the percentage of times the arrangement occurred in 1000 randomly generated trees. The sequences used to construct the trees are indicated in the legends to Tables 3 and 4.

so closely related as to be considered a single species (Kamiyama *et al.*, 1989; Sullivan *et al.*, 1995).

PCR-based identification of *C. dubliniensis*

Because of the many phenotypic similarities shared by *C. albicans* and *C. dubliniensis* it is not easy to discriminate between isolates of these species in the clinical laboratory. However, examination of an alignment of the *ACT1*-associated intron sequences of these two species (Fig. 2) and the observation that they differ by 16·6% (Table 4) suggested that this significant sequence divergence could be exploited as a means to identify *C. dubliniensis* accurately and rapidly in combination with a rapid template DNA preparation procedure. PCR primers specific for the *C. dubliniensis* intron (DUBF/DUBR; Table 1, Fig. 1) were synthesized and used to amplify a DNA fragment of 288 bp from *C. dubliniensis* template DNA obtained by boiling cells from a single 48 h colony suspended in 50 µl water for 10 min. PCR reactions also contained the fungal universal primers RNAF/RNAR (Fell, 1993), which amplify a product of approximately 610 bp from the fungal large-subunit rRNA gene and serve as an internal positive control. While all fungal species should produce a product of approximately 610 bp with the RNAF/RNAR primers, only *C. dubliniensis* isolates should yield the 288 bp amplimer with the DUBF/DUBR primer set. The *C. dubliniensis*-specific primer pair DUBF/DUBR was tested in a blind trial using template DNA

Table 5. Yeast species used in PCR identification experiments with the *C. dubliniensis*-specific primers DUBF/DUBR

Species	No. of isolates	Reference(s)
C. albicans	53	This study
		Pinjon *et al.* (1998); Jabra-Rizk *et al.* (1999)
*C. dubliniensis**	122	This study; Sullivan *et al.* (1995); Sullivan *et al.* (1997); Coleman *et al.* (1997a); Moran *et al.* (1997, 1998); Pinjon *et al.* (1998); Jabra-Rizk *et al.* (1999); Pujol *et al.* (1997)
C. glabrata	1	Haynes & Westerneng (1996)
C. kefyr	1	NCPF 3234
C. krusei	1	Haynes & Westerneng (1996)
C. norvegensis	1	NCPF 3860
C. parapsilosis	4	This study
C. sake	1	NCPF 8360
C. stellatoidea	1	ATCC 11006
	9	This study
C. tropicalis	1	NCPF 3111
T. beigelii	1	NCPF 3857

* One hundred and fourteen of the *C. dubliniensis* isolates were recovered from oral specimens, five were recovered from faecal specimens and one each was recovered from a vaginal, sputum and a post-mortem lung specimen. The isolates were recovered from different countries as follows: Argentina, 1 isolate; Australia, 2; Belgium, 5; Canada, 6; France, 4; Germany, 4; Greece, 1; Ireland, 48; Scandinavia, 4; Spain, 5; Switzerland, 4; UK, 17; USA, 21.

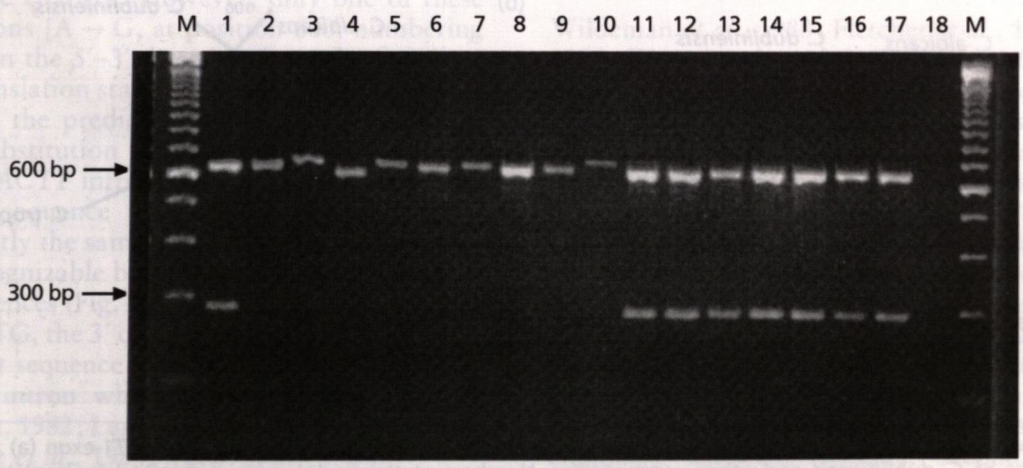

Fig. 4. Agarose gel electrophoresis of PCR-amplified DNA products generated using the *C. dubliniensis*-specific primers DUBF/DUBR (Table 1, Fig. 2) and the universal fungal primers RNAF/RNAR (Fell, 1993) with template DNA from yeast strains. The profiles shown correspond to: the *C. dubliniensis* type strain CD36 (lane 1); *C. albicans* (lane 2); *C. glabrata* (lane 3); *C. kefyr* (lane 4); *C. krusei* (lane 5); *C. norvegensis* (lane 6); *C. sake* (lane 7); *C. stellatoidea* (lane 8); *C. tropicalis* (lane 9); *Trichosporon beigelii* (lane 10); *C. dubliniensis* American isolate (lane 11); *C. dubliniensis* Argentinian isolate (lane 12); *C. dubliniensis* Australian isolate (lane 13); *C. dubliniensis* Canadian isolate (lane 14); *C. dubliniensis* French isolate (lane 15); *C. dubliniensis* German isolate (lane 16); *C. dubliniensis* UK isolate (lane 17). A negative control in which no template DNA was used in the PCR reaction was also included (lane 18). The 288 bp *C. dubliniensis*-specific amplimer generated by the DUBF/DUBR primers is present in lane 1 and lanes 11–17. Lanes marked M contain 100 bp size reference markers.

from the yeast isolates listed in Table 5 as follows: *C. albicans* ($n=53$), *C. dubliniensis* ($n=122$), *C. glabrata* ($n=1$), *C. kefyr* ($n=1$), *C. krusei* ($n=1$), *C. norvegensis* ($n=1$), *C. parapsilosis* ($n=4$), *C. sake* ($n=1$), *C. stellatoidea* ($n=10$), *C. tropicalis* ($n=1$) and *Trichosporon beigelii* ($n=1$). All 196 yeast isolates yielded an amplimer of approximately 610 bp, but only the *C. dubliniensis* isolates yielded the 288 bp amplimer. Fig. 4 shows examples of the PCR amplimers obtained with representative strains belonging to a variety of different yeast species, including epidemiologically unrelated *C. dubliniensis* isolates from disparate geographical locations. Use of this PCR test in conjunction with the rapid template DNA preparation procedure used here means that a *C. dubliniensis* isolate can be identified unequivocally in less than 4 h.

DISCUSSION

Because C. *dubliniensis* was only described recently it is important to further investigate and confirm its taxonomic and phylogenetic relationship to other medically important yeast species, especially the closely related C. *albicans*. The close relationship between C. *dubliniensis* and C. *albicans* has resulted in difficulties in developing rapid and reliable identification techniques capable of definitively discriminating between the two species. As a result, in-depth epidemiological studies on the prevalence of this organism have been hampered. There is an urgent requirement for a test which can rapidly and definitively identify C. *dubliniensis* directly following primary culture from clinical specimens. Such a test must be simple to use, inexpensive, easily accessible to clinical diagnostic laboratories and suitable for application to large numbers of samples.

The objectives of this study were to investigate the phylogenetic relationship of C. *dubliniensis* to other yeast species using non-rRNA gene sequences, and to develop a rapid identification technique for this organism. To achieve these objectives the *ACT1* gene was chosen for investigation, firstly because it is ideal for inferring phylogenetic relationships due to its high degree of sequence conservation in all eukaryotes, and secondly because it is unusual among yeast genes in that it is interrupted by an intron in most yeasts studied. Cloning and gene sequence analysis revealed that the overall similarity between the C. *dubliniensis* and C. *albicans ACT1* genes is 90·6%. Comparison of the spliced coding sequences of the two species revealed that the exon sequences are 97·9% identical. This level of homology is comparable to the percentage sequence identity between the two species reported previously for the V3 variable region of the large-subunit rRNA gene (97·52–97·75%; Sullivan *et al.*, 1995, 1997) and the small-subunit rRNA gene (98·6%; Gilfillan *et al.*, 1998). The predicted C. *dubliniensis* ACT1 protein sequence was identical to that of C. *albicans*, apart from a single conservative substitution. A phylogenetic tree generated from nucleotide comparisons of *ACT1* coding sequences from C. *dubliniensis* and a variety of yeast species showed that C. *dubliniensis* was grouped separately from C. *albicans* and the other yeast species in 100% of trees generated (Fig. 3a). These studies represent the first phylogenetic investigation of C. *dubliniensis* based on non-rRNA gene sequences, and they unequivocally confirm its unique position as a separate taxon within the genus *Candida* as determined previously by comparative rRNA gene sequence analysis (Sullivan *et al.*, 1995, 1997; Gilfillan *et al.*, 1998). In contrast to the highly conserved nature of the C. *dubliniensis* and C. *albicans ACT1* exon sequences there was considerable divergence (16·6%) between the *ACT1*-associated introns of the two species. When these and the *ACT1*-associated intron sequences from a number of other yeast species were used to generate a second phylogenetic tree (Fig. 3b) the unique position of C. *dubliniensis* as a separate taxon within the genus *Candida* was affirmed. These results also confirmed that C. *dubliniensis* is most closely related to C. *albicans*. In addition, the C. *albicans* and C. *stellatoidea ACT1*-associated introns were found to differ by one basepair substitution, corresponding to a 0·2% sequence divergence. These findings provided further evidence that C. *albicans* and C. *stellatoidea* should be considered as the same species. This situation is analogous to that found between *S. cerevisiae* and *S. carlsbergensis*, where the *ACT1*-associated introns differ by one basepair deletion and one basepair substitution, and it is accepted that these two organisms are in fact the same species (Nellen *et al.*, 1981). The C. *tropicalis* intron sequence differs from that of C. *albicans* by 43·4%, confirming that it is more distantly related to C. *albicans* than C. *dubliniensis* (Table 4, Fig. 3b). All of these findings indicate that the *ACT1*-associated intron sequences are not subject to the same level of evolutionary constraint as the *ACT1* coding sequences.

The *ACT1* genes of fungal species, in general, are noteworthy because of the presence of introns (Gallwitz & Sures, 1980; Fidel *et al.*, 1988; Wildeman *et al.*, 1988; Deshler *et al.*, 1989; Losberger & Ernst, 1989; Fletcher *et al.*, 1994; Cox *et al.*, 1995; Matheucci *et al.*, 1995; Wery *et al.*, 1996). At present, most known introns can be assigned unambiguously to one of four classes, depending on the intron structure and location (Krainer & Maniatis, 1988). *ACT1*-associated introns belong to class IV, which are nuclear pre-mRNA introns. The C. *dubliniensis ACT1*-associated intron is located at the 5′ end of the gene, where it interrupts the fourth codon. The *ACT1* genes of C. *albicans*, C. *glabrata*, *S. cerevisiae* and *K. lactis* all contain introns located at this codon (Losberger & Ernst, 1989; O. Kurzai and others, unpublished data submitted directly to GenBank – accession no. AF069746); Gallwitz & Sures, 1980; Deshler *et al.*, 1989). This position is conserved amongst fungi, as all fungal actin genes that contain an intron do so at the third, fourth or fifth codon. Three conserved sequence elements have also been identified in the nuclear pre-mRNA introns of yeasts, at the 5′ and 3′ splice sites and at a site within the intron near the 3′ splice site, known as the branchpoint sequence. All three conserved elements have been shown to be important for the accurate and efficient splicing of introns in *S. cerevisiae* (Langford *et al.*, 1984; Leer *et al.*, 1984; Molenaar *et al.*, 1984; Teem *et al.*, 1984; Mount, 1982). The C. *dubliniensis*, C. *albicans*, C. *stellatoidea* and C. *tropicalis ACT1*-associated introns possess all three conserved elements, namely GTATG (5′ consensus), TAG (3′ consensus) and TACTAAC (branchpoint), (this study, Fig. 2; Losberger & Ernst, 1989). These sequences are also present in C. *glabrata* and *K. lactis* although the 3′ consensus sequence is CAG (Deshler *et al.*, 1989; see GenBank accession no. AF069746 for the C. *glabrata ACT1*-intron sequence).

