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Comparative growth analysis of *Candida dubliniensis* and *Candida albicans in vitro* and in selected infection models

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

April 2006

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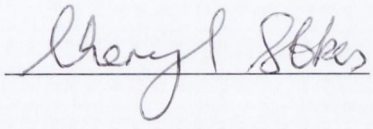

Cheryl Stokes

"Science is a wonderful thing if one does not have to earn one's living at it."

Albert Einstein

Summary

Candida dubliniensis is the species most closely related to *C. albicans* and is primarily associated with colonisation and oral candidiasis in Human Immunodeficiency Virus (HIV)-infected individuals and AIDS patients. Despite the similarities between the two, epidemiological data and results from *in vivo* infection models indicate that *C. albicans* is significantly more pathogenic than *C. dubliniensis*. The purpose of this study was to investigate if differences in growth might explain this apparent difference in the ability of *C. albicans* and *C. dubliniensis* to cause disease.

Candida dubliniensis strains demonstrated a significantly slower growth rate than *C. albicans* strains in both Sabouraud liquid broth, modified antibiotic medium 13 (SDB) and yeast nitrogen base medium (YNB). Kirkpatrick *et al.*, have previously reported that *C. albicans* has the capacity to out-compete *C. dubliniensis* when grown in co-culture, and *C. dubliniensis* was only present in two out of eleven pairings after 96 h of growth in SDB medium using CHROMagar Candida medium to differentiate between the two species (Kirkpatrick *et al.*, 2000). In the present study CHROMagar Candida medium was deemed an inefficient means of discriminating between the species, therefore in order to facilitate efficient discrimination, strains were tagged with a molecular cassette containing genes encoding mycophenolic acid resistance (MPA^R) and green fluorescent protein (GFP). The two species were examined in co-culture in both SDB and YNB and differentiation was performed on MPA-containing and drug-free media. *Candida dubliniensis* strains were always found to be present in significantly lower amounts than *C. albicans* strains, which is most likely due to their slower growth rate.

The kinetics of hyphal induction was examined in both *C. albicans* and *C. dubliniensis* using thirteen separate environmental conditions, including both solid and liquid media, which are known to induce the yeast to hyphal transition. *Candida albicans* strains were significantly more efficient at producing hyphae under all conditions tested except on both Pal's and Staib medium where *C. dubliniensis* strains produced abundant hyphae, pseudohyphae and chlamydospores whereas *C. albicans* remained in the yeast phase.

A homologue of the gene encoding the hyphal wall protein (Hwp1) protein, which is exclusively expressed by the hyphal form of *C. albicans* was found to contain deletions in *C. dubliniensis*. These deletions span the domains, which are proposed to be involved in

adhesion. Surprisingly, *CdHWP1* and *CaHWP1* were only 45.3% homologous at the nucleotide sequence level and 49.3% homologous at the amino acid level. However, comparison of the available *C. albicans* and *C. dubliniensis* genome sequence data revealed that all of the genes implicated in the regulation of hyphal induction or expressed by hyphae in *C. albicans* have very closely related homologues in the *C. dubliniensis* genome.

Comparative growth of *C. albicans* and *C. dubliniensis* was also compared using an *ex vivo* infection model, Reconstituted Human Oral Epithelium (RHOE). Using this model *C. albicans* was significantly more efficient at adhering to the surface of the epithelium in contrast to *C. dubliniensis* where very few cells attached. *Candida dubliniensis* cells present were solely in the yeast phase on the surface of the epithelium whereas *C. albicans* cells consisted of both yeast and hyphal cells which penetrated through the epithelium. Despite the lack of hyphae, damage to the epithelium occurred during *C. dubliniensis* infection, however it occurred later during infection when compared with *C. albicans* (i.e. 36 h in contrast to 12 h).

An infant mouse model which has previously been used to examine the ability of *C. albicans* to colonise and disseminate to various organs was also used in this study to compare *C. albicans* and *C. dubliniensis* when inoculated alone and in co-inoculation experiments. *Candida dubliniensis* like *C. albicans* was capable of colonising the stomach of infant mice however at significantly lower levels than *C. albicans*, (i.e. 50% in contrast to 100%). Dissemination to the liver was also evident in both species after immunocompromising treatment. However, as before significantly lower colonisation levels were evident in *C. dubliniensis* in contrast to *C. albicans* (i.e. 50% in contrast to 90%). Similar results were obtained in co-infection experiments whereby 2.4 x 10^2 cfu/ml of *C. dubliniensis* derivative CD36 (M/G) was detected in the stomach in comparison with *C. albicans* strain SC5314 where 1.7 X 10^7 cfu/ml was detected. Furthermore, in the liver 1.5 x 10^1 cfu/ml of *C. dubliniensis* derivative CD36 (M/G) (which had been tagged with the molecular marker cassette) was detected while 1.9 x 10^3 cfu/ml of *C. albicans* strain SC5314 was detected.

The results of this study demonstrate considerable differences in growth rate and the ability of *C. dubliniensis* strains to adhere, colonise and produce hyphae in comparison with *C. albicans* strains *in vivo*, *in vitro* and *ex vivo*. These findings may partly explain why *C. albicans* is significantly more pathogenic than *C. dubliniensis*.

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Abbreviations

AIDS	acquired immunodeficiency syndrome
A480, A600, A485,A535	absorption at 480 nm, 600 nm
ATP	adenosine 5'-triphosphate
Azole	synthetic N-substituted azoles, including the imidazole and triazole andtifungal antibiotics
BEC	buccal epithelial cells
bp	base pairs
BSA	bovine serum albumin
Bq	becquerel
cfu	colony forming units
Ci	curie
CMC	chronic mucocutaneous candidiasis
CSH	cell surface hydrophobicity
Δ	deletion (of a gene)
dpm	disintegrations per minute
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
dNTP	dideoxynucleoside
ECM	extracellular matrix proteins
EDTA	ethylenediamine tetraacetic acid
e. g.	for example
et al.	and others
g	gravity

GFP	green fluorescent protein
GI	gastrointestinal
GPI	glycophosphatidylinositol
h	hour(s)
HIV	human immunodeficiency virus
HUVEC	human umbilical vein endothelial cells
IPTG	isopropyl-β-D-thiogalactopyranoside
ICU	itensive care unit
i.e.	that is
iv	intravenous
kb	kilobase pairs
L agar	Luria agar
L broth	Luria broth
LDH	lactate dehydrogenase
l	litre
M	molar
Mb	megabase
mg	milligram
µg	microgram
ml	millilitre
µl	microlitre
µm	micron
mM	millimolar
min	minute

NCCL	National Committee for Clinical Laboratory Standards
nm	nanometre
no.	number
OPC	oropharyngeal candidiasis
$O.D._x$	optical density at x nm
PAS	Periodic-Acid Schiff
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDA	potato dextrose agar
PFGE	pulsed-field gel electrophoresis
RAPD	random amplified polymorphic DNA
rDNA	ribosomal DNA
RFLP	restriction fragment length poymorphism
RHE	reconstituted human epithelium
RHVE	reconstituted human vaginal epithelium
RNA	ribonucleic acid
RNase	ribonuclease
s	seconds
SDA	sabouraud dextrose agar
SDB	sabouraud liquid broth modified antibiotic medium 13
SDS	sodium dodecyl sulphate
SSC	salt, sodium citrate
TBE	tris-borate EDTA
Tris	tris (hydroxymethyl) aminoethane

Tgase	transglutaminase
U	Units
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
VVC	vulvovaginal candidiasis
w/v	% weight in volume expresses the number of grams of an active constituent in 100 millilitres of solution
X-gal	5-bromo-4-chloro-indoyl-β-D-galactoside
YNB	yeast nitrogen base
YPD	yeast-extract-peptone-dextrose
YPS	yeast extract-peptone-sucrose
>	greater than
<	less than
≥	greater than or equal to
≤	less than or equal to

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.