One striking feature of the C. *dubliniensis* introns was that they showed little intraspecies variation, even among isolates from geographically divergent locations. The small changes which were recorded consisted of single base changes, some of which were shared by more

than one strain, and deletions which occurred at the end of poly(T) and poly(A) stretches. Introns containing these deletions were sequenced on separate occasions using different preparations of template DNA to rule out the possibility of sequencing or amplification artefacts. We concluded that these deletions are genuine and are probably the result of slipped-strand mispairing during replication. Similar intraspecies sequence conservation was observed with the *ACT1*-associated introns from *C. albicans* and *C. stellatoidea*. Boucher *et al.* (1996) made similar findings with their analysis of the group I self-splicing intron present in the large-subunit rRNA gene, in which the intron is present in a similar location in *C. albicans*, *C. stellatoidea* and *C. dubliniensis*. Again there was no significant intraspecies variation in the intron sequence. Furthermore, the *C. albicans* self-splicing intron and that of *C. stellatoidea* showed a high degree of homology, differing only by three single basepair substitutions. They also found that the homology between the *C. albicans* and *C. dubliniensis* group I introns (*CaLSU* and *CdLSU*, respectively) was quite high except for two regions of divergence contained in two stem–loop regions, both of which are much longer in *C. dubliniensis* than in *C. albicans*. These two regions lie outside the catalytic core, and although they are predicted to have a more complex secondary structure than those of *C. albicans* they do not affect the self-splicing ability of the intron. Our analysis of the *C. dubliniensis* and *C. albicans ACT1*-associated introns showed that although identical conserved elements are present in both species, nucleotide differences accounting for a 16·6% sequence divergence were dispersed throughout the length of the intron (Fig. 2). With group I introns, conservation of the nucleotide sequence may be important as it dictates the secondary structure of the intron and therefore its self-splicing ability. However, with group IV introns, such as the *C. dubliniensis* and *C. albicans ACT1*-associated introns, the splicing event is mediated by the spliceosome and although maintenance of the three conserved elements is important for splicing there do not appear to be any other constraints upon conservation of the nucleotide sequence. This may explain why divergence between the *C. dubliniensis* and *C. albicans ACT1*-associated introns sequences is dispersed throughout the intron.

Genotypic tests such as DNA fingerprinting analysis, karyotype analysis and RFLP analysis have been used in the differentiation of *C. dubliniensis* and *C. albicans* isolates. However, these techniques cannot be easily applied to the analysis of large numbers of clinical isolates. In contrast, PCR, which may be applied to the detection of genetic differences, is rapid and relatively inexpensive. The low level of intraspecies sequence variation in the *C. dubliniensis ACT1*-associated intron, and the extent of divergence from the *C. albicans ACT1*-associated intron sequence, suggested that this region could provide the basis for the design of oligonucleotide primers capable of readily discriminating between isolates of both species using PCR. In order to facilitate the rapid processing of large numbers of samples and to decrease the time required, template DNA was prepared by boiling a single fresh *Candida* colony suspended in 50 µl water. With template DNA preparation taking approximately 15 min, PCR amplification 2·5 h and electrophoresis of amplimers 1 h, *C. dubliniensis* colonies can be identified in as little as 4 h. Thus *C. dubliniensis* colonies can be identified directly from primary isolation plates without the requirement for additional subculture. This technique is particularly effective when used for the identification of presumptive *C. dubliniensis* isolates cultured from clinical specimens on CHROMagar Candida medium. The *C. dubliniensis*-specific primers were tested with a collection of 122 isolates of *C. dubliniensis* in a blind trial with isolates of 10 other yeast species, including 53 isolates of *C. albicans* and 10 isolates of *C. stellatoidea* (Table 5). The primers correctly identified only the *C. dubliniensis* isolates, with 100% accuracy. Recently, Mannarelli & Kurtzman (1998) also developed a PCR-based identification test for discriminating between *C. albicans* and *C. dubliniensis* isolates; however, these primers were only tested with seven *C. dubliniensis* isolates. Another study by Elie *et al.* (1998) reported the development of a *C. dubliniensis* probe, specific for the internal transcribed spacer region (ITS2) of the ribosomal gene cluster. However, this probe has been tested with a very small sample number ($n=5$) and the method itself involves a PCR-enzyme-linked immunoassay, which is relatively time consuming. In contrast, our method is a simple and rapid technique capable of identifying suspect colonies directly from a primary isolation plate. In addition it has been evaluated against a large number of isolates from diverse geographical locations. Our findings clearly demonstrate that PCR identification based upon the *ACT1*-associated intron sequence is a definitive and rapid technique for the identification of *C. dubliniensis*.

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REFERENCES

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* 215, 403–410.

Bennett, D. E., McCreary, C. E. & Coleman, D. C. (1998). Genetic characterization of a phospholipase C gene from *Candida*

albicans: presence of homologous sequences in *Candida* species other than *C. albicans*. *Microbiology* **144**, 55–72.

Boerlin, P., Boerlin-Petzold, F., Durussel, C., Addo, M., Pagani, J.-L., Chave, J.-P. & Bille, J. (1995). Cluster of atypical *Candida* isolates in a group of human immunodeficiency virus-positive drug users. *J Clin Microbiol* **33**, 1129–1135.

Boucher, H., Mercure, S., Montplaisir, S. & Lemay, G. (1996). A novel group I intron in *Candida dubliniensis* is homologous to a *Candida albicans* intron. *Gene* **180**, 189–196.

Coleman, D. C., Sullivan, D. J., Bennett, D. E., Moran, G. P., Barry, H. J. & Shanley, D. B. (1997a). Candidiasis: the emergence of a novel species, *Candida dubliniensis*. *AIDS* **11**, 557–567.

Coleman, D., Sullivan, D., Harrington, B., Henman, M., Shanley, D., Bennett, D., Moran, G., McCreary, C. & O'Neill, L. (1997b). Molecular and phenotypic analysis of *Candida dubliniensis*: a recently identified species linked with oral candidosis in HIV-infected and AIDS patients. *Oral Dis* **3** (Supplement 1), S96–S101.

Cox, G. M., Rude, T. H., Dykstra, C. C. & Perfect, J. R. (1995). The actin gene from *Cryptococcus neoformans*: structure and phylogenetic analysis. *J Med Vet Mycol* **33**, 261–266.

Deshler, J. O., Garrett, L. P. & Rossi, J. J. (1989). *Kluyveromyces lactis* maintains *Saccharomyces cerevisiae* intron-encoded splicing signals. *Mol Cell Biol* **9**, 2208–2213.

Elie, C. M., Lott, T. J., Reiss, E. & Morrison, C. J. (1998). Rapid identification of *Candida* species with species-specific DNA probes. *J Clin Microbiol* **36**, 3260–3265.

Fell, J. (1993). Rapid identification of yeast species using three primers in a polymerase chain reaction. *Mol Mar Biol Biotechnol* **3**, 174–180.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.

Fidel, S., Doonan, J. H. & Morris, N. R. (1988). *Aspergillus nidulans* contains a single actin gene which has unique intron locations and encodes a gamma actin. *Gene* **70**, 283–293.

Fletcher, L. D., McDowell, J. M., Tidwell, R. R., Meagher, R. B. & Dykstra, C. C. (1994). Structure, expression and phylogenetic analysis of the gene encoding actin 1 in *Pneumocystis carinii*. *Genetics* **137**, 743–750.

Frischauf, A. M., Lehrach, H., Poustka, A. & Murray, N. (1983). Lambda replacement vectors carrying polylinker sequences. *J Mol Biol* **170**, 827–842.

Gallagher, P. J., Bennett, D. E., Henman, M. C., Russell, R. J., Flint, S. R., Shanley, D. B. & Coleman, D. C. (1992). Reduced azole susceptibility of *Candida albicans* from HIV-positive patients and a derivative exhibiting colony morphology variation. *J Gen Microbiol* **138**, 1901–1911.

Gallwitz, D. & Sures, I. (1980). Structure of a split yeast gene: complete nucleotide sequence of the actin gene in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci USA* **77**, 2546–2550.

Gilfillan, G. D., Sullivan, D. J., Haynes, K., Parkinson, T., Coleman, D. C. & Gow, N. A. R. (1998). *Candida dubliniensis*: phylogeny and putative virulence factors. *Microbiology* **144**, 829–838.

Haynes, K. A. & Westerneng, T. J. (1996). Rapid identification of *Candida albicans*, *C. glabrata*, *C. parapsilosis* and *C. krusei* by species-specific PCR of large subunit ribosomal DNA. *J Med Microbiol* **44**, 390–396.

Hennessy, E. S., Drummond, D. R. & Sparrow, J. C. (1993). Molecular genetics of actin function. *Biochem J* **282**, 657–671.

Hightower, R. C. & Meagher, R. B. (1986). The molecular evolution of actin. *Genetics* **114**, 315–332.

Jabra-Rizk, M. A., Baqui, A. A., Kelley, J. I., Falkler, W. A. J., Merz, W. G. & Meiller, T. F. (1999). Identification of *Candida dubliniensis* in a prospective study of patients in the United States. *J Clin Microbiol* **37**, 321–326.

Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, vol. 3, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.

Kamiyama, A., Niimi, M., Tokunaga, M. & Nakayama, H. (1989). DNA homology between *Candida albicans* strains: evidence to justify the synonymous status of *Candida stellatoidea*. *Mycopathologia* **107**, 3–7.

Kirkpatrick, W. R., Revankar, S. G., McAtee, R. K. & 7 other authors (1998). Detection of *Candida dubliniensis* in oropharyngeal samples from human immunodeficiency virus-infected patients in North America by primary CHROMagar Candida screening and susceptibility testing of isolates. *J Clin Microbiol* **36**, 3007–3012.

Korn, E. D. (1978). Biochemistry of actomyosin-dependent cell motility (a review). *Proc Natl Acad Sci USA* **75**, 588–599.

Krainer, A. R. & Maniatis, T. (1988). RNA splicing. In *Transcription and Splicing*, pp. 131–206. Edited by B. D. Hames & D. M. Glover. Washington DC: IRL Press.

Kwon-Chung, K. J., Riggsby, W. S., Uphoff, R. A., Hicks, J. B., Whelan, W. L., Reiss, E., Magee, B. B. & Wickes, B. L. (1989). Genetic differences between type I and type II *Candida stellatoidea*. *Infect Immun* **57**, 527–532.

Langford, C. J., Klinz, F., Donath, C., Gallwitz, D. (1984). Point mutations identify the conserved intron-contained TACTAAC box as an essential splicing signal sequence in yeast. *Cell* **36**, 645–653.

Leer, R. J., van Raamsdonk-Duin, M. M. C., Hagendoorn, M. J. M., Mager, W. H. & Planta, R. J. (1984). Structural comparison of yeast ribosomal protein genes. *Nucleic Acids Res* **12**, 6685–6700.

Losberger, C. & Ernst, J. F. (1989). Sequence of the *Candida albicans* gene encoding actin. *Nucleic Acids Res* **17**, 9488.

Mannarelli, B. M. & Kurtzman, C. P. (1998). Rapid identification of *Candida albicans* and other human pathogenic yeasts by using short oligonucleotides in a PCR. *J Clin Microbiol* **36**, 1634–1641.

Matheucci, E., Jr, Henrique-Silva, F., El-Gogary, S., Rossini, C. H. B., Leite, A., Escobar Vera, J., Carle Urioste, C., Crivellaro, O. & El-Dorry, H. (1995). Structure, organization and promoter expression of the actin-encoding gene in *Trichoderma reesei*. *Gene* **161**, 103–106.

Meis, J. F., Ruhnke, M., De Pauw, B. E., Odds, F. C., Siegert, W. & Verweij, P. E. (1999). *Candida dubliniensis* candidemia in patients with chemotherapy-induced neutropenia and bone marrow transplantation. *Emerging Infect Dis* **5**, 150–153.

Mertins, P. & Gallwitz, D. (1987). A single intronless actin gene in the fission yeast *Schizosaccharomyces pombe*: nucleotide sequence and transcripts formed in homologous and heterologous yeast. *Nucleic Acids Res* **15**, 7369–7379.

Molenaar, C. M. T., Woudt, L. P., Jansen, A. E. M., Mager, W. H., Planta, R. J., Donovan, D. M. & Pearson, N. J. (1984). Structure and organization of two linked ribosomal protein genes in yeast. *Nucleic Acids Res* **12**, 7245–7358.

Moran, G. P., Sullivan, D. J., Henman, M. C., McCreary, C. E., Harrington, B. J., Shanley, D. B. & Coleman, D. C. (1997). Antifungal drug susceptibilities of oral *Candida dubliniensis* isolates from human immunodeficiency virus (HIV)-infected and non-HIV-infected subjects and generation of stable fluconazole-resistant derivatives *in vitro*. *Antimicrob Agents Chemother* **41**, 617–623.