Moran, G., Stokes, C., Thewes, S., Hube, B., Coleman, D. C. & Sullivan, D. (2004). Comparative genomics using *Candida albicans* DNA microarrays reveals absence and divergence of virulence-associated genes in *Candida dubliniensis*. *Microbiology* **150,** 3363-3382.

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Chapter 1
General Introduction

1.1 General Introduction

During the last two decades the prevalence of fungal infections has increased steadily. The reasons for this shift in the epidemiology of human fungal disease is the significant increase in the number of immunocompromised patients and the increasing use of invasive medical procedures and broad spectrum antimicrobial agents (Fridkin & Jarvis, 1996). These infections are primarily opportunistic in origin and their increase has mainly been due to a rise in the number of susceptible patients. These include patients in intensive care units following major medical procedures, patients receiving anti-cancer therapy, patients with chemotherapy induced neutropenia and those receiving organ transplant associated immunosuppressive therapy (Verduyn Lunel *et al.*, 1999; Yang & Lo, 2001). In addition, HIV-infected individuals and AIDS patients are also particularly susceptible to fungal infection (Sullivan *et al.*, 2004). *Candida* species are amongst the most important fungal pathogens, have emerged as significant human pathogens during the past two decades and are currently the fourth most common cause of nosocomial bloodstream infection in the USA, with a mortality rate of 40% to 60% (Eggimann & Pittet, 2001; Ellis, 2002). In nenonatal intensive care units, they are an even more frequent cause of bloodstream infections (Edmond *et al.*, 1999; Pittet & Wenzel, 1995; Saiman *et al.*, 2000), however, the relative prevalence of *Candida* infections may vary between institutions. Disease caused by *Candida* species range from superficial mucousal or cutaneous to systemic infection.

1.1.1 Superficial candidiasisis

The most common mucousal infections include oropharyngeal (OPC), oesophageal, vaginal and chronic mucocutaneous candidiasis. Several clinical forms of OPC have been described, the most prevalent form of which is pseudomembranous candidiasis which is commonly referred to as thrush. Other forms of OPC include erythematous candidiasis, *Candida* leukoplakia and angular cheilitis. Oropharyngeal candidiasis is the most common mycosis in AIDS patients and is often the first clinical manifestation of HIV infection (Ruhnke, 2002). Indeed, more than 90% of individuals with progressive HIV disease develop OPC at some time during the course of their disease (Garber, 2001). The majority of patients (77 to 100%) with OPC are infected with *C. albicans*, while the remaining patients

are infected with one or more non-*C. albicans* species of *Candida*, either alone or in combination with *C. albicans* (Lasker *et al.*, 2001; Powderly, 1992; Redding *et al.*, 1997; Sangeorzan *et al.*, 1994; Vargas & Joly, 2002). Oesophageal candidiasis mainly occurs in patients with advanced HIV infection (Ruhnke, 2002) and it has been suggested that oesophageal candidiasis in HIV-positive patients may be the first manifestation of AIDS (Fidel *et al.*, 1999). However, oral *Candida* infections can also occur in neonates, the elderly, diabetics and individuals using topical steroids (Challacombe, 1994). Angular cheilitis, erythema, maceration and ulceration of the skin adjacent at the angles of the mouth and denture stomatitis, are also associated with *Candida* species.

Vaginal carriage of *Candida* species and other yeasts is reported to range from 10 to 20% in healthy women. Although vulvovaginal candidiasis (VVC) is more common in HIV-infected women, it is estimated that as many as 75% of women, regardless of their HIV status, will experience at least one episode of VVC during their life time (Ruhnke, 2002). While most women suffer from sporadic, mild to moderate vaginitis, approximately 10% of women suffer from more severe and recurrent symptoms. Women with predisposing factors such as immunosuppression and uncontrolled diabetes are at a higher risk for this type of infection (Fidel *et al.*, 1999). In addition, nearly three-quarters of all healthy women experience at least one vaginal yeast infection and about 5% endure recurrent bouts of disease (Sobel, 1998).

Chronic mucocutaneous candidiasis (CMC) is a collective syndrome in which patients have chronic and/or recurrent infections of the skin, nails, and mucous membranes with *Candida* species. Individuals at risk of CMC are those unable to develop effective cell-mediated immune responses against *Candida*.

While superficial *Candida* infections may be relatively common, their symptoms while debilitating rarely are life-threatening. However, when the fungus penetrates into the bloodstream and results in candidemia and systemic disease the effect on the patient is often fatal.

1.1.2 Invasive candidiasis

Invasive candidiasis accounted for 17% of hospital-acquired infections reported during the European study on the prevalence of nosocomial infections in critically ill patients

(EPIC), which included 10,038 patients in 1417 intensive care units (ICUs) in 17 countries in 1992 (Vincent *et al.*, 1995). Invasive candidiasis refers to *Candida* infections, other than those of the skin and mucous membranes, whereby *Candida* cells penetrate through the epithelial barrier. Invasive candidiasis includes candidemia (colonisation of the bloodstream by *Candida* species), acute or chronic disseminated candidiasis, and less frequently, infection of a single deep organ. Invasive candidiasis is generally the result of penetration of *Candida* cells into the bloodstream and the subsequent hematogenous spread of the infective organism (hematogenous candidiasis), but can also occur from direct inoculation following surgery (non-hematogenous candidiasis). Invasive candidiasis is associated with high mortality. The incidence of candidemia among hospitalised patients has doubled during the past 20 years (Asmundsdottir *et al.*, 2002; Kullberg & Filler, 2002). Candidemia has been shown to be associated with underlying conditions such as cancer, abdominal surgery, diabetes mellitus and HIV (Kao *et al.*, 1999). Risk factors for candidemia and disseminated candidiasis also include the use of antibiotics prior to infection (Pfaller *et al.*, 1998a), the presence of intravenous catheters, neutropenia and prior colonisation with *Candida* species (Kullberg & Filler, 2002). It has been shown that broad-spectrum antibiotics eliminate the endogenous bacterial flora and allow the overgrowth of *Candida* and other yeast species (Pfaller *et al.*, 1998a; Samonis *et al.*, 1994). The vast majority of invasive infections are caused by endogenous *Candida* strains that belong to the patient's normal flora. Indeed, several studies have highlighted the correlation between previous gastrointestinal colonisation by *Candida* species and candidemia (Kullberg & Filler, 2002; Pittet *et al.*, 1994).

Chronic disseminated candidiasis is predominantly observed in neutropenic patients, especially those suffering from leukemia. In these patients, invasive candidiasis develops in multiple organs over a period of several weeks or even several months as a result of a candidemia acquired during an episode of neutropenia and is frequently not detected until after neutropenia has resolved.

1.2 Epidemiology of *candidiasis*

The genus *Candida* consists of more than 200 species, although only a dozen or so of these have been associated with human infection. Numerous surfaces of the body have been shown to be colonised by *Candida* species including the vaginal wall, surfaces in the oral cavity, and the anorectal surface (Soll *et al.*, 1991). *Candida* species are considered to be a normal part of the oropharyngeal and gastrointestinal flora (Ruhnke, 2002). *Candida* species usually reside as commensal organisms as part of an individuals normal microflora and can be detected in approximately 50% of the population in this form. However, when host defenses are compromised *Candida* species may become opportunistic pathogens and establish an infection. While *C. albicans* is generally considered to be the most pathogenic of the *Candida* species, a variety of other members of the genus notably *C. krusei*, *C. glabrata*, *C. tropicalis C. parapsilosis* and *C. dubliniensis* are also increasingly recognised as human pathogens (Edmond *et al.*, 1999; Gilfillan *et al.*, 1998; Pittet & Wenzel, 1995). The emergence of these species is thought to be due to the widespread therapeutic use of prophylactic antifungals as they have an inherent reduced susceptibility relative to *C. albicans* (Chavanet *et al.*, 1994; De Bernardis *et al.*, 1996; Johnson *et al.*, 1995; Moran *et al.*, 1997; Powderly, 1992). The reasons for this shift in the epidemiology of candidiasis may also be linked to the general increase in the incidence of fungal infections and the increased longevity of severely immunocompromised individuals which has resulted in a larger population of extremely debilitated patients, thus allowing many of these species to emerge and cause disease.