Moran, G. P., Sanglard, D., Donnelly, S. M., Shanley, D. B.,

Sullivan, D. J. & Coleman, D. C. (1998). Identification and expression of multidrug transporters responsible for fluconazole resistance in *Candida dubliniensis*. *Antimicrob Agents Chemother* **42**, 1819–1830.

Mount, S. M. (1982). A catalogue of splice junction sequences. *Nucleic Acids Res* **10**, 459–472.

Nellen, W., Donath, C., Moos, M. & Gallwitz, D. (1981). The nucleotide sequences of the actin genes from *Saccharomyces carlsbergensis* and *Saccharomyces cerevisiae* are identical except for the introns. *J Mol Appl Genet* **1**, 239–244.

Odds, F. C., Van Nuffel, L. & Dams, G. (1998). Prevalence of *Candida dubliniensis* isolates in a yeast stock collection. *J Clin Microbiol* **6**, 2869–2873.

Pfaller, M. A., Messer, S. A., Gee, S., Joly, S., Pujol, C., Sullivan, D. J., Coleman, D. C. & Soll, D. R. (1999). *In vitro* susceptibilities of *Candida dubliniensis* isolates tested against the new triazole and echinocandin antifungal agents. *J Clin Microbiol* **37**, 870–872.

Pinjon, E., Sullivan, D., Salkin, I., Shanley, D. & Coleman, D. (1998). Simple, inexpensive, reliable method for differentiation of *Candida dubliniensis* from *Candida albicans*. *J Clin Microbiol* **36**, 2093–2095.

Pollard, T. D. (1990). Actin. *Curr Opin Cell Biol* **2**, 33–40.

Pujol, C., Renaud, F., Mallie, M., de Meeus, T. & Bastide, J.-M. (1997). Atypical strains of *Candida albicans* recovered from AIDS patients. *J Med Vet Mycol* **35**, 115–121.

Saitou, N. & Nei, M. (1987). The neighbour joining method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Salkin, I. F., Pruitt, W. R., Padhye, A. A., Sullivan, D., Coleman, D. & Pincus, D. H. (1998). Distinctive carbohydrate assimilation profiles used to identify the first clinical isolates of *Candida dubliniensis* recovered from the United States. *J Clin Microbiol* **36**, 1467.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**, 5463–5467.

Schoofs, A., Odds, F. C., Colebunders, R., Ieven, M. & Goosens, H. (1997). Use of specialised isolation media for recognition and identification of *Candida dubliniensis* isolates from HIV-infected patients. *Eur J Clin Microbiol Infect Dis* **16**, 296–300.

Sullivan, D. & Coleman, D. (1998). *Candida dubliniensis*: characteristics and identification. *J Clin Microbiol* **36**, 329–334.

Sullivan, D. J., Westerneng, T. J., Haynes, K. A., Bennett, D. E. & Coleman, D. C. (1995). *Candida dubliniensis* sp. nov.: phenotypic and molecular characterisation of a novel species associated with oral candidosis in HIV-infected individuals. *Microbiology* **141**, 1507–1521.

Sullivan, D., Haynes, K., Bille, J., Boerlin, P., Rodero, L., Lloyd, S., Henman, M. & Coleman, D. (1997). Widespread geographic distribution of oral *Candida dubliniensis* strains in human immunodeficiency virus-infected individuals. *J Clin Microbiol* **35**, 960–964.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.

Teem, J. L., Abovich, N., Kaufer, N. F. & 12 other authors (1984). A comparison of yeast ribosomal protein gene DNA sequences. *Nucleic Acids Res* **12**, 8295–8312.

Welch, M. D., Holtzman, D. A. & Drubin, D. G. (1994). The yeast actin cytoskeleton. *Curr Opin Cell Biol* **6**, 110–119.

Wery, J., Dalderup, M. J., Ter Linde, J., Boekhout, T. & Van Ooyen, A. J. J. (1996). Structural and phylogenetic analysis of the actin gene from the yeast *Phaffia rhodozyma*. *Yeast* **12**, 641–651.

Wildeman, A. G. (1988). A putative ancestral actin gene present in a thermophilic eukaryote: novel combination of intron positions. *Nucleic Acids Res* **16**, 2553–2564.

Zakut, R., Shanai, M., Givol, D., Neuman, S., Yaffe, D. & Nudel, U. (1982). Nucletoide sequence of the rat skeletal muscle actin gene. *Nature* **298**, 857–859.

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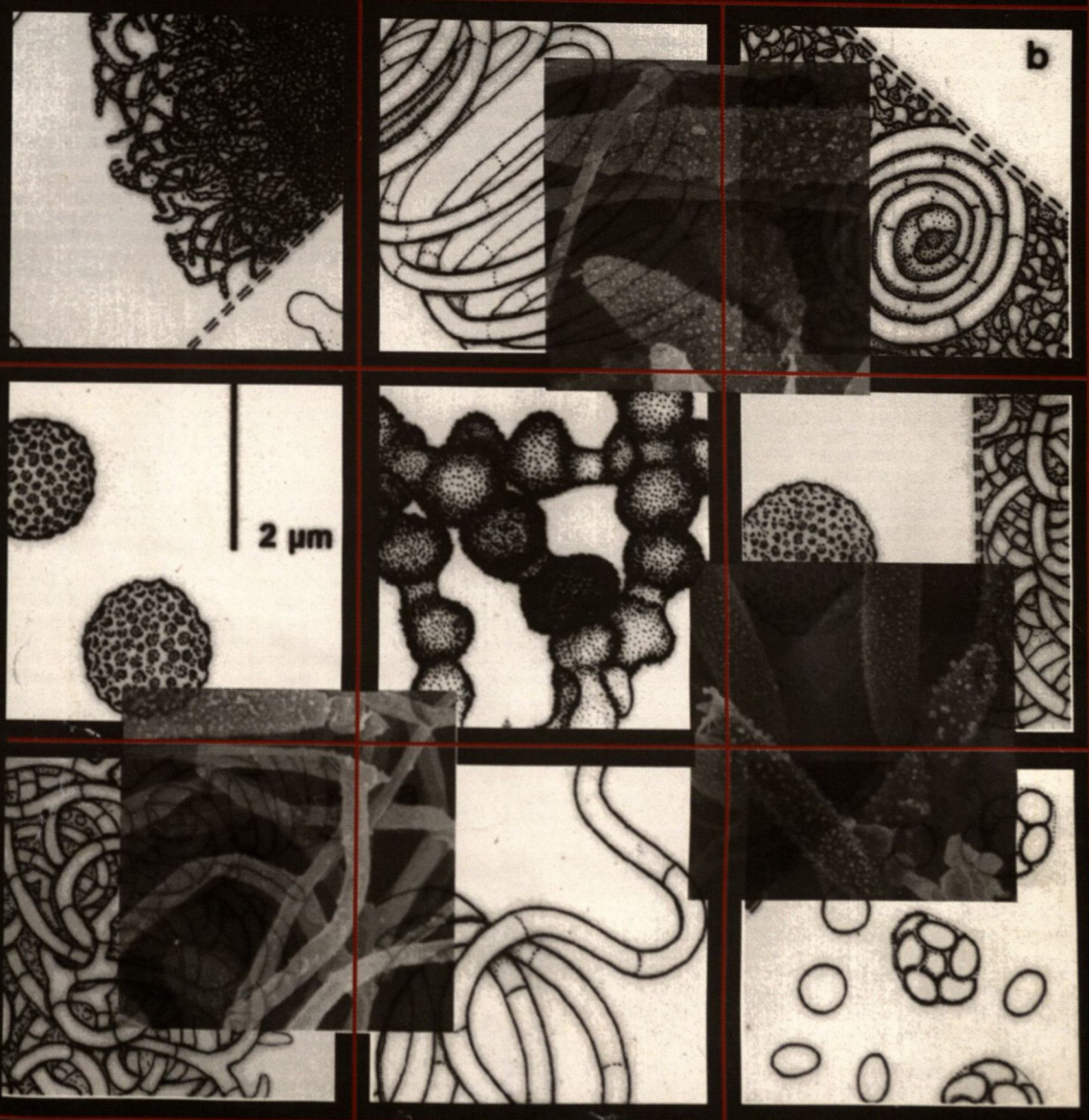

References

1. Collins LA, Samore MH, Roberts, *et al.* Risk factors for invasive fungal infections complicating orthotopic liver transplantation. J Infect Dis 1994; 170: 644-652
2. Paya CV. Fungal infections in solid-organ transplantation. Clin Infect Dis 1993; 16: 677-688.
3. Singh N, Arnow PM, Bonham A, *et al.* Invasive aspergillosis in liver transplant recipients in the 1990s. Transplantation 1997; 64: 716-720.
4. Patel R, Portela D, Badley AD, *et al.* Risk factors of invasive *Candida* and *non-Candida* fungal infections after liver transplantation. Transplantation 1996; 62: 926-934.
5. Georgopapadakou NH, Walsh TJ. Human mycoses: drugs and targets for emerging pathogens. Science 1994; 264: 371-373
6. Manez R, Martin M, Raman D, *et al.* Fluconazol therapy in transplant recipients receiving FK506. Transplantation 1994; 57: 1521-35.
7. Hibberd PL, Rubin RH. Clinical aspects of fungal infection in organ transplant recipients. Clin Infect Dis 1994; 19 (Suppl.1): S33-S40.
8. Kullberg, B.J. Trends in immunotherapy of fungal infections. Eur J Clin Microbiol Infect Dis 1997; 16: 51-55.
9. Adler-Moore JP, Proffitt RT. Development, characterization, efficacy and mode of action of AmBisome, a unilamellar liposomal formulation of amphotericin B. J Liposom Res 1993; 3: 429-450.
10. Boswell GW, Buell D, Bekersky I. AmBisome (liposomal amphotericin B): a comparative review. J Clin Pharmacol 1998; 38: 583-592.
11. Braun F, Rüchel R, Lorf T, *et al.* Is liposomal amphotericin B (AmBisome) an effective prophylaxis of mycotic infections after liver transplantation? Transplant Proc 1998; 30: 1481-1483.
12. Castaldo P, Stratta RJ, Wood P, *et al.* Clinical spectrum of fungal infections after orthotopic liver transplantation. Arch Surg 1991; 126: 149-156.
13. Mora NP, Cofer JB, Solomon H, *et al.* Analysis of severe infections (INF) after 180 consecutive liver transplants: the impact of amphotericin B prophylaxis for reducing the incidence and severity of fungal infections. Transplant Proc 1991; 23: 1528-1530.
14. Mora NP, Klintmalm G, Solomon H, Goldstein RM, Gonwa TA, Husberg BS. Selective amphotericin B prophylaxis in the reduction of fungal infections after liver transplant. Transplant Proc 1992; 24: 154-155.
15. Singh N, Mieles L, Yu VL, Gayowski T. Invasive aspergillosis in liver transplant recipients: association with candidemia and consumption coagulopathy and failure of prophylaxis with low-dose amphotericin B. Clin Infect Dis 1993; 17: 906-908.
16. Tollemar J, Hockerstedt K, Ericzon BG, Jalanko H, Ringden O. Liposomal amphotericin B prevents invasive fungal infections in liver transplant recipients. A randomized, placebo-controlled study. Transplantation 1995; 59: 45-50.
17. Verweij PE, Dompeling EC, Donnelly JP, Schattenberg AV, Meis JF. Serial monitoring of *Aspergillus* antigen in the early diagnosis of invasive aspergillosis. Preliminary investigations with two examples. Infection 1997; 25: 86-89.
18. Bretagne S, Marmorat-Khuong A, Kuentz M, Latgé JP, Bart-Delabesse E, Cordonnier C. Serum *Aspergillus* galactomannan antigen testing by sandwich ELISA: Practical Use in Neutropenic Patients. J Infect 1997;35:7-15.
19. Singh N, Gayowski T, Wagener M, Marino IR, Yu VL. Pulmonary infections in liver transplant recipients receiving tacrolimus. Transplantation 1996; 61: 396-401.
20. Hadley S, Samore MH, Lewis WD, Jenkins RL, Karchmer AW, Hammer SM. Major infectious complications after orthotopic liver transplantation and comparison of outcomes in patients receiving cyclosporine or FK506 as primary immunosuppression. Transplantation 1995, 59: 851-859.
21. Patel R, Paya CV. Infections in solid-organ transplant recipients. Clin Microbiol Rev 1997; 10: 86-124.
22. Ringdén O, Tollemar J. Liposomal amphotericin B (AmBisome®) treatment of invasive fungal infections in immuncompromised children. Mycoses 1993; 36: 187-192.
23. Simon C, Stille W.Antimykotika. In: Antibiotika-Therapie in Klinik und Praxis. (9 Ed.) Stuttgart, Schattauer,1997.
24. Prentice H.G , Hann I M, Herbrecht, R *et al.* A randomized comparison of liposomal versus conventional amphotericin B for the treatment of pyrexia of unknown origin in neutropenic patients. Br J Haematol 1997; 98: 711-718.