1.3 Characterisation of *Candida dubliniensis*

1.3.1 Discovery of *C. dubliniensis*

Candida dubliniensis is a member of the genus *Candida*, which emerged in the 1990's and was originally thought to be an atypical isolate of *C. albicans*. Although these isolates shared many of the characteristic features of *C. albicans* isolates, they exhibited a number of atypical properties. These atypical isolates hybridised poorly to the *C. albicans*-specific

DNA probe 27A (Boerlin *et al.*, 1995; McCullough *et al.*, 1995; Schmid *et al.*, 1992; Sullivan *et al.*, 1993). Subsequently, extensive analysis was undertaken on these isolates including, phenotypic, genotypic and phylogenetic analysis of nucleotide sequences of the V3 region of the large rRNA subunit gene. On the basis of the results from this extensive analysis Sullivan *et al.*, concluded in 1995 that the atypical isolates constituted a novel, distinct taxon within the genus *Candida* which was named *Candida dubliniensis* (Sullivan *et al.*, 1995).

1.3.2 Phenotypic characteristics used for the identification of *C. dubliniensis*

1.3.2.1 Chlamydospore and germ tube production

It was originally thought that the ability to produce chlamydospores was a characteristic of *C. albicans* only and was thus used a means of identification of this species from clinical specimens. However, *C. dubliniensis* also possesses the ability to produce chlamydospores (Sullivan *et al.*, 1995). However, *C. albicans* tends to produce single chlamydospores attached terminally to hyphae by a single suspensor cell, in contrast to *C. dubliniensis,* which tends to produce abundant chlamydospores, frequently arranged in contiguous pairs, triplets or larger groups attached to a single suspensor cell (Sullivan *et al.*, 1995). Unlike other *Candida* species, both *C. dubliniensis* and *C. albicans* have the ability to produce germ tubes when incubated in serum (Sullivan *et al.*, 1995). However, unlike *C. albicans* isolates, *C. dubliniensis* isolates do not produce germ tubes when incubated in medium containing *N*-acetyl glucosamine (Gilfillan *et al.*, 1998).

1.3.2.2 Lack of growth at 42°C

Isolates of *C. dubliniensis* grow well at 30°C and 37°C on all mycological culture media used for the culture of *Candida* species (Sullivan *et al.*, 1995). However, *C. dubliniensis* isolates were observed to grow poorly or not at all at 42°C, unlike *C. albicans* isolates which grow well at this temperature (Sullivan *et al.*, 1995). In a study of 120 *C. dubliniensis* isolates and 98 *C. albicans* isolates Pinjon *et al.* observed that *C. dubliniensis* isolates do not grow at 45°C, in contrast the majority of *C. albicans* isolates are capable of growth at 45°C (Pinjon *et al.*, 1998). However, other methods have shown that not all *C.*

albicans isolates are capable of growing at this elevated temperature and therefore this method cannot be reliably used to differentiate between the two species (Gales *et al.*, 1999)

1.3.2.3 Carbon and nitrogen assimilation profiles

Commercially available yeast identification systems such as the bioMerieux API ID 32C and API 20C AUX systems are routinely used to identify *Candida* species in diagnostic laboratories. These kits generate a numerical code or profile based on the assimilation patterns of a specific range of substrates. Comparison of the profile generated for an unknown isolate with database profiles allows its identification. The range of carbohydrates assimilated by *C. albicans* and *C. dubliniensis* has been shown to be significantly different (Boerlin *et al.*, 1995; Gales *et al.*, 1999; Jabra-Rizk *et al.*, 1999; Kirkpatrick *et al.*, 1998; Pincus *et al.*, 1999; Sullivan *et al.*, 1995; Tintelnot *et al.*, 2000). Previous studies have shown that identification of *C. dubliniensis* using substrate assimilation profile analysis can be problematic, mainly because the *C. dubliniensis* assimilation profiles included in the database of commercially available yeast identification systems such as the API ID 32C system do not include all *C. dubliniensis* isolate profiles (Pincus *et al.*, 1999). *Candida dubliniensis* is unable to readily assimilate xylose or α-methyl-D-glucoside, which has been shown to be useful in discriminating between *C. albicans* and *C. dubliniensis* (Gales *et al.*, 1999; Pincus *et al.*, 1999).

1.3.2.4 Growth on differential agar media

On routinely used mycological media such as Potato dextrose agar (PDA) and Sabouraud dextrose agar (SDA), *C. dubliniensis* and *C. albicans* produce similar colonies of a creamy white colour. However, the commercially available medium CHROMagar® Candida medium can be used for the preliminary identification of clinically important *Candida* species (Odds & Bernaerts, 1994). On primary isolation on this medium, *C. dubliniensis* colonies are dark green in colour whereas *C. albicans* colonies are light blue-green in colour (Coleman *et al.*, 1997b; Koehler *et al.*, 1999; Schoofs *et al.*, 1997; Sullivan & Coleman, 1998). However, *C. dubliniensis* isolates can lose their distinctive dark green coloration following prolonged storage or subculture (Schoofs *et al.*, 1997). Recently studies

have found that on media supplemented with particular plant extracts namely Staib agar (birdseed agar) (Al Mosaid *et al.*, 2001; Staib & Morschhauser, 1999), Pal's agar (sunflower seed agar) (Al Mosaid *et al.*, 2003) and Tobacco agar (Khan *et al.*, 2004) phenotypic differences can be observed between *C. albicans* and *C. dubliniensis*. *Candida albicans* colonies are smooth and consist of blastospores whereas *C. dubliniensis* colonies are rough and consist of hyphae, pseudohyphae and chlamydospores on these media. While it had been reported that *C. dubliniensis* isolates produce hyphae and chlamydospores on Staib medium (Staib & Morschhauser, 1999), a more comprehensive study found that 14.6% *C. dubliniensis* isolates did not produce them and concluded that differentiation of *C. dubliniensis* from *C. albicans* on Staib agar should be based on colonial morphology rather than hyphae and chlamydospore production (Al Mosaid *et al.*, 2001). A study by Al Mosaid *et al.* have reported 100% accuracy in differentiating *C. albicans* from *C. dubliniensis* on Pal's agar (Al Mosaid *et al.*, 2003).

1.3.3 Genotypic characteristics of *C. dubliniensis*

1.3.3.1 DNA fingerprinting

The first evidence that suggested that *C. dubliniensis* isolates were genetically distinct from other *Candida* species came from DNA fingerprinting studies with the moderately repetitive *C. albicans* fingerprinting probes 27A and Ca3 (Boerlin *et al.*, 1995; Boyle *et al.*, 2002; McCullough *et al.*, 1995; Sullivan *et al.*, 1995). While the 27A probe hybridised strongly to *C. albicans* and to type I and type II *C. stellatoidea* *Eco*RI-digested DNA, producing complex patterns of bands (Scherer & Stevens, 1988), the fingerprint patterns obtained for *C. dubliniensis* isolates consisted of a small number of weakly hybridising bands (Coleman *et al.*, 1997a; McCullough *et al.*, 1995; Sullivan *et al.*, 1995). Similarly, the Ca3 fingerprinting probe hybridised weakly to *C. dubliniensis* genomic DNA while it gave strong hybridisation bands with *C. albicans* genomic DNA (Boerlin *et al.*, 1995). In contrast, Joly *et al.* identified and cloned a *C. dubliniensis*-specific DNA repetitive element termed Cd25 which could readily distinguish clinical isolates of *C. dubliniensis* in DNA fingerprinting studies (Joly *et al.*, 1999). Based on fingerprint profiles obtained with the Cd25 probe, Joly *et al.* showed that *C. dubliniensis* isolates could be divided into two distinct groups termed Cd25 group I and Cd25 group II (Joly *et al.*, 1999). Isolates within Cd25 group I were more

homogeneous or more closely related to one another than isolates within Cd25 group II. This dichotomy in *C. dubliniensis* isolates was confirmed by the extensive study of Gee *et al.* (Gee *et al.*, 2002). In addition, Gee *et al.* found that Cd25 group I isolates were predominant worldwide and mainly recovered from HIV-infected patients while Cd25 group II isolates were mainly recovered from HIV-negative individuals (Gee *et al.*, 2002). Joly *et al.* also cloned two other DNA repetitive elements (Cd1 and Cd24) from *C. dubliniensis* (Joly *et al.*, 1999) which can be used for DNA fingerprinting, although they cross react with *C. albicans* genomic DNA. Because the patterns generated with the Cd1 and Cd24 probes are less stable over time both *in vivo* and *in vitro* than patterns generated with the Cd25 probe, these two probes are more suited to the investigation of microevolution within strains. Recently Al Mosaid *et al.* while investigating *C. dubliniensis* isolates recovered from Saudi Arabia, Egypt and Israel performed Cd25 fingerprinting analysis and found a novel clade of isolates determined Cd25-group III (Al Mosaid *et al.*, 2005). These findings thus determined that the population structure in Saudi Arabia and Egypt is different from populations of isolates from the rest of the world.

1.3.3.2 Karyotype profiles

The presence of one or more chromosome-sized bands <1 Mb has been found to be a characteristic feature of the karyotype profiles of *C. dubliniensis* isolates (Gee, 2002; Sullivan *et al.*, 1995). A study of karyotype profiles of 89 *C. dubliniensis* isolates by Gee *et al.* showed that two major karyotypes patterns could be identified on the basis of the presence or absence of chromosome-sized bands at approximately 1.7 and 1.75 Mb (Gee *et al.*, 2002).

1.3.3.3 Identification of *C. dubliniensis* isolates by PCR

Several PCR based techniques have been developed to facilitate the identification of *C. dubliniensis* and its differentiation from *C. albicans.* Donnelly *et al.* developed a *C. dubliniensis*-specific PCR test using primers based on the *ACT1*-associated intron sequence of *C. dubliniensis* which shares 83.4% identity with the *C. albicans* sequence (Donnelly *et al.*, 1999). An extensive evaluation of this PCR method carried out using 122 *C. dubliniensis* isolates, 53 *C. albicans* isolates and a number of isolates from other *Candida* species showed that positive identification of *C. dubliniensis* could be obtained in 4 h (Donnelly *et al.*, 1999).

A number of other PCR methods based on the sequence differences between *C. albicans* and *C. dubliniensis* in the internal transcribed spacer (ITS) regions ITS1 and ITS2 of the ribosomal gene cluster have been developed (Irobi *et al.*, 1999; Martin *et al.*, 2000; McCullough *et al.*, 1999; Park *et al.*, 2000; Williams *et al.*, 2001). In one study, the sequence of the ITS2 region was used to develop a molecular beacon probe for identification of *C. dubliniensis* as well as *C. albicans* (Park *et al.*, 2000). Recently, another PCR-based test for the identification of *C. dubliniensis* was developed based on the sequence of the mitochondrial cytochrome b gene (Biswas *et al.*, 2001). The most definitive methods of identifying *C. dubliniensis* are based on differential amplification of species-specific sequences using the polymerase chain reaction (PCR) and real time PCR (Elie *et al.*, 1998; Kurzai *et al.*, 1999; Tamura *et al.*, 2000). PCR based tests have an advantage over phenotypic and molecular typing methods in that they are rapid, easy to perform and are amenable to large throughput volume.

1.3.3.4 Genotyping of *C. dubliniensis* isolates on the basis of ITS sequence analysis

Gee *et al.* carried out sequence analysis of the ITS region of *C. dubliniensis* isolates and identified four distinct genotypes among the basis of the sequence variations in the ITS1 and ITS2 regions (Gee *et al.*, 2002). All *C. dubliniensis* isolates belonging to Cd25 group I were found to belong to genotype 1 while the Cd25 group II isolates comprised of three distinct genotypes (genotypes 2, 3 and 4) (Gee *et al.*, 2002). Furthermore, it was observed that genotype 1 isolates appear to be associated with carriage and infection in HIV-infected patients, whereas genotypes 2-4 isolates were mainly recovered from HIV-negative individuals. Gee *et al.* also showed that the *C. dubliniensis* genome undergoes microevolutionary genomic re-arrangements at a far higher rate than *C. albicans* (Gee *et al.*, 2002). A recent study by Al-Mosaid *et al.*, showed that when investigating *C. dubliniensis* isolates from Saudi Arabia, Egypt and Israel that 20/30 (66.6%) of isolates from Saudi Arabia and Egypt belonged to genotypes 3 and 4, with the rest of the isolates belonging to genotype 1 (Al Mosaid *et al.*, 2005).

To facilitate epidemiological studies, Gee *et al.* developed a genotype-specific PCR assay based on sequence differences in the ITS region between the four genotypes (Gee *et al.*, 2002). Using this assay, the genotype and hence the Cd25 fingerprint group of an isolate

can be determined in a matter of 5 h. This simple assay should greatly facilitate the investigation of the epidemiological relevance of each genotype group of *C. dubliniensis* isolates.

1.3.3.5 Phylogenetic analysis of *C. dubliniensis*

Molecular analysis has revealed that *C. dubliniensis* is genetically distinct from *C. albicans* and other yeast species. Previously Sullivan *et al.* sequenced 500 bp of the V3 variable region of the large ribosomal subunit gene of nine separate *C. dubliniensis* isolates from Ireland, the UK and Australia and compared them to the corresponding sequences of seven other *Candida* species (Sullivan *et al.*, 1995). A phylogenetic tree was produced based on these sequences of the V3 variable region and the *C. dubliniensis* isolates formed a homogeneous cluster that was significantly different from the other *Candida* species analysed (Sullivan *et al.*, 1995). Gilfillan *et al.* confirmed the close relationship between the two species by comparing the nucleotide sequences of their small rRNA genes and determined that the difference between the two sequences was 1.4% (Gilfillan *et al.*, 1998). *Candida albicans* and *C. dubliniensis* showed 97.75% identity based on this region. A recent study by Moran *et al.* using comparative genomics with *C. albicans* DNA microarrays found only 4.4% of *C. albicans* sequences were absent or highly divergent in *C. dubliniensis* which suggests that the two species have diverged relatively recently (Moran *et al.*, 2004).