Candida dubliniensis: An update

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The increased incidence of fungal infections during the last decade has been well-documented [1-4]. Given that one of the most important factors contributing to this phenomenon is the increased numbers of immunocompromised individuals, it is perhaps not surprising that species previously not associated with human disease and novel species previously unknown to science have been identified as potential pathogens (e.g., *Penicillium marneffei* [5], *Emmonsia pasteuriana* [6] and *Candida dubliniensis* [7]).

C. dubliniensis was first identified as a new species in 1995 [7]. As its name suggests this species was originally described in Dublin, Ireland. While performing an epidemiological investigation of oral candidosis in Irish HIV-infected individuals and AIDS patients in the early 1990s it was discovered that some germ tube- and chlamydospore-positive isolates, which were identified as *Candida albicans* on the basis of these characteristics, failed to hybridize efficiently with the *C. albicans*-specific DNA fingerprinting probe 27A [7,8]. Subsequent in-depth analysis of these organisms revealed that they constituted a distinct species clearly separate from, but closely related to, *C. albicans* [7]. In the intervening four years *C. dubliniensis* isolates have been identified in a range of clinical settings by many laboratories throughout the world [9-19].

The purpose of this short article is to review briefly the most recent data available on *C. dubliniensis*. In particular we wish to highlight the advances being made in the development of rapid and accurate tests to allow the discrimination of *C. dubliniensis* from other *Candida* species, especially *C. albicans*. With the introduction of these tests we hope that many other laboratories will be encouraged to search for this species in clinical specimens and culture collections and thus provide further information concerning the epidemiology and the true clinical significance of this newly identified opportunistic pathogen.

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Phenotypic characteristics

C. dubliniensis is closely related to and shares many phenotypic characteristics with *C. albicans* [7]. This close similarity has hindered differentiation between the two species in the clinical laboratory. Both species produce germ tubes and chlamydospores, features previously associated solely with, and used for the definitive identification of, *C. albicans*. It has been reported that *C. dubliniensis* strains can differ from *C. albicans* in that they often produce chlamydospores more readily and more abundantly on Rice agar Tween (RAT), Tween 80-oxgall-caffeic acid (TOC) or cornmeal agar [7,13,20]. However, this unusual chlamydospore presentation has not been shown to be reproducible in some laboratories [14,21]. In a recent study describing North American *C. dubliniensis* isolates it was shown that 16 of 23 (70%) *C. dubliniensis* isolates produced abundant chlamydospores, however, 1 of 28 (3.6%) *C. albicans* isolates examined also exhibited a similar phenotype [14]. Thus, while examination of chlamydospore production may be of some use as a confirmatory identification test for *C. dubliniensis* it should not be used as a primary means of identification. Comparative growth analysis at elevated temperatures such as 42°C and 45°C has also been suggested as a means of discriminating *C. dubliniensis* from *C. albicans* [7,22]. While all *C. dubliniensis* isolates tested so far do not grow at 45°C there is some confusion as to what proportion of *C. albicans* isolates can grow at this temperature. In our laboratory we have found that only 1 of 100 *C. albicans* isolates tested failed to grow at 45°C [22]. However, in another study it has been shown that 10 out of the 28 (36%) *C. albicans* isolates tested also failed to grow at this temperature [14]. The reason for this discrepancy is not clear, but may be a reflection of the inaccuracy of temperature readings and heat distribution in many laboratory incubators. Whatever the reasons, it would again appear that absence of growth at 45°C should only be used as a confirmatory test in conjunction with one or more other identification tests.

The recent introduction of the chromogenic medium CHROMagar Candida has proven to be particularly helpful in the identification of *C. dubliniensis* isolates, particularly following primary culture from clinical specimens. While *C. albicans* colonies are a light blue/green colour on this medium *C. dubliniensis* colonies are a much darker green colour [20,21,23]. This colour is particularly pronounced if plates are incubated for longer than 48 h (e.g., up to 72 h). Although CHROMagar Candida has been widely used in the identification of primary clinical isolates of *C. dubliniensis* it has been reported that the ability of *C. dubliniensis* to produce its distinctive dark green colour can be lost following subculture and storage [21]. One of the earliest observations

which suggested that *C. dubliniensis* was distinct from *C. albicans* was based on comparative analysis of substrate assimilation profiles using commercially available yeast identification kits such as the bioMérieux API ID 32C and API 20C AUX systems [7]. The data generated using these kits revealed that the range of carbohydrates assimilated by *C. albicans* and *C. dubliniensis* was significantly different. From these and other studies it is evident that *C. dubliniensis* isolates, unlike the great majority of *C. albicans* isolates, are unable to assimilate methyl-α-D-glucoside, lactate or xylose [7,14,24]. In addition, *C. dubliniensis* grows much more slowly than *C. albicans* when trehalose is the only source of carbon. The recent inclusion of many specific *C. dubliniensis* carbohydrate assimilation profiles in the databases of the API ID 32C and API 20C AUX kits will certainly aid the identification of this species. *C. dubliniensis* and *C. albicans* can also be distinguished using a variety of other commercially available yeast identification techniques, including the RapID Yeast Plus, VITEK YBC and VITEK 2 ID-YST systems [25]. One interesting characteristic exhibited by *C. dubliniensis* is that cells grown at 37°C on Sabouraud's dextrose agar have the ability to coaggregate *in vitro* with cells of the oral bacterial species *Fusobacterium nucleatum* [26]. *C. albicans* cells grown under the same conditions fail to coaggregate with this species. The clinical significance of this finding is not clear, however, the authors who first described this phenomenon suggest that a test which they have developed to distinguish *C. dubliniensis* from *C. albicans* based on this phenomenon is rapid, specific and inexpensive [26].

C. dubliniensis isolates have also been discriminated from *C. albicans* using a number of more sophisticated techniques. Firstly, Bikandi *et al.* have developed a *C. dubliniensis*-specific antiserum [9]. In this study, antiserum raised against *C. dubliniensis* was adsorbed with *C. albicans* blastospores and subsequently used in an indirect immunofluorescence assay. In this test the antiserum reacted with blastospores and germ tubes of *C. dubliniensis*, but not with *C. albicans* blastospores, suggesting that there are differences in the cell wall architecture of the two species. Interestingly, the antiserum also reacted, albeit weakly, with *C. albicans* germ tubes and hyphae. However, this did not interfere with the results obtained in a blind trial when the antiserum correctly discriminated between 83 *C. dubliniensis* and 43 *C. albicans* isolates. This test is very rapid and specific, however, its potential for widespread use is limited by the availability of the antiserum and the necessity to use immunofluorescence microscopy. Other tests which allow the discrimination of *C. dubliniensis* and *C. albicans* include pyrolysis mass spectrometry (PyMS) and Fourier transform infrared (FT-IR) spectroscopy [27]. However, the technology required to perform these techniques is not widely available thus precluding their usefulness in routine clinical diagnostic laboratories.

Genotypic characteristics

The first isolates now known to be *C. dubliniensis* were first noticed and distinguished from *C. albicans* isolates because of their unusual DNA fingerprint patterns generated using the *C. albicans*-specific DNA fingerprinting probe 27A [7,8]. That there are significant differences in the chromosomal arrangement of sequences in each species was confirmed using a wide range of DNA profiling techniques, including fingerprinting with oligonucleotides homologous to microsatellite sequences, pulsed-field gel electrophoresis (PFGE) and randomly amplified polymorphic DNA (RAPD) PCR analysis [7]. These data indicated that the genomic organisation of *C. dubliniensis* is readily distinguishable from that of *C. albicans*. Recently, a species-specific repetitive DNA element has been identified in *C. dubliniensis* which shows promise for use as a specific fingerprinting probe for this species and will greatly aid in the epidemiological analysis of *C. dubliniensis* infections [28]. Interestingly, preliminary data using this probe suggest that *C. dubliniensis* isolates can be subdivided into two distinct groups, one of which forms a cluster of closely related strains [28]. However, DNA fingerprinting techniques, such as restriction endonuclease (REA) analysis, PFGE analysis and DNA fingerprinting using specific probes are expensive, time consuming and not readily applicable to routine use for identification purposes in most clinical microbiology diagnostic laboratories.

Demonstrating that *C. dubliniensis* has a distinct genomic organisation was insufficient for the delineation of *C. dubliniensis* as a species separate from *C. albicans*. To determine the phylogenetic relationship of these organisms it was necessary to demonstrate that, in addition to differences in genomic organisation, there is a significant nucleotide sequence divergence between the two species. The final and most conclusive evidence that *C. dubliniensis* is a *bona fide* species came from the comparative analysis of ribosomal RNA (rRNA) gene sequences from a variety of *Candida* species. In the original paper describing *C. dubliniensis* it was found that a 600 bp region encompassing the V3 variable region of the large rRNA (lrRNA) genes of *C. dubliniensis* and *C. albicans* differed by 2.3% [7]. Similar analysis of the D1/D2 region of the lrRNA genes of both species also revealed a significant degree of nucleotide divergence [29]. In addition, comparison of the sequence of the self-splicing group I introns present in the lrRNA genes of both species revealed that the *C. dubliniensis* intron is almost identical to that of *C. albicans* except for two widely divergent stem-loop regions [11]. The unique phylogenetic position of *C. dubliniensis* was further established by comparison of the sequences of the entire small rRNA genes (approximately 1.8 kb) of *C. dubliniensis* and *C. albicans* which revealed a difference of 1.4% [30]. In addition to ribosomal RNA sequences, the *ACT1* gene, which encodes the structural protein actin, has been used extensively in phylogenetic studies. Comparison of the *ACT1* genes from *C. albicans* and *C. dubliniensis* showed that the coding sequences differ by 2.1% while the less highly conserved *ACT1*-associated introns differ by 16.6% [31]. These findings strongly suggest that *C. albicans* and *C. dubliniensis* diverged from each other in the distant past.

As well as direct evidence of significant sequence divergence in specific genes there is also evidence of genome-wide sequence divergence based on data obtained using multilocus enzyme electrophoresis (MLEE) analysis. This technique, which measures the relative electrophoretic mobility of specific proteins, was used to differentiate a subgroup of Swiss atypical *Candida* isolates, which were later identified as *C. dubliniensis*, from *C. albicans* [10]. In the original study by Boerlin *et al.* it was observed that, in contrast with *C. albicans*, *C. dubliniensis* isolates did not appear to produce β-glucosidase activity. This led to the design of a simple method to differentiate between the two species based on the ability of *C. albicans* to generate fluorescence in the presence of methyl-umbelliferyl-labelled β-glucoside [10]. This technique has been used quite successfully in a number of studies, although in a recent analysis of an archival stock collection 67 of 537 (12.5%) *C. albicans* isolates were

found to be β-glucosidase negative [17]. Another technique based on genetic sequence divergence that shows great potential for use in the rapid identification of *C. dubliniensis* is the polymerase chain reaction (PCR). To date *C. dubliniensis*-specific primers have been designed on the basis of the sequence of the D1/D2 region of the lrRNA gene [29] and the *ACT1*-intron [31]. In the latter study, the *ACT1 C. dubliniensis*-specific primers have been tested successfully in an extensive blind trial including greater than 120 *C. dubliniensis* and 50 *C. albicans* isolates from a range of clinical specimens recovered from patients around the world (Figure 1). Using this test *C. dubliniensis* isolates can be identified accurately in less than 4 h. *C. albicans*-specific primers have also been designed based on *PHR1* sequences which do not yield amplimers when used with *C. dubliniensis* template DNA [32]. Restriction fragment length polymorphism analysis of amplimers obtained using PCR primers flanking various regions of the rRNA locus have also been demonstrated to allow the discrimination of *C. dubliniensis* from *C. albicans* [33]. In addition, a PCR enzyme immunoassay (PCR-EIA) using a *C. dubliniensis*-specific DNA probe derived from the ITS2 region of the rRNA locus has also been developed [12]. These techniques are specific, rapid, easy to perform and applicable to large numbers of isolates and should enhance the rapid and accurate identification of *C. dubliniensis* in the future.