1.3.4 Epidemiological analysis of *C. dubliniensis*

Epidemiological studies indicate the prevalence of *C. dubliniensis* throughout the world including Argentina, Australia, Belgium, Brazil, Canada, Egypt, France, Germany, Greece, India, Israel, Italy, Kuwait, Japan, Norway, Saudi Arabia, South Africa, Spain, Switzerland, UK and the USA and also its association with carriage and infection in the oral cavities of HIV infected and AIDS patients (Ahmad *et al.*, 2004; Al Mosaid *et al.*, 2005; Alves *et al.*, 2001; Boerlin *et al.*, 1995; Boucher *et al.*, 1996; Fisher *et al.*, 2001; Giammanco *et al.*, 2000; Gugnani *et al.*, 2003; Hannula *et al.*, 1997; Kurtzman & Robnett, 1997; Le Guennec *et al.*, 1995; McCullough *et al.*, 1994; Polacheck *et al.*, 2000; Pujol *et al.*, 1997; Schmid *et al.*, 1992; Schoofs *et al.*, 1997; Sullivan *et al.*, 1997; Sullivan *et al.*, 1995; Tamura *et al.*, 2000). This species may be particularly adapted to colonising the oral cavity although

it has also been isolated from sputum, blood, vaginal flora and faeces (Odds, 1988; Pfaller *et al.*, 1998b; Pinjon *et al.*, 1998). Furthermore, studies have reported the association of *C. dubliniensis* with systemic infections in patients with severe underlying medical conditions (Arthington-Skaggs *et al.*, 2002; Boyle *et al.*, 2002; Gottlieb *et al.*, 2001; Kibbler *et al.*, 2003; Meis *et al.*, 1999). Although the true incidence of candidemia caused by *C. dubliniensis* is not known, a recent study carried out in a Dutch hospital found that this species was present in 0.8% of fungal surveillance cultures (Meis *et al.*, 2000). Other studies found the prevalence of oral *C. dubliniensis* in HIV-infected individuals to be between 11% and 25% (Brown *et al.*, 2000; Graybill *et al.*, 1998; Jabra-Rizk *et al.*, 1999; Meiller *et al.*, 1999). The first reported case of *C. dubliniensis* endocarditis was recently described (Carr *et al.*, 2005). *Candida dubliniensis* has also been found in the oral microflora of healthy individuals (Mosca *et al.*, 2005). Relatively high prevalence rates have also been found in patients suffering from diabetes (Willis *et al.*, 2000) and also in the subgingival sites of HIV-positive children (Portela *et al.*, 2004). *Candida dubliniensis* isolates from the oral cavity are most commonly recovered in mixed culture with other *Candida* species, especially *C. albicans* (Coleman *et al.*, 1997b). However, pure cultures of *C. dubliniensis* have been recovered from individuals exhibiting clinical symptoms of oral candidiasis, suggesting that this organism can be responsible for oral infections (Al Mosaid *et al.*, 2003; Al Mosaid *et al.*, 2005; Coleman *et al.*, 1997b; Moran *et al.*, 1998).

In a study on an Irish population of normal healthy individuals only 3.5% of individuals were found to carry *C. dubliniensis* (Ponton *et al.*, 2000). *Candida dubliniensis* is only rarely associated with systemic infections and is responsible for as few as 2% of cases of candidemia which is in contrast to *C. albicans* which accounts for approximately 65% of the *Candida* isolates recovered from blood samples (Sullivan *et al.*, 2004). This result is surprising considering the similarities between the two species.

1.4 *Candida* Virulence

Although *C. albicans* is an opportunistic pathogen, once it breaches the impaired immune system, it is a very successful and versatile pathogen. Since *C. albicans* has the ability to colonise and grow in a wide variety of anatomic sites, it clearly has the ability to

quickly adapt to changes in its local environmental conditions which is demonstrated in the neutral pH of the bloodstream or tissues *C. albicans* expresses *PHR1* whose function is associated with cell wall synthesis and whose expression is optimal at neutral pH. At acidic pH, for example in the vaginal canal, *PHR1* is switched off and a second pH regulated gene, *PHR2,* which provides a similar function is expressed (Ghannoum *et al.*, 1995; Muhlschlegel & Fonzi, 1997; Saporito-Irwin *et al.*, 1995). The homologues of *PHR1* and *PHR2* in *C. dubliniensis* have been cloned and their functions are similar to that of *C. albicans* (Heinz *et al.*, 2000). The ability of *C. albicans* to colonise, penetrate, and damage host tissues depends on imbalances between *Candida* virulence attributes and specific defects in host immune defences. Host recognition is another important virulence factor of *C. albicans* which involves binding of the organism to host cells, host cell proteins or microbial competitors (co-aggregation) which most likely prevents or at least reduces the ability of the host to clear the fungus. *Candida dubliniensis* has been shown to grow well at 37°C *in vitro* and produce biofilms under specific conditions (Kirkpatrick *et al.*, 2000). Studies have also shown that the growth rate of *C. dubliniensis* is less than that of *C. albicans* (Vilela *et al.*, 2002). The difference in growth rate between the two species has been observed to be more pronounced under conditions of environmental stress and *C. dubliniensis* was found to be less tolerant of elevated temperatures (e.g. 42°C; (Pinjon *et al.*, 1998)), osmotic pressure (e.g. 6.5% (w/v) NaCl (Alves *et al.*, 2002)) and also oxidative stress (e.g. 10 mM H_2O_2 (Vilela *et al.*, 2002)). Kirkpatrick *et al.* have also examined what happens when *C. albicans* and *C. dubliniensis* are inoculated in mixed culture and found that in nine out of eleven pairings *C. albicans* was only present after 96 h, thus out-competing *C. dubliniensis* (Kirkpatrick *et al.*, 2000).

The mechanisms used by *Candida* species to colonise and infect human tissues are poorly understood, however, several attributes have been proposed as virulence factors in *C. albicans.* The most important virulence factors identified to date include the production of secreted hydrolytic enzymes, dimorphic transition, the ability to switch between different cell phenotypes and adhesion to inert and biological substrates. Interestingly many of these attributes are shared by *C. dubliniensis* (Gilfillan *et al.*, 1998; Hannula *et al.*, 2000; Vilela *et al.*, 2002). However, despite the similarities between *C. albicans* and *C. dubliniensis*, epidemiological data and the results of experiments using *in vivo* infection models indicate that *C. dubliniensis* is significantly less pathogenic than *C. albicans* (Gilfillan *et al.*, 1998;

Vilela *et al.*, 2002). The sum of the effects of many *Candida* virulence factors lead to the establishment of infection in a suitable compromised host. In some host niches, particular fungal proteins (e.g. adhesins) are important, while in other niches, these proteins are less important and other factors play a dominant role. By being adapted for growth in many possible host niches, *C. albicans* is able to cause the wide spectrum of clinical manifestations for which it is known.

1.4.1 Adherence

Adherence is one of the first and most important stages of the infection process for *Candida* species. There are a number of *Candida* proteins that bind to several extracellular matrix proteins (ECM) of mammalian cells, such as fibronectin (FN), laminin, fibrinogen and collagen type I and IV (Chaffin *et al.*, 1998; Gaur *et al.*, 1999; Sturtevant & Calderone, 1997). *Candida* species have also been observed to adhere to other bacteria, medical devices such as intra-venous catheters and to mammalian cells (Odds, 1988). Studies have shown that there are variations in the adherence capabilities of *Candida* species, which explains why some are found more frequently colonising mucousal surfaces (Ellepola & Samaranayake, 1998; King *et al.*, 1980).

Factors known to have an effect on the retention or distribution of oral *Candida* species are many, including acidic pH, sucrose- or glucose-rich diets, and *Candida* cell surface mannoprotein and cell surface hydrophobicity all tend to enhance adhesion (Chaffin *et al.*, 1998; Marcotte & Lavoie, 1998; Webb *et al.*, 1998). The cell wall of *C. albicans* is the site of contact for adherence of the fungus to its environment (Calderone & Braun, 1991; Glee *et al.*, 1995; Hazen, 1989). Initial attachment of *C. albicans* to surfaces is primarily mediated by mannoprotein adhesins on the fibrils of the outermost layer of the yeast cell surface (Hazen, 1989; Hazen, 1995). The mechanism of adherence of *C. albicans* to human tissues are varied and as a result the *Candida*-host cell recognition systems are extremely complex and involve a variety of ligand-receptor components (Sturtevant & Calderone, 1997). Depending on the types of receptors involved, different kinds of interactions between the fungus and host tissues have been described, such as protein-protein interactions, protein-carbohydrate interactions and *Candida* mannoprotein ligands which are recognsied by unknown host receptors (Calderone & Braun, 1991; Cannon & Chaffin, 1999). The

expression of cell surface components on *C. albicans* is, in part, growth phase specific. For example, hyphal forms of the organism bind fibrinogen, laminin, and the complement (C3) conversion product C2d, while yeast cells do not (Bouali *et al.*, 1987; Bouali *et al.*, 1988; Bouchara *et al.*, 1990). Cannon & Chaffin have shown that certain lipid classes such as phospholipids are also involved in adherence, with a glycosphingolipid of buccal epithelial cells (BEC) shown to be the adherence target for *C. albicans* (Cannon & Chaffin, 1999).

It is widely known that mucous O-linked glycoproteins (mucins) are produced and secreted from the mucous cells of stomach, oesophagus, small and large intestine, salivary glands as well as the gall bladder and pancreatic ducts. Mucins play an important role in host defence against infection (de Repentigny *et al.*, 2000). In the same study de Repentigny *et al.* demonstrated that binding of *Candida* species to purified small intestinal mucin showed a close correlation with their virulence hierarchy determined previously from animal models. They showed that *C. dubliniensis* adhered strongly to mucin and significant differences were found among *Candida* species, adhering highly (*C. dubliniensis*, *C. tropicalis*, and *C. albicans*), moderately, (*C. parapsilosis* and *C. lusitaniae*) or weakly (*C. krusei* and *C. glabrata*) to mucin (de Repentigny *et al.*, 2000).

Candida species can also adhere to the surfaces of medical devices and form biofilms, which results in an increase in candidemia and antifungal resistance related to catheter insertion (Chandra *et al.*, 2001; Hawser & Douglas, 1994). Yeast species, mainly *C. albicans* are the third leading cause of catheter-related infections, and they have the second highest colonisation to infection rate (Crump & Collignon, 2000). Hawser & Douglas have also shown that there is a positive association between the degree of virulence and the ability to form biofilms (Hawser & Douglas, 1994). Kirkpatrick *et al.* showed that *C. dubliniensis* like *C. albicans* forms biofilms *in vitro* and that *C. albicans* demonstrated superior growth characteristics over *C. dubliniensis* in growth competition experiments under planktonic conditions. However, *C. dubliniensis* was better able to withstand the competitive pressures of *C. albicans* using biofilm conditions and the presence of a supporting structure polyvinylchloride (PVC). To date little is known about interspecies interactions between *C. albicans* and *C. dubliniensis*, yet these species have been frequently isolated concomitantly from the oral cavities of immunocompromised patients (Coleman *et al.*, 1997a).

Although the strongest mechanism for adherence involves a mannoprotein adhesin on *C. albicans*, cell surface hydrophobicity (CSH) has been described by many investigators as being involved in adherence (Calderone & Braun, 1991; Cannon & Chaffin, 1999; Hazen, 1989). Cell surface hydrophobicity plays an important role in the adherence process by providing hydrophobic interactions between hydrophobic proteins embedded in the matrix of the *C. albicans* cell wall which turn the initial attachment between the yeast and a surface into a strong bond (Calderone & Braun, 1991; Cannon & Chaffin, 1999; Hazen, 1989; Jabra-Rizk *et al.*, 2001). Recent studies have shown that *C. dubliniensis*, unlike *C. albicans* (Cannon & Chaffin, 1999) possesses cell surface variations that allow it to be constantly hydrophobic, regardless of environmental conditions at 37°C (Hazen, 2004). It is suggested that *C. albicans* may be hydrophilic as a commensal and converts to the hydrophobic surface phenotype during parasitism as CSH has been shown to precede the yeast-to-hyphae conversion (Cannon & Chaffin, 1999; Hazen & Hazen, 1992). Mice challenged with hydrophobic yeast cells died faster than those challenged with hydrophilic cells (Hazen & Hazen, 1988). Furthermore, hydrophobic cells grown at 25°C germinated more rapidly than hydrophilic cells after engulfment by polymorphonuclear neutrophils, providing them with an escape mechanism, making them less susceptible to killing by phagocytes (Glee *et al.*, 1995; Hazen *et al.*, 1991)

Gilfillan *et al.,* have shown that oral *C. dubliniensis* isolates have been shown to adhere to BEC to a greater extent than *C. albicans* isolates when grown in the presence of glucose (Gilfillan *et al.*, 1998) which may be relevant to the observation that *C. dubliniensis* is found almost exclusively in the oral cavity (Coleman *et al.*, 1997b). In the same study however, no difference was observed between the two species when grown in galactose medium (Gilfillan *et al.*, 1998). The reasons for the increased adherence has been suggested to be due to the elevated expression of receptors, such as the glucose-responsive *C. albicans* analogue to the mammalian neutrophil integrin protein, CD11b/CD18 (Gustafson *et al.*, 1991). In addition molecules such as aspartyl proteinases (Saps) and phosphatases have been shown to play a crucial role in the ability of yeast to adhere to human (BECs) and other substrates (Cannon & Chaffin, 1999; Sturtevant & Calderone, 1997). Jabra-Rizk *et al.* have shown that *C. dubliniensis* isolates have greater adherence to BEC than do typical *C. albicans* strains and that it is thought that hydrophobic interactions are involved in this

mechanism of adherence (Jabra-Rizk *et al.*, 2001). However Vidotto *et al.*, compared the ability of *C. albicans* and *C. dubliniensis* to adhere to BEC and vaginal epithelial cells (VEC) and found that *C. albicans* adhered better than *C. dubliniensis* (Vidotto *et al.*, 2003). Interestingly, Borg-von Zepelin *et al.* have shown that *C. dubliniensis* isolates adhered better to an epithelial cell line (Vero) which was used to mimick mucousal infections in the presence of fluconazole in comparison with controls without fluconazole (Borg-Von Zepelin *et al.*, 2002). This was in contrast to *C. albicans* which showed a decreased adherence in the presence of fluconazole independently of their *in vitro* susceptibility to this drug (Borg-Von Zepelin *et al.*, 2002).

1.4.1.1 *ALS* (Agglutinin-like sequence) genes

Adhesion is a critical step for colonisation and establishment of infection. The *ALS* (agglutinin-like sequence) gene family of *C. albicans* encodes cell-surface glycoproteins implicated in adhesion of the organism to host surfaces. The Als family which consist of a family of eight glycosylated proteins have shown homology to the *S. cerevisiae* α-agglutinin protein which is required for cell-cell recognition during mating. Common features of the Als proteins are the presence of three domains; a relatively conserved 5' domain of 1299-1308 nucleotides; a central domain of variable length; tandem repeats each consisting of 108 bp; and a 3' domain that is variable in length encoding a serine-threonine rich protein. The Als family also possess features typical of a secreted protein, which includes a hydrophobic carboxyl terminus that suggests a glycophosphatidylinositol (GPI) anchor. Heterologous expression of *ALS* genes in *S. cerevisiae* confers an adherence phenotype on the organism suggesting Als proteins function in adhesion to host surfaces, a property that is positively correlated with *Candida* pathogenesis (Calderone & Braun, 1991; Fu *et al.*, 1998; Gaur & Klotz, 1997). The *ALS* genes are also differentially expressed under a variety of conditions that include morphological form, growth medium composition, growth phase, and strain of *C. albicans* (Hoyer, 2001; Hoyer *et al.*, 1998). In *C. albicans* Als1p and Als5p have been shown to provide an adhesive function (Chandra *et al.*, 2001; Fu *et al.*, 1998; Gaur & Klotz, 1997; Gaur *et al.*, 1999). The Als1 protein is also important for the adherence of the organism to the oral mucousa during the early stage of infection and is also expressed by