Epidemiology

Originally identified in specimens recovered from the oral cavities of HIV-infected individuals with recurrent oral candidosis in Ireland, *C. dubliniensis* has since been identified in a wide variety of clinical settings throughout the world. Details of the isolation of this species from different subject cohorts in our own study population are presented in Table 1. In addition to the recovery of *C. dubliniensis* in Ireland, there have been many recent reports of the identification of this species in laboratories around the world [9-19,24,28,33,34]. Most of these isolates have been recovered from cases of oral candidosis in HIV-infected individuals. From our own experience

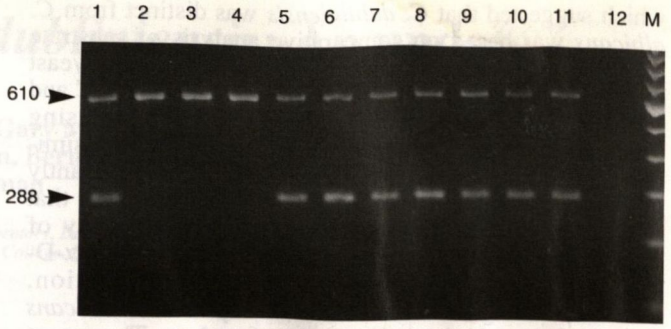

Figure 1. Agarose gel with ethidium bromide-stained amplimers from PCR reactions using fungal-specific primers (610 bp product) and *C. dubliniensis*-specific primers derived from the *ACT1* intron sequence (288 bp product) [31]. Lane 1; *C. dubliniensis* type strain, CD36, Lane 2; *C. albicans* 132A, Lane 3; *C. albicans* SC5314, Lane 4; Type 1 *C. stellatoidea* ATCC11006, Lanes 5-11; clinical isolates of *C. dubliniensis*. Lane 12; negative control lacking template DNA. Lane M; 100 bp molecular weight ladder.

C. dubliniensis appears to be most often associated with recurrent episodes of the erythematous form of oral candidosis. Interestingly, in a recent study, *C. dubliniensis* was implicated in an unusual form of linear gingival erythematous candidosis [35]. We have also identified this species as a cause of oral disease in non-HIV-infected individuals and have detected it at low incidence levels in normal healthy individuals (Table 1). In addition, there have also been reports of the recovery of *C. dubliniensis* isolates from vaginal and faecal samples [7,17]. Isolates have also been recovered from cases of systemic disease in non-HIV-infected patients [16,22]. In a recent report one patient receiving cytotoxic chemotherapy for relapsed rhabdomyosarcoma and two patients following allogeneic haematopoietic stem cell transplants yielded *C. dubliniensis*-positive blood cultures [16].

The earliest known isolates of *C. dubliniensis* predate the AIDS epidemic. One isolate deposited in the British National Collection for Pathogenic Fungi as *C. stellatoidea* in 1957 [7] and another deposited in the Centraal Bureau voor Schimmelcultures in Holland as *C. albicans* in 1952 have recently been identified as *C. dubliniensis* [16]. This highlights the problem of misidentification of

Table 1. Recovery of oral *C. dubliniensis* isolates from different cohorts of Irish individuals.

Group	No. of subjects	Clinical symptoms of oral candidiasis	No. subjects yielding *C. dubliniensis*	Other *Candida* species co-isolated*
HIV-positive	185	Symptomatic	48 (26%)	12 *C. dubliniensis* only 36 *C. dubliniensis* & other *Candida* species
HIV-positive	216	Asymptomatic	39 (18%)	7 *C. dubliniensis* only 32 *C. dubliniensis* & other *Candida* species
AIDS	82	Symptomatic	26 (31.7%)	8 *C. dubliniensis* only 18 *C. dubliniensis* & other *Candida* species
AIDS	36	Asymptomatic	9 (25%)	3 *C. dubliniensis* only 6 *C. dubliniensis* & other *Candida* species
HIV-negative¶	72	Symptomatic	10 (13.9%)	3 *C. dubliniensis* only 7 *C. dubliniensis* & other *Candida* species
HIV-negative§	56	Symptomatic	6 (10.7%)	2 *C. dubliniensis* only 4 *C. dubliniensis* & other *Candida* species
HIV-negative#	202	Asymptomatic	7 (3.5%)	1 *C. dubliniensis* only 6 *C. dubliniensis* & other *Candida* species

Data from Coleman *et al.* [36] and [D. Coleman unpublished].
C. albicans was the species most commonly co-isolated with *C. dubliniensis*, followed by (in decreasing order of frequency) *C. glabrata*, *C. tropicalis*, *C. krusei* and, infrequently, several other non-*C. albicans Candida* species and other yeast species.
¶ HIV-negative subjects with denture-associated oral candidosis.
§ HIV-negative subjects with non-denture-associated oral candidosis.
# Normal healthy oral *Candida* carriers.

C. dubliniensis due to its phenotypic similarity with *C. albicans* (and *C. stellatoidea*). In two separate studies approximately 2% of germ tube- and chlamydospore-positive isolates of *Candida* originally identified as *C. albicans* were found to be *C. dubliniensis* [17,36]. When isolates recovered from HIV-infected individuals alone were taken into account the proportion of misidentified isolates assumed even greater significance.

Antifungal drug resistance and virulence

Since *C. dubliniensis* is most often associated with recurrent episodes of disease in HIV-infected individuals it has been suggested that its recent emergence as a human pathogen may have resulted from selection due to the widespread use of antifungal drug therapy [36]. However, a number of studies have revealed that the great majority of *C. dubliniensis* isolates are susceptible to commonly used and novel antifungal agents [14,17,37,38]. In the most comprehensive study performed to date 97% of the 71 *C. dubliniensis* isolates tested were susceptible to fluconazole [38] , the agent which has been used most commonly in the treatment of oral candidosis in HIV-infected individuals. In this study, resistance (e.g., the MIC interpretative breakpoint concentration) was defined as MIC ≥64 μg/ml as recommended by the NCCLS [39]. However, a number of isolates with dose-dependent susceptibility (MIC 16-32 μg/ml) have also been described in several other studies [14,17,37]. Notably, comparison of the geometric mean MICs for fluconazole, itraconazole and ketoconazole for 58 isolates each of *C. albicans* and *C. dubliniensis* revealed that the MIC values of *C. dubliniensis* were significantly and consistently higher than those of the *C. albicans* isolates [17]. Thus although the vast majority of *C. dubliniensis* isolates are susceptible to fluconazole they may be slightly less so than most *C. albicans*, perhaps allowing them a limited selective advantage in patients treated extensively with this drug. Another interesting phenomenon concerning *C. dubliniensis* is the comparative ease with which it is possible to induce stable fluconazole resistance *in vitro*. Simply growing colonies on agar medium containing sequentially increasing concentrations of fluconazole results in the development of resistance [37]. Analysis of the resistance mechanisms in both clinical and *in vitro*-generated resistant organisms has revealed that overexpression of the major facilitator protein Mdr1p appears to be largely responsible for the resistance phenotype [40]. This is in contrast to the situation in *C. albicans* where it has been suggested that overexpression of the ABC transporter protein Cdr1p is a more common mechanism of fluconazole-resistance [41,42]. To date, resistance to antifungal agents other than fluconazole (e.g., itraconazole, ketoconazole, amphotericin B, voriconazole and a range of novel agents including triazoles and echinocandins) has not been observed in *C. dubliniensis*.

Despite the fact that *C. dubliniensis* is a significant cause of human disease, very few studies have been performed to investigate virulence factors in this species. Given the close phenotypic similarity between *C. dubliniensis* and *C. albicans* it might be expected that they may share the ability to produce certain putative virulence factors. Both species are dimorphic, although in one limited study, it has been suggested that the kinetics of hyphal production in *C. dubliniensis* is slower than that observed for reference *C. albicans* strains [30]. This may have a bearing on the ability of *C. dubliniensis* isolates to invade tissue and may contribute to the apparent lower virulence of this species. In the same study it was also shown that *C. dubliniensis* possesses homologues of seven *C. albicans* secretory aspartyl proteinase genes (*SAP*). Contrary to expectation, an early study on five atypical *Candida* isolates, which were later identified as *C. dubliniensis*, suggested that these isolates produced higher levels of proteinase activity than reference isolates of *C. albicans* [15]. Both of these studies also suggested that *C. dubliniensis* isolates are more adherent to buccal epithelial cells than the *C. albicans* strains tested [15,30]. Interestingly, SAPs have been proposed to play a role in adherence to tissue. Clearly the pathogenicity of *C. dubliniensis* is a complex subject and the data from these two studies have yet to be confirmed. The only available published data from an animal model is also equivocal. In a limited study, the *in vivo* virulence of four *C. dubliniensis* isolates (one vaginal and three oral) and one reference *C. albicans* isolate was tested in a systemic mouse model of infection. With an inoculum size of 2×10^6 cells per mouse the *C. dubliniensis* strains were clearly less virulent than the reference *C. albicans* isolate, however, when the inoculum was increased to 1×10^7 cells per mouse the results were less clear cut [30]. These data are clearly very preliminary and are based on limited numbers of strains. In addition, a systemic infection model is not ideal for the analysis of virulence of organisms implicated in superficial infections.

Conclusions

C. dubliniensis has emerged as a significant cause of candidosis. Although it is primarily associated with recurrent oral infections in HIV-infected individuals, it has also been implicated in cases of superficial and systemic disease in non-HIV-infected individuals. In order to confirm the true clinical significance of *C. dubliniensis* there is a clear need for a thorough investigation of its epidemiology. This should be facilitated by the recent development of a number of reliable identification tests. We recommend the use of CHROMagar Candida medium as a primary means for the presumptive identification of *C. dubliniensis* in clinical samples following primary culture. Any colonies showing a dark green colour should be examined using one or more of the following simple tests; carbohydrate assimilation (particularly xylose, α-methyl-D-glucoside and lactate), absence of growth at 45°C, fluorescence with methyl-umbelliferyl-β-glucoside or PCR using species-specific primers. In the future, further studies should also be performed to determine the frequency of antifungal drug resistance in clinical isolates and the mechanisms of resistance used by this species. Such studies should help to determine some of the reasons for the recent emergence of *C. dubliniensis* as a cause of human disease. Finally, the analysis of virulence mechanisms in *C. dubliniensis* and their comparison with those of *C. albicans* should help our understanding of how both of these organisms cause disease.