filamenting cells and is important for adherence of *C. albicans* to human umbilical vein endothelial cells (HUVEC), but not to BEC (Fu *et al.*, 2002; Kamai *et al.*, 2002; Zhao *et al.*, 2004). The Als3 protein promotes adherence to both HUVECs and BECs (Zhao *et al.*, 2004). Three distinct *ALS*-like sequences have been identified in *C. dubliniensis* (*ALSD1-3*) (Hoyer *et al.*, 2001). These genes however, show significant divergence in comparison with *C. albicans* most notably at the 3' ends (Hoyer *et al.*, 2001). Hoyer *et al.* also suggested that in *C. dubliniensis* there was a difference in regulation of the gene family and in production of cell wall proteins (Hoyer *et al.*, 2001).

1.4.1.2 Hwp1 (Hyphal wall protein 1)

Germ tubes are the initial projections observed when *Candida* switches from a yeast form to hyphal growth. Hyphal wall protein 1 was originally isolated as a hyphal and germ tube-specific protein from a differential screen (Staab *et al.*, 1996; Staab *et al.*, 1999). Hyphal wall protein 1 encodes an outer surface mannoprotein that is believed to be oriented with its amino-terminal domain surface-exposed and the carboxyl terminus most probably covalently integrated with cell wall-β-glucan (Staab *et al.*, 1996). The amino terminal sequence was found to resemble mammalian transglutaminase (Tgase) substrates and furthermore that Hwp1 serves as a substrate for Tgases creating a stable and covalent attachment to epithelial cells. The N-terminal domain of Hwp1 is cell surface exposed and the C-terminal is covalently integrated into the cell wall (Staab *et al.*, 1996). It has also been shown by Tsuchimori *et al.*, that a *hwp1Δ* null mutant of *C. albicans* caused reduced mortality in mice, germinated less readily in the kidneys of infected mice and caused less endothelial cell damage (Tsuchimori *et al.*, 2000). Other microbial adhesins operate through lectin-like or hydrophobic interactions and therefore the ability of Hwp1 to become cross-linked to host proteins makes it an unconventional adhesin. It has been shown that Hwp1 plays a role in adherence to BEC (Staab *et al.*, 1999). It has been suggested that Hwp1 helps to retain desquamating BECs on the mucousal surface, thereby embedding hyphae in an agglomeration of non-viable cells, and permitting growth in a typical fungal, absorptive heterotrophic manner (Sundstrom, 2002). Hyphal wall protein 1 has also been shown to be

selectively expressed on the wall of conjugation tubes formed by a/a cells, but not α/α cells, in the mating process (Daniels *et al.*, 2003).

1.4.1.3 Int1 (Integrin like protein)

Studies by Klotz and Smith have shown that the integrin like proteins of fungi play a role in adherence to intact collagen (Klotz & Smith, 1991). Integrins are expressed as heterodimeric transmembrane proteins (α and β subunits) on a wide variety of cells, and possess divalent cation binding sites that are required for ligand binding (Gale *et al.*, 1996; Hostetter, 1996; Hostetter, 1999). It is thought that α and β subunits co-operate in the recognition and binding of integrin ligands in the extracellular matrix (Gale *et al.*, 1996). The Int1 protein is similar to vertebrate leukocyte adhesions, which bind extracellular matrix proteins and induce morphological changes in response to extracellular signals (Calderone, 1998; Kinneberg *et al.*, 1999). Disruption of *INT1* in *C. albicans* has been shown to reduce adhesion to human epithelial cells by 40% and reduced virulence in *C. albicans* in a mouse model (Kinneberg *et al.*, 1999). A homologue of *INT1* in *C. dubliniensis* has thus far not been detected.

1.4.1.4 *MNT* gene family (α-1,2-mannosyltransferase)

The *MNT1* gene of *C. albicans* is involved in *O*-glycosylation of cell wall and secreted proteins and is important for both adherence to host surfaces and therefore for virulence (Buurman *et al.*, 1998; Thomson *et al.*, 2000). The outer cell wall of *C. albicans* is enriched in mannoproteins which are embedded in a matrix of structural polysaccharides consisting of β-1,3- and β-1,6-linked glucan and chitin (Kapteyn *et al.*, 2000). Both the protein and carbohydrate components of mannoproteins have been implicated in adhesion to the host (Calderone, 1993; Frieman *et al.*, 2002; Fukazawa & Kagaya, 1997; Gaur & Klotz, 2004). A second member of the *CaMNT* gene family has been discovered which encodes a protein with significant sequence similarity to the α-1,2-mannosyltransferase, CaMnt1 (Munro *et al.*, 2005). This protein is also a α-1,2-mannosyltransferase, which along with Mnt1 is responsible for the addition of the second and third mannose residues in *C. albicans*

O-glycans. It has been shown that deletion of both copies of Mnt1 and Mnt2 resulted in truncation of *O*-linked glycans and reduction in virulence indicating that these enzymes are important for normal interactions of *C. albicans* with its host (Munro *et al.*, 2005). Deletion mutants of both Mnt1 or Mnt2 were significantly reduced in adherence to BECs and Matrigel-coated surfaces, indicating a role for *O*-glycosylated cell wall proteins or *O*-mannan itself in adhesion to host surfaces (Munro *et al.*, 2005). Homologues of the *MNT* gene family in *C. dubliniensis* have thus far not been detected.

1.4.2 Morphology

One of the most important and intensively studied virulence attributes of *C. albicans* is its ability to grow as a yeast and filamentous form. While *C. albicans* is often referred to as being dimorphic it is more correctly described as polymorphic being able to grow as unicellular yeast, pseudohyphae, true hyphae (with parallel-sided walls) and chlamydospores. The hyphal and pseudohyphal forms are fundamentally different and can be distinguished by a number of characteristics (Sudbery *et al.*, 2004). The ability to grow in the hyphal form has been particularly associated with virulence. The direction of hyphal growth is guided by grooves, ridges and pores in the substratum and it has been suggested that this contact guidance (thigmotropism) may facilitate hyphal invasion of host epithelia through membrane invaginations, wounds or points of weakened surface integrity (Gow *et al.*, 1994; Sherwood *et al.*, 1992). Numerous attempts have tried to forge a relationship between the filamentous form and virulence (Odds, 1988). However, both yeast and hyphal cells have been isolated from infection sites suggesting that both have a role in the development and progression of disease. Despite this the importance of hyphae in virulence has been emphasised in animal model experiments comparing the virulence of mutant strains that are defective in hyphal production and their parental strains. At least two important functions of hyphae have been suggested previously; the hyphal form may be important for penetrating tissue surfaces and also that the hyphal form may be important for escaping from host cells following internalisation (Douglas, 2002; Kumamoto & Vinces, 2005; Whiteway & Oberholzer, 2004). Hyphae are important for tissue invasion because they allow application of mechanical force and because they allow *C. albicans* cells to escape from and damage host cells following uptake by endocytosis.