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References

1. Fischer-Hoch SP, Hutwagner L. Opportunistic candidiasis: an epidemic of the 1980s. Clin Infect Dis 1995; 21:897-904.
2. Fridkin SK, Jarvis WR. Epidemiology of nosocomial fungal infections. Clin Microbiol Rev 1996; 9:499-511.
3. Hazen KC. New and emerging yeast pathogens. Clin Microbiol Rev 1995; 8:462-478.
4. Pfaller MA. Nosocomial candidiasis: Emerging species, reservoirs, and modes of transmission. Clin Infect Dis 1996; 22(Suppl. 2):S89-S94.
5. Cooper CR. From bamboo rats to humans: the odyssey of *Penicillium marneffei*. ASM News 1998; 64: 390-397.
6. Gori S, Drouhet E, Gueho E, *et al.* Mycological, ultrastructural, and experimental aspects of a new dimorphic fungus, *Emmonsia pasteuriana* sp. nov. isolated from a cutaneous disseminated mycosis in AIDS. J Mycol Med 1998; 8:64-77.
7. Sullivan DJ, Westerneng TJ, Haynes KA, Bennett DE, Coleman DC. *Candida dubliniensis* sp. nov.: phenotypic and molecular characterisation of a novel species associated with oral candidosis in HIV-infected individuals. Microbiology 1995; 141:1507-1521.
8. Sullivan D, Bennett D, Henman M, *et al.* Oligonucleotide fingerprinting of isolates of *Candida* species other than *C. albicans* and of atypical *Candida* species from human immunodeficiency virus-positive and AIDS patients. J Clin Microbiol 1993; 31: 2124-2133.
9. Bikandi J, San Millán R, Moragues MD, *et al.* Rapid identification of *Candida dubliniensis* by indirect immunofluorescence based on differential localization of antigens on *C. dubliniensis* blastospores and *Candida albicans* germ tubes. J Clin Microbiol 1998; 36: 2428-2433.
10. Boerlin P, Boerlin-Petzold F, Durussel C, *et al.* Cluster of atypical *Candida* isolates in a group of human immunodeficiency virus-positive drug users. J Clin Microbiol 1995; 33:1129-1135.
11. Boucher H, Mercure S, Montplaisir S, Lemay G. A novel group I intron in *Candida dubliniensis* is homologous to a *Candida albicans* intron. Gene 1996; 180:189-196.
12. Elie CM, Lott TJ, Reiss E, Morrison CJ. Rapid identification of *Candida* species with species-specific DNA probes. J Clin Microbiol 1998; 36:3260-3265.
13. Jabra-Rizk MA, Baqui AA, Kelley JI, Falkler WA, Merz WG, Meiller TF. Identification of *Candida dubliniensis* in a prospective study of patients in the United States. J Clin Microbiol 1999; 37:321-326.
14. Kirkpatrick WR, Revankar SG, McAtee RK, *et al.* Dectection of *Candida dubliniensis* in oropharyngeal samples from human immunodeficiency virus-infected patients in North America by primary CHROMagar Candida screening and susceptibility testing of isolates. J Clin Microbiol 1998; 36:3007-3012.
15. McCullough M, Ross B, Reade P. Characterization of genetically distinct subgroup of *Candida albicans* strains from oral cavities of patients infected with human immunodeficiency virus. J Clin Microbiol 1995; 33:696-700.
16. Meis JF, Ruhnke M, De Pauw BE, Odds FC, Siegert W, Verweij PE. *Candida dubliniensis* candidemia in patients with chemotherapy-induced neutropenia and bone marrow transplantation. Emerg Infect Dis 1999; 5:150-153.
17. Odds FC, Van Nuffel L, Dams G. Prevalence of *Candida dubliniensis* isolates in a yeast stock collection. J Clin Microbiol 1998; 6:2869-2873.
18. Sullivan D, Haynes K, Bille J, *et al.* Widespread geographic distribution of oral *Candida dubliniensis* strains in human immunodeficiency virus-infected individuals. J Clin Microbiol 1997; 35:960-964.
19. Velegraki A, Logotheti M. Presumptive identification of an emerging yeast pathogen: *Candida dubliniensis* (sp. nov.) reduces 2,3,5-triphenyltetrazolium chloride. FEMS Immunol Med Microbiol 1998; 20:239-241.
20. Koehler AP, Chu K-C, Houang ETS, Cheng AFB. Simple, reliable, and cost-effective yeast identification scheme for the clinical laboratory. J Clin Microbiol 1999; 37:422-426.
21. Schoofs A, Odds FC, Colebunders R, Ieven M, Goosens H. Use of specialised isolation media for recognition and identification of *Candida dubliniensis* isolates from HIV-infected patients. Eur J Clin Microbiol Infect Dis 1997; 16:296-300.
22. Pinjon E, Sullivan D, Salkin I, Shanley D, Coleman D. Simple, inexpensive, reliable method for differentiation of *Candida dubliniensis* from *Candida albicans*. J Clin Microbiol 1998; 36:2093-2095.
23. Sullivan D, Coleman D. *Candida dubliniensis*: Characteristics and identification. J Clin Microbiol 1998; 36:329-334.
24. Salkin IF, Pruitt WR, Padhye AA, Sullivan D, Coleman D, Pincus DH. Distinctive carbohydrate assimilation profiles used to identify the first clinical isolates of *Candida dubliniensis* recovered from the United States. J Clin Microbiol 1998; 36:1467.
25. Pincus DH, Coleman DC, Pruitt WR, *et al.* Rapid identification of *Candida dubliniensis* with commercial yeast identification systems. J Clin Microbiol 1999; *submitted.*
26. Jabra-Rizk MA, Falkler WA, Merz WG, Kelley JI, Baqui AA, Meiller TF. Coaggregation of *Candida dubliniensis* with *Fusobacterium nucleatum*. J Clin Microbiol 1999; 37:1464-1468.
27. Timmins EM, Howell SA, Alsberg BK, Noble WC, Goodacre R. Rapid differentiation of closely related *Candida* species and strains by pyrolysis-mass spectrometry and Fourier transform-infrared spectroscopy. J Clin Microbiol 1998; 36:367-374.
28. Joly S, Pujol C, Rysz M, Vargas K, Soll DR. Development and characterization of complex DNA fingerprinting probes for the infectious yeast *Candida dubliniensis*. J Clin Microbiol 1999; 37:1035-1044.
29. Mannarelli BM, Kurtzman CP. Rapid identification of *Candida albicans* and other human pathogenic yeasts by using short oligonucleotides in a PCR. J Clin Microbiol 1998; 36:1634-1641.
30. Gilfillan GD, Sullivan DJ, Haynes K, Parkinson T, Coleman DC, Gow NAR. *Candida dubliniensis*: phylogeny and putative virulence factors. Microbiology 1998; 144:829-838.
31. Donnelly SA, Sullivan DJ, Shanley DB, Coleman DC. Phylogenetic analysis and rapid identification of *Candida dubliniensis* based on analysis of *ACT1* intron and exon sequences. Microbiology 1999; 145: 1871-1882
32. Kurzai O, Heinz WJ, Sullivan DJ, Coleman DC, Frosch M, Mühlschlegel FA. Rapid PCR test for discriminating between *Candida albicans* and *Candida dubliniensis* isolates using primers derived from the pH-regulated *PHR1* and *PHR2* genes of *C. albicans*. J Clin Microbiol 1999; 37:1587-1590.
33. McCullough MJ, Clemons KV, Stevens DA. Molecular and phenotypic characterization of genotypic *Candida albicans* subgroups and comparison with *Candida dubliniensis* and *Candida stellatoidea*. J Clin Microbiol 1999; 37:417-421.
34. Pujol C, Renaud F, Mallie M, de Meeus T, Bastide J-M. Atypical strains of *Candida albicans* recovered from AIDS patients. J Med Vet Mycol 1997; 35:115-121.
35. Velegraki A, Nicolatau O, Theodoridou M, Mostrou G, Legakis NJ. Paediatric AIDS-related linear gingival erythema: a form of erythematous candidiasis. J Oral Pathol Med 1999; 28:178-182.
36. Coleman DC, Sullivan DJ, Bennett DE, Moran GP, Barry HJ, Shanley DB. Candidiasis: the emergence of a novel species, *Candida dubliniensis*. AIDS 1997; 11:557-567.
37. Moran GP, Sullivan DJ, Henman MC, *et al.* Antifungal drug susceptibilities of oral *Candida dubliniensis* isolates from human immunodeficiency virus (HIV)-infected and non-HIV-infected subjects and generation of stable fluconazole-resistant derivatives *in vitro*. Antimicrob Agents Chemother 1997; 41:617-623.
38. Pfaller MA, Messer SA, Gee S, *et al.* *In vitro* susceptibilities of *Candida dubliniensis* isolates tested against the new triazole and echinocandin antifungal agents. J Clin Microbiol 1999; 37:870-872.
39. National Committee for Clinical Laboratory Standards. Reference method for broth dilution antifungal susceptibility testing of yeast. approved standard M27-A. National Committee or Clinical Laboratory Standards, Wayne, Pa, 1997.
40. Moran GP, Sanglard D, Donnelly SM, Shanley DB, Sullivan DJ, Coleman DC. Identification and expression of multidrug transporters responsible for fluconazole resistance in *Candida dubliniensis*. Antimicrob Agents Chemother 1998; 42:1819-1830.
41. Albertson GD, Niimi M, Cannon RD, Jenkinson HF. Multiple efflux mechanisms are involved in *Candida albicans* fluconazole resistance. Antimicrob Agents Chemother 1996; 40:2835-2841.
42. Sanglard D, Kuchler K, Ischer F, Pagani J-L, Monod M, Bille J. Mechanisms of resistance to azole antifungal agents in *Candida albicans* isolates from AIDS patients involve specific multidrug transporters. Antimicrob Agents Chemother 1995; 39:2378-2386.

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Recovery of *Candida dubliniensis* from Non-Human Immunodeficiency Virus-Infected Patients in Israel

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***Candida dubliniensis* is a recently discovered yeast species principally associated with carriage and disease in the oral cavities of human immunodeficiency virus (HIV)-infected individuals. To date the majority of isolates of this species have been identified in Europe and North America. In this study, five *Candida* isolates recovered from separate HIV-negative hospitalized patients in Jerusalem, Israel, were presumptively identified as *C. dubliniensis* on the basis of their dark green coloration when grown on CHROMagar Candida medium. Their identification was confirmed by a variety of techniques, including carbohydrate assimilation profiles, absence of growth at 45°C, positive reaction with *C. dubliniensis*-specific antibodies as determined by indirect immunofluorescence analysis, and positive amplification with *C. dubliniensis*-specific PCR primers. All five strains were shown to be susceptible to a range of antifungal agents, including fluconazole. One of the five isolates was recovered from urine specimens, while the remaining four were recovered from upper respiratory tract and oral samples. While none of the patients was HIV positive, all were receiving broad-spectrum antibacterials at the time isolates of *C. dubliniensis* were obtained from clinical specimens. This study describes the first isolates of *C. dubliniensis* from the Middle East and confirms that this yeast can be associated with carriage and infection in the absence of HIV infection.**

Candida dubliniensis, which was first established as a novel yeast species in 1995, is phenotypically and genotypically closely related to the most frequently identified human fungal pathogen, *Candida albicans* (25). This close similarity between the two species has proved problematic in the identification of *C. dubliniensis* in clinical samples and in retrospective analyses of laboratory stock collections, with many isolates being misidentified as *C. albicans* (5, 18). However, the recent description of reliable and rapid identification tests, including the observation of differentially colored primary colonies on CHROMagar Candida medium and the use of *C. dubliniensis*-specific PCR primers, will greatly facilitate the identification of this species in clinical samples and establish its epidemiologic significance (6, 24). In addition, accurate species identification has been aided by the inclusion of *C. dubliniensis*-specific carbohydrate assimilation profiles in the databases of commercially available yeast identification kits, such as the API ID 32C and the API 20C AUX systems (20, 24). To date the majority of *C. dubliniensis* isolates have been identified in Western Europe and North America (1–3, 7, 10–14, 17, 18, 23, 25, 26). Most of these isolates were associated with oral carriage and oropharyngeal infection in human immunodeficiency virus (HIV)-infected individuals. In a recent study of an Irish subject group, 26% (48 of 185) of HIV-positive individuals and 32% (26 of 82) of AIDS patients with oral candidiasis yielded *C. dubliniensis*. In approximately 25% of these cases *C. dubliniensis* was the only species detected (5, 24). However, *C. dubliniensis* is not exclusively associated with HIV-infected individuals. In the same study *C. dubliniensis* was also identified in clinical specimens recovered from HIV-negative individuals, both with and without symptoms of oral candidiasis. In an analysis of oral samples taken from healthy individuals without any signs of oral disease, 3.5% (7 of 202) of subjects yielded *C. dubliniensis*, suggesting that this species is a minor constituent of the normal human oral flora. *C. dubliniensis* was also identified in 12.5% (16 of 128) of cases of oral candidiasis in HIV-negative individuals (5, 24). Although the oral cavity is the human niche from which *C. dubliniensis* has been recovered most frequently, there have been reports of isolates being recovered from other anatomical sites and specimens, including the vagina, the lung, feces, and sputum (16, 18, 21). Furthermore, *C. dubliniensis* was recently identified as the cause of three cases of systemic disease in HIV-negative Dutch patients receiving post-bone marrow transplantation immunosuppressive treatment or cytotoxic chemotherapy for the treatment of rhabdomyosarcoma (14). In this study we describe the application of routine phenotypic and rapid molecular methods to the identification of *C. dubliniensis* isolates from five separate HIV-negative hospitalized patients in Israel. All five isolates were shown to be susceptible to a range of antifungal agents, including fluconazole. This is the first report of the identification of this novel species in the Middle East.

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CASE REPORTS

Case 1. A 39-year-old female with a past history of thalassemia major, splenectomy, and transfusion associated hemochromatosis initially presented with acute respiratory tract infection. The physical examination on admission was notable only for the presence of a purulent postnasal drip. Given the patient's asplenic condition, she was placed on intravenous cefuroxime, 750 mg every 8 h. Oral and upper respiratory

specimens were inoculated onto blood agar (5% [vol/vol] defibrinated sheep blood), chocolate agar, and MacConkey agar media and incubated at 35°C for 48 h in an atmosphere of 5% (vol/vol) CO_2. With the recovery of beta-hemolytic streptococcus (Lancefield group G), *Staphylococcus aureus*, *C. albicans*, and *Candida krusei*, treatment was altered to cephalexin, 500 mg every 6 h, for a total duration of 14 days.

While the patient's respiratory symptoms slowly resolved, initial symptoms of oropharyngeal candidiasis were observed 4 days after the initiation of antibiotic therapy. As a result, the patient was placed on clotrimazole troches (10 mg), five times per day. Five days after the initiation of antifungal therapy and while the patient was still being treated for her respiratory infection, additional oral and upper respiratory specimens were obtained and cultured at 30°C for 48 h on Emmon's modified Sabouraud glucose agar (SGA) supplemented with 50 μg of chloramphenicol and 5 μg of gentamicin/ml. Pure cultures of *C. dubliniensis* were recovered from all specimens, resulting in the alteration of the antifungal therapy to oral fluconazole, 200 mg daily for 5 days. Cultures inoculated with respiratory specimens collected 10 days after completion of antifungal therapy were negative for yeasts, and a physical examination at this same time disclosed only mild glossitis and perlèche.

Case 2. A 19-year-old man was admitted to the hospital due to a urinary tract infection complicating type I neurofibromatosis. Massive retroperitoneal tumors were known to obstruct the ureters and deform the bladder, with secondary renal failure and recurrent episodes of urinary tract infection. In view of previous infection with resistant bacteria (prior to hospitalization the patient was repeatedly treated for urinary tract infection, with eventual culture of antibiotic-resistant bacteria), the patient was treated with broad-spectrum antibiotics, including vancomycin, meropenem, and co-trimoxazole, and was subsequently referred for surgical revision of his urinary tract. Peri- and postoperatively, during removal of drains, the patient was frequently given broad-spectrum antibiotics as a prophylactic measure, including ampicillin, ofloxacin, and metronidazole. Urine cultures during that period grew numerous bacteria, including *Strenotrophomonas maltophilia*, *Citrobacter koseri*, *S. aureus*, and *Enterococcus faecalis*. Forty days after admission, when a suprapubic catheter was removed without adequate alternative urinary drainage, the patient became febrile. A urine culture was positive for *Enterococcus faecium* and *Trichosporon beigelii*. Administration of antibacterial agents was continued, drainage of the urinary tract was restored by intermittent catheterizations, and the patient defervesced. During this time the patient did not receive any antifungal agent. Repeated urine cultures in the following 10 days were positive for *T. beigelii*, and antibiotic treatment was eventually stopped. Follow-up urine culture 5 days later yielded *T. beigelii* and *C. dubliniensis*. Because the patient was asymptomatic, he was not treated. A urine culture 2 weeks later was negative for fungi.

Case 3. A 21-year-old woman with cystic fibrosis and thalassemia minor was admitted to the hospital due to worsening dyspnea, productive sputum, and fever. The patient had recurrent episodes of *Pseudomonas aeruginosa* respiratory tract infection, the last being 1 month prior to this admission, for which she was treated with ceftazidime and amikacin and later switched to ciprofloxacin. Bronchoscopy revealed purulent secretions, with a Gram stain of lavage disclosing gram-positive cocci and gram-negative rods. Cultures of this lavage fluid were positive for *S. aureus*, *P. aeruginosa*, and *C. dubliniensis*. The patient was treated with ceftazidime and amikacin and improved from the bacterial infection within 5 days. Previous bronchoalveolar lavage and follow-up sputum cultures were persistently positive only for *S. aureus* and *P. aeruginosa*.

Case 4. A 52-year-old woman was admitted for right-side pneumonia leading to acute respiratory failure. Her past medical history was significant for indicating type II polyglandular autoimmune syndrome (Schmidt's syndrome) treated with prednisone (7.5 mg daily), insulin, and thyroxine; ischemic heart disease; and perforated appendicitis 5 months prior to admission. The patient was treated with cefuroxime and ciprofloxacin, and prednisone was replaced by hydrocortisone (300 mg daily). Bronchial washings performed on admission through the endotracheal tube disclosed few granulocytes without bacteria and no significant growth. Bronchoscopy done 4 days later disclosed a minute amount of purulent secretions. A Gram stain was negative, while a culture was positive for *C. dubliniensis*. Thereafter, acute renal failure developed, and peritoneal dialysis was initiated. The patient died 19 days later with evidence of *C. albicans* peritonitis and bloodstream infection (specimens from both sources yielded *C. albicans*). An autopsy was not performed. HIV serology was negative.

Case 5. A 31-year-old otherwise healthy woman was treated for postpartum endometritis with ampicillin, gentamicin, and metronidazole for 10 days. One week after completion of therapy she noticed a burning sensation on the tongue accompanied by a whitish discoloration that persisted. Three weeks later a diagnosis of oral candidiasis was made. A superficial tongue specimen disclosed mixed bacterial morphotypes with few leukocytes. A culture was positive for mixed bacteria and *C. dubliniensis*. She was not treated, and at a follow-up examination 4 weeks later all symptoms and signs had resolved. Microscopic analysis and culture of specimens taken from the endometrium were both negative for fungi.

MATERIALS AND METHODS

Yeast isolates. Yeast isolates from clinical specimens were recovered following primary culture for 48 h at 30°C on Emmon's modified SGA supplemented with 50 μg of chloramphenicol and 5 μg of gentamicin/ml. Following incubation, confluent or semiconfluent areas of yeast growth were sampled with a sterile wire loop and streaked on CHROMagar Candida medium (CHROMagar, Paris, France) to yield single colonies. Selected colonies exhibiting different colony colors were transferred to and maintained on SGA at 30°C. A single isolated colony of each species grown on SGA was transferred after 48 h of incubation at 30°C to CHROMagar medium, incubated at 30°C, and examined for colony color after 24 and 48 h. Colonies from 48-h SGA cultures were similarly used as inocula in the following standard morphological and physiological tests: (i) chlamydoconidia formation on corn meal agar supplemented with 1% (wt/vol) Tween 80, (ii) germ tube development in human serum incubated for 3 h at 37°C, (iii) sensitivity to cycloheximide as determined by growth on Mycosel agar (BBL, Cockeysville, Md.), and (iv) growth at 37, 42, and 45°C on SGA. Carbohydrate source and nitrogen source assimilation patterns were evaluated by using the API ID 32C and the API 20C AUX yeast assimilation systems (bioMérieux, Marcy l'Etoile, France), according to the manufacturer's instructions, with an inoculum derived from 48-h SGA cultures.

Serotyping. *C. dubliniensis* isolates were serotyped on the basis of agglutination reactions with antiserum raised against *Candida* antigenic factor no. 6 (Iatron Laboratories, Inc., Tokyo, Japan) as described previously (25).

Chemicals, enzymes, and oligonucleotides. Analytical-grade or molecular biology grade chemicals were purchased from Sigma-Aldrich, BDH (Poole, Dorset, United Kingdom) or Boehringer Mannheim (Lewes, East Sussex, United Kingdom). Enzymes were purchased from Boehringer Mannheim or the Promega Corporation (Madison, Wis.) and used according to the manufacturer's instructions. Custom-synthesized oligonucleotides were purchased from Genosys Biotechnologies (Pampisford, Cambridgeshire, United Kingdom).

In vitro antifungal susceptibility tests. The in vitro antifungal susceptibilities of *C. dubliniensis* isolates were determined by using the Etest system (AB Biodisk, Solna, Sweden) according to the manufacturer's instructions. The MIC was defined as the lowest concentration of antifungal agent at which the border of the elliptical inhibition zone intercepted the readable scale on the strip (4). All tests were quality controlled by using *C. krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019.

Immunofluorescence. An indirect immunofluorescence assay (IFA) was performed as described previously (1). Briefly, the blastospores from *C. dubliniensis* isolates, including the *C. dubliniensis* type strain, CD36 (CBS 7989) (25), and

TABLE 1. Substrate assimilation profiles[b] of Israeli *C. dubliniensis* isolates

Strain or isolate	Strain type (reference) or date of isolation	API ID 32C results			API 20C AUX results		
		Profile	Species	Predictive value (%)[a]	Profile	Species	Predictive value (%)[a]
Reference strains							
C. dubliniensis CD36	Type strain (25)	7142140015	*C. dubliniensis*	99.3	6172134	*C. dubliniensis*	99.9
C. albicans 179A	Serotype A strain (9)	7347340015	*C. albicans*	99.9	2776174	*C. albicans*	98.3
C. albicans 132A	Serotype B strain (9)	7347140015	*C. albicans*	99.9	2376174	*C. albicans*	99.2
Clinical isolates							
P-6265	December 1998	7042140011	*C. dubliniensis*	99.9	6152034	*C. dubliniensis*	99.9
P-6785	March 1999	7042140011	*C. dubliniensis*	99.9	6142034	*C. dubliniensis*	99.5
P-7073	May 1999	7142140015	*C. dubliniensis*	99.3	2172174[c]	*C. albicans*	97.6
P-7266	June 1999	7142100015	*C. dubliniensis*	99.9	6172134	*C. dubliniensis*	99.9
P-7276	June 1999	7042140015	*C. dubliniensis*	99.8	6152034	*C. dubliniensis*	99.9

[a] bioMérieux APILAB Plus database, version 3.3.3.
[b] All profiles were read following 48 h incubation at 30°C.
[c] Isolate P7073 is *C. dubliniensis* as determined by IFA and by PCR amplification with the primer set of DUBF and DUBR (this study). This isolate assimilated trehalose. However, in the current database the expected percentage for trehalose assimilation by *C. dubliniensis* is 0%. A recent in-depth study has demonstrated that many proven *C. dubliniensis* isolates were misidentified or unidentified by the API 20C AUX system due to positive trehalose reactions and that incorporation of this variability in a future database would permit correct identification of such isolates as *C. dubliniensis* (20).

reference oral *C. albicans* isolate 132A (9) were grown on Sabouraud agar (Oxoid, Poole, Dorset, United Kingdom) plates for 48 h at 37°C, resuspended in phosphate-buffered saline (PBS) at a cell density of 10^6 cells/ml, and placed on Teflon-coated immunofluorescence slides. The slides were incubated with anti-*C. dubliniensis* rabbit serum (1) diluted 1:5 in PBS supplemented with Evans blue (0.05% [wt/vol]) and Tween 20 (0.05% [vol/vol]) and washed, and the reacting antibodies were revealed by incubation with fluorescein-conjugated goat anti-rabbit immunoglobulin G (Sigma).

PCR identification of *C. dubliniensis*. PCR identification of *C. dubliniensis* with the *C. dubliniensis*-specific primer pair DUBF and DUBR (6) was carried out in a 50-μl final volume containing 10 pmol each of the forward and reverse primers, 2.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 9.0 at 25°C), 10 mM KCl, 0.1% (vol/vol) Triton X-100, 2.5 U of *Taq* DNA polymerase (Promega), and 25 μl of template DNA-containing cell supernatant (prepared as described below). The primer pair of DUBF and DUBR is complementary to sequences within the *ACT1*-associated intron sequence of *C. dubliniensis* and yields an amplimer of 288 bp. Each reaction mixture also contained 10 pmol each of the universal fungal primers RNAF and RNAR (8), which amplify a fragment of approximately 610 bp from all fungal large-subunit rRNA genes, as an internal positive control. Cycling conditions consisted of 6 min at 95°C, followed by 30 cycles of 30 s at 94°C, 30 s at 58°C, and 30 s at 72°C, followed by 72°C for 10 min. Amplification products were separated by electrophoresis through 2.0% (wt/vol) agarose gels containing 0.5 μg of ethidium bromide/ml and were visualized on a UV transilluminator.

Preparation of template DNA. *Candida* template DNA for use in PCR experiments with the *C. dubliniensis*-specific primer pair DUBF and DUBR was prepared as described by Donnelly et al. (6). Briefly, a single colony from a culture grown for 48 h at 37°C on potato dextrose agar or CHROMagar Candida medium was suspended in 50 μl of sterile distilled water. Cell suspensions were boiled for 10 min, and the lysed cells were subjected to a clearing spin for 5 min at 20.000 × *g*. Template DNA contained in 25 μl of supernatant was used for PCR amplification.

RESULTS AND DISCUSSION

Phenotypic characterization of putative *C. dubliniensis* isolates. Clinical specimens from five separate hospitalized Israeli patients yielded yeast colonies which were dark blue-green in color on CHROMagar medium. These were all found to produce germ tubes in normal human serum, to form abundant chlamydoconidia following growth on corn meal agar, and to grow in the presence of cycloheximide on Mycosel agar. Based on these findings and in accordance with previous studies (5, 22, 25) these isolates were presumptively identified as *C. dubliniensis*. In order to confirm this identification, all five isolates were then subjected to substrate assimilation profile analysis with the API ID 32C and 20C AUX yeast identification systems. The profiles of four of the five corresponded to excellent identification of *C. dubliniensis* (Table 1). The profile of one isolate corresponded to good identification of *C. albicans*. Since *C. dubliniensis* was only first described as a new species in 1995, it has only recently been added to the API 20C AUX and the API ID 32C databases. The results of a recent study show that these systems have excellent potential as a means of identifying this yeast but that database modifications are required to avoid its misidentification as *C. albicans* or unidentified results (20). The largest discrepancy observed was the positive trehalose assimilation results found with 15 and 30% of the 80 *C. dubliniensis* isolates tested with the API 20C AUX and the API ID 32C systems, respectively. In that study the authors concluded that it is reasonable to assume that incorporation of this variability in a future database would correct this problem (20). Indeed, if isolate P-7073 had not assimilated trehalose, it would have been identified as *C. dubliniensis* with the API 20C AUX system with the current database.

All five presumptive Israeli *C. dubliniensis* isolates and the type strain grew on SGA at 37 and 42°C, but none grew at 45°C. In contrast, isolates and reference strains of *C. albicans* grew well at all temperatures. These findings provided supporting evidence that the five Israeli isolates were *C. dubliniensis*. Previous studies have shown that *C. dubliniensis* isolates do not grow at 45°C and that many grow poorly or not at all at 42°C (21, 25). Furthermore, all five presumptive *C. dubliniensis* isolates and the type strain belonged to *C. albicans* serotype A, as determined by latex agglutination with rabbit antiserum raised against *Candida* antigenic factor no. 6. To date all *C. dubliniensis* isolates tested belong exclusively to *C. albicans* serotype A (22, 23, 25). All of these findings strongly suggested that the five Israeli isolates were *C. dubliniensis*.

To further investigate the identity of the five putative *C. dubliniensis* isolates, blastospores of each were tested by IFA with anti-*C. dubliniensis* serum adsorbed with *C. albicans* blastospores. The adsorbed serum had been shown in previous studies to differentially label *C. dubliniensis* isolates (1). The antiserum reacted with blastospores of all five putative *C. dubliniensis* isolates and the type strain, CD36, but did not label blastospores of reference *C. albicans* isolate 132A. These results confirmed that the five Israeli isolates were *C. dubliniensis*.

All five of the Israeli *C. dubliniensis* isolates were susceptible to antifungal drugs as determined with Etest strips and yielded

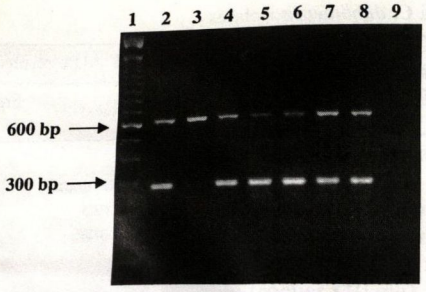

FIG. 1. Agarose gel showing ethidium bromide-stained amplimers from PCRs with the *C. dubliniensis*-specific primers DUBF and DUBR (288-bp product) and the fungal universal primers RNAF and RNAR (610-bp product). The amplimers shown in the lanes were obtained from template DNA from yeast strains and isolates as follows: lane 2, *C. dubliniensis* type strain CD36; lane 3, *C. albicans* reference strain 132A, lanes 4 to 8, Israeli *C. dubliniensis* isolates P-6265, P-6785, P-7073, P-7266, and P-7276, respectively. Lane 1, molecular weight size markers (100-bp ladder); lane 9, negative control lacking template DNA.

MICs in the range of 0.004 to 0.047 (amphotericin B), 0.008 to 0.032 (5-fluorocytosine), 0.008 to 1.0 (itraconazole), 0.008 to 0.032 (ketoconazole), and 0.38 to 1.0 μg/ml (fluconazole). These results are in agreement with previous studies, which demonstrated that the majority of *C. dubliniensis* clinical isolates are susceptible to commonly used antifungal drugs (16, 19).

PCR-based identification of *C. dubliniensis* isolates. In order to confirm the definitive identification of the five Israeli *C. dubliniensis* isolates, template DNA from each was subjected to PCR analysis with a set of primers (DUBF and DUBR) complementary to *C. dubliniensis ACT1*-associated intron sequences (6). These primers amplify a DNA fragment of 288 bp from *C. dubliniensis*, but do not yield an amplimer from *C. albicans*, *Candida stellatoidea*, or any other *Candida* species. Each PCR mixture also contained the fungal universal primer pair RNAF and RNAR (8), which amplify a product of approximately 610 bp from the fungal large-subunit ribosomal RNA gene and which served as an internal positive amplification control. All five Israeli *C. dubliniensis* isolates and the *C. dubliniensis* type strain, CD36, yielded amplimers of 288 bp and approximately 610 bp (Fig. 1). In contrast, *C. albicans* reference strain 132A yielded an amplimer of approximately 610 bp only (Fig. 1). These findings unequivocally confirmed the results of the phenotypic tests that the five isolates were *C. dubliniensis*.

This study constitutes the first report of the isolation of *C. dubliniensis* in the Middle East and broadens our knowledge of the widespread geographic distribution of this organism (23). Additionally, the results confirm previous findings that *C. dubliniensis* is usually an opportunistic pathogen which is mainly associated with colonization and infection of the oral cavity and upper respiratory tract (5, 22). In three of these cases *C. dubliniensis* appears to have been responsible for infection. Most of the patients had underlying disease, but all were treated with broad-spectrum antibiotics, which may have been a contributory factor in the outgrowth of *C. dubliniensis*. All five isolates of *C. dubliniensis* were susceptible to commonly used antifungal drugs, including fluconazole. This was not surprising since fluconazole resistance has only been reported previously for clinical isolates of *C. dubliniensis* from HIV-infected individuals previously treated with the drug (15, 16, 19). Furthermore, all of these data add to the present limited evidence (14, 16, 18, 25) that infection and colonization with *C. dubliniensis* are not confined to HIV-infected individuals. The use of CHROMagar Candida medium (12) as a means of preemptively identifying *C. dubliniensis* in clinical specimens on primary culture should help, when combined with other phenotypic techniques described in this study, to facilitate the detection and identification of additional cases of *C. dubliniensis* colonization and infection.

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ADDENDUM IN PROOF

Since this article was submitted for publication, an additional six isolates recovered from six separate non-HIV-infected patients have been definitively identified as *C. dubliniensis* in the same hospital in Jerusalem, Israel. Two isolates were recovered from vaginal tract specimens, two were recovered from respiratory tract specimens, and one each was recovered from a wound specimen and a sputum specimen.

REFERENCES

1. **Bikandi, J., R. San Millán, M. D. Moragues, G. Cebas, M. Clarke, D. C. Coleman, D. J. Sullivan, G. Quindós, and J. Pontón.** 1998. Rapid identification of *Candida dubliniensis* by indirect immunofluorescence based on differential localization of antigens on *C. dubliniensis* blastospores and *Candida albicans* germ tubes. J. Clin. Microbiol. **36:**2428–2433.
2. **Boerlin, P., F. Boerlin-Petzold, C. Durussel, M. Addo, J.-L. Pagani, J.-P. Chave, and J. Bille.** 1995. Cluster of atypical *Candida* isolates in a group of human immunodeficiency virus-positive drug users. J. Clin. Microbiol. **33:** 1129–1135.
3. **Boucher, H., S. Mercure, S. Montplaisir, and G. Lemay.** 1996. A novel group I intron in *Candida dubliniensis* is homologous to a *Candida albicans* intron. Gene **180:**189–196.
4. **Clancy, C. J., and M. H. Nguyen.** 1999. Correlation between in vitro susceptibility determined by E-test and response to therapy with amphotericin B: results from a multicenter prospective study of candidemia. Antimicrob. Agents Chemother. **43:**1289–1290.
5. **Coleman, D. C., D. J. Sullivan, D. E. Bennett, G. P. Moran, H. J. Barry, and D. B. Shanley.** 1997. Candidiasis: the emergence of a novel species, *Candida dubliniensis*. AIDS **11:**557–567.
6. **Donnelly, S. M., D. J. Sullivan, D. B. Shanley, and D. C. Coleman.** 1999. Phylogenetic analysis and rapid identification of *Candida dubliniensis* based on analysis of *ACT1* intron and exon sequences. Microbiology **145:**1871–1882.
7. **Elie, C. M., T. J. Lott, E. Reiss, and C. J. Morrison.** 1998. Rapid identification of *Candida* species with species-specific DNA probes. J. Clin. Microbiol. **36:**3260–3265.
8. **Fell, J.** 1993. Rapid identification of yeast species using three primers in a polymerase chain reaction. Mol. Mar. Biol. Biotechnol. **2:**174–180.
9. **Gallagher, P. J., D. E. Bennett, M. C. Henman, R. J. Russell, S. R. Flint, D. B. Shanley, and D. C. Coleman.** 1992. Reduced azole susceptibility of *Candida albicans* from HIV-positive patients and a derivative exhibiting colony morphology variation. J. Gen. Microbiol. **138:**1901–1911.
10. **Jabra-Rizk, M. A., A. A. Baqui, J. I. Kelley, W. A. Falkler, W. G. Merz, and T. F. Meiller.** 1999. Identification of *Candida dubliniensis* in a prospective study of patients in the United States. J. Clin. Microbiol. **37:**321–326.
11. **Joly, S., C. Pujol, M. Rysz, K. Vargas, and D. R. Soll.** 1999. Development and characterization of complex DNA fingerprinting probes for the infectious yeast *Candida dubliniensis*. J. Clin. Microbiol. **37:**1035–1044.
12. **Kirkpatrick, W. R., S. G. Revankar, R. K. McAtee, J. L. Lopez-Ribot, A. W. Fothergill, D. I. McCarthy, S. E. Sanche, R. A. Cantu, M. G. Rinaldi, and T. F. Patterson.** 1998. Detection of *Candida dubliniensis* in oropharyngeal samples from human immunodeficiency virus-infected patients in North America by primary CHROMagar Candida screening and susceptibility testing of isolates. J. Clin. Microbiol. **36:**3007–3012.
13. **McCullough, M., B. Ross, and P. Reade.** 1995. Characterization of genetically distinct subgroup of *Candida albicans* strains isolated from oral cavities of patients infected with human immunodeficiency virus. J. Clin. Microbiol. **33:**696–700.
14. **Meis, J. F., M. Ruhnke, B. E. De Pauw, F. C. Odds, W. Siegert, and P. E. Verweij.** 1999. *Candida dubliniensis* candidemia in patients with chemotherapy-induced neutropenia and bone marrow transplantation. Emerg. Infect. Dis. **5:**150–153.
15. **Moran, G. P., D. Sanglard, S. M. Donnelly, D. B. Shanley, D. J. Sullivan, and D. C. Coleman.** 1998. Identification and expression of multidrug transporters

responsible for fluconazole resistance in *Candida dubliniensis*. Antimicrob. Agents Chemother. **42:**1819–1830.

16. **Moran, G. P., D. J. Sullivan, M. C. Henman, C. E. McCreary, B. J. Harrington, D. B. Shanley, and D. C. Coleman.** 1997. Antifungal drug susceptibilities of oral *Candida dubliniensis* isolates from human immunodeficiency virus (HIV)-infected and non-HIV-infected subjects and generation of stable fluconazole-resistant derivatives in vitro. Antimicrob. Agents Chemother. **41:**617–623.
17. **Morschauser, J., M. Ruhnke, S. Michel, and J. Hacker.** 1999. Identification of CARE-2-negative *Candida albicans* as *Candida dubliniensis*. Mycoses **42:** 29–32.
18. **Odds, F. C., L. Van Nuffel, and G. Dams.** 1998. Prevalence of *Candida dubliniensis* isolates in a yeast stock collection. J. Clin. Microbiol. **36:**2869–2873.
19. **Pfaller, M. A., S. A. Messer, S. Gee, S. Joly, C. Pujol, D. J. Sullivan, D. C. Coleman, and D. R. Soll.** 1999. *In vitro* susceptibilities of *Candida dubliniensis* isolates tested against the new triazole and echinocandin antifungal agents. J. Clin. Microbiol. **37:**870–872.
20. **Pincus, D. H., D. C. Coleman, W. R. Pruitt, A. A. Padhye, I. F. Salkin, M. Geimer, A. Bassel, D. J. Sullivan, M. Clarke, and V. Hearn.** 1999. Rapid identification of *Candida dubliniensis* with commercial yeast identification systems. J. Clin. Microbiol. **37:**3533–3539.
21. **Pinjon, E., D. Sullivan, I. Salkin, D. Shanley, and D. Coleman.** 1998. Simple, inexpensive, reliable method for differentiation of *Candida dubliniensis* from *Candida albicans*. J. Clin. Microbiol. **36:**2093–2095.
22. **Sullivan, D., and D. Coleman.** 1998. *Candida dubliniensis*: characteristics and identification. J. Clin. Microbiol. **36:**329–334.
23. **Sullivan, D., K. Haynes, J. Bille, P. Boerlin, L. Rodero, S. Lloyd, M. Henman, and D. Coleman.** 1997. Widespread geographic distribution of oral *Candida dubliniensis* strains in human immunodeficiency virus-infected individuals. J. Clin. Microbiol. **35:**960–964.
24. **Sullivan, D. J., G. Moran, S. Donnelly, S. Gee, E. Pinjon, B. McCarton, D. B. Shanley, and D. C. Coleman.** 1999. *Candida dubliniensis*: an update. Rev. Iberoam. Micol. **16:**72–76.
25. **Sullivan, D. J., T. J. Westerneng, K. A. Haynes, D. E. Bennett, and D. C. Coleman.** 1995. *Candida dubliniensis* sp. nov.: phenotypic and molecular characterisation of a novel species associated with oral candidosis in HIV-infected individuals. Microbiology **141:**1507–1521.
26. **Velegraki, A., and M. Logotheti.** 1998. Presumptive identification of an emerging yeast pathogen: *Candida dubliniensis* (sp. nov.) reduces 2,3,5-triphenyltetrazolium chloride. FEMS Immunol. Med. Microbiol. **20:**239–241.