Changes in environmental conditions such as pH and temperature, presence of serum, CO_2 concentrations and nutrient depletion induce this transition *in vitro*. This dimorphism is thought to contribute to colonisation and dissemination within host tissues and thereby to promote infection. The filamentation process has been shown by Ramage *et al.*, to be inhibited by farnesol which is a qurom sensing molecule (Enjalbert & Whiteway, 2005; Ramage *et al.*, 2002). The transition from a yeast to hyphal form may alter the host inflammatory response and the hyphal tip is also the site of apical secretion of enzymes able to degrade proteins, lipids and other cellular components that further facilitate infiltration into solid substrates and tissues (Hube & Naglik, 2001). Both the yeast and hyphal forms of *C. albicans* also produce their own repertoire of (secreted aspartyl proteinases) Saps, each of which has been implicated in virulence (Hube & Naglik, 2001). The ability to exist in a hyphal form is required for virulence partially because hyphal extension is suited to penetrate epithelial tissues and rupture phagocytes (Zheng *et al.*, 2003). It has been shown that organisms locked into a single morphology uniformly have reduced virulence in animal models of candidiasis (Bahn & Sundstrom, 2001; Laguna *et al.*, 1997; Murad *et al.*, 2001).

A study by Vilela *et al.* suggested that *C. dubliniensis* has a higher survival rate when injected intravenously into mice than *C. albicans* and also that *C. dubliniensis* remains mostly in the yeast form in the infected organs whereas in contrast, *C. albicans* changed to the mycelial form (Vilela *et al.*, 2002). Unlike other pathogenic fungi the morphological response to environmental changes is rapid in *C. albicans* and germ tubes are produced within 1 hour of placing cells in appropriate conditions (Bahn & Sundstrom, 2001). The analysis of the kinetics of hyphal production by *C. albicans* and *C. dubliniensis* under a limited set of induction conditions has been examined and suggests that hyphal production is less efficient in *C. dubliniensis* in comparison with *C. albicans* (Gilfillan *et al.*, 1998).

Studies of the regulatory proteins that control hyphal morphogenesis demonstrate that co-regulation of virulence factors and morphogenesis genes is accomplished through the activity of specific transcription factors. Signal transduction pathways play a role in morphogenesis and in *C. albicans* the transcription factors Efg1, Cph1 and Czf1 have been shown to play a crucial role in forming hyphae in response to environmental factors (Brown & Gow, 1999; Lengeler *et al.*, 2000; Lo *et al.*, 1997; Stoldt *et al.*, 1997). The best characterised regulator of hyphal morphogenesis is the transcription factor Efg1, which is a

member of a family of basic helix-loop-helix transcriptional regulators (Stoldt *et al.*, 1997). Although Efg1 acts as a positive regulator of filamentous growth, however, there are certain conditions in which filamentation is negatively regulated by Efg1 (Giusani *et al.*, 2002; Sonneborn *et al.*, 1999). Double mutants of Efg1 and Cph1 fail to produce germ tubes and hyphae under any condition tested and show reduced virulence in an animal model (Lo *et al.*, 1997). Other proteins are involved in the repression of the yeast-hyphal phase including Tup1 and Rbf1 (Braun & Johnson, 1997; Ishii *et al.*, 1997). The putative transcription factor Cph1, homologous to *S. cerevisiae* Ste12, also plays a role in regulating hyphal morphogenesis (Liu *et al.*, 1994). Both *EFG1* and *CPH1* were identified by virtue of their ability to promote filamentous growth in the non-pathogenic yeast *S. cerevisiae. Candida albicans* deletion mutants lacking either *EFG1* or *CPH1* exhibit defects in hypha formation, although the effects of deleting Cph1 are minor compared with those of deleting Efg1. In combination, the Efg1/Cph1 double mutant is extremely defective for filamentous growth under most laboratory conditions tested (Lo *et al.*, 1997). Lo *et al.* also demonstrated that the Efg1/Cph1 double mutant fails to cause mortality following intravenous inoculation of mice and is thus completely avirulent in the systemic model (Lo *et al.*, 1997). Efg1 regulates functions as diverse as cell wall dynamics and metabolism. It is also clear that Efg1 regulates the expression of numerous genes including *SAP4*, *SAP5* and *SAP6* (Korting *et al.*, 2003; Staib *et al.*, 2002).

Expression of the adhesins Als1, Als3 and Hwp1 is dependent on Efg1 (Fu *et al.*, 2002; Hoyer *et al.*, 1998; Sharkey *et al.*, 1999). Other pathways have been shown to have an involvement in morphogenesis including the cell wall integrity protein Kinase C (PKC) and the osmoregulation (two component high osmolarity glycerol (Hog1) pathways (Alonso-monge *et al.*, 1999; Calera & Calderone, 2001; Calera *et al.*, 2000; Navarro-Garcia *et al.*, 1998). Despite the numerous pathways involved in the regulation of the yeast to hyphal transition they all converge to regulate a common set of genes termed the hyphal regulon, which includes the *ALS3, HWP1, RBT1, RBT4, SAP4, SAP5* and *SAP6* genes (Cowen *et al.*, 2002; Kadosh & Johnson, 2005; Lane *et al.*, 2001). This makes it possible for *C. albicans* to monitor its environment closely and to respond to many cues provided by the host.

1.4.3 Enzymes that contribute to invasiveness

1.4.3.1 Secreted Aspartyl Proteinases (SAPs)

The three most significant extracellular hydrolytic enzymes produced by *C. albicans* are the secreted aspartyl proteinases (Sap), phospholipase enzymes, and lipases. Of these three the Sap proteins have been the most extensively studied (Borg-Von Zepelin *et al.*, 1998; Fusek *et al.*, 1994; Hube & Naglik, 2001; Monod *et al.*, 19994; Naglik *et al.*, 2003; Schaller *et al.*, 2000; Schaller *et al.*, 1999). So far ten different *SAP* genes (*SAP1-10*) have been identified in *C. albicans,* eight of these proteinases (Sap1-8) are secreted into the extracellular space while while Sap9 and Sap10 are membrane-anchored GPI proteins (Schaller *et al.*, 2005). *Candida albicans* is not the only *Candida* species known to produce extracellular proteinases, many of the pathogenic species including, *C. dubliniensis* (Gilfillan *et al.*, 1998), *C. tropicalis* (Fusek *et al.*, 1994; Zaugg *et al.*, 2001), and *C. parapsilosis* (de Viragh *et al.*, 1993) have been shown to produce active extracellular proteinases *in vitro*. *Candida albicans SAP* genes encode preproenzymes approximately 60 amino acids longer than the mature enzyme, which are processed when transported via the secretory pathway.

Secreted aspartyl proteinases degrade human proteins such as albumin, haemoglobin, keratin and secretory immunoglobulin A (Hube *et al.*, 1998). The production of Saps is associated with a number of other putative virulence attributes of *C. albicans* including hyphal formation, adhesion and phenotypic switching. Extracellular proteinases are believed to contribute to candidal virulence by hydrolysing host proteins to provide nutrients, to facilitate adhesion and to assist in the evasion of the host cells by digesting proteins of host cell membranes (Borg-Von Zepelin *et al.*, 2002; Klemba & Goldber, 2002; Naglik *et al.*, 2003). It has been shown that the virulence of *Candida* species correlates with the level of Sap activity *in vitro* as the most virulent species such as *C. albicans*, *C. tropicalis* and *C. parapsilosis* produce more proteinases *in vitro* than do less virulent species (Ruchel *et al.*, 1992). The reason for the presence of so many *SAP* genes in *C. albicans* is not clear. However, it has been shown that subsets of *SAP* genes are expressed under specific conditions. Unlike Sap1-8, which are thought to be all secreted, Sap9 and Sap10 both have C-terminal concensus sequences typical for glycophosphatidyl inositol (GPI) proteins (Borg-Von Zepelin *et al.*, 1998).

The expression of Sap1-3 have been detected in yeast cells, Sap4-6 have been detected when cells are undergoing the yeast to hyphae transition at neutral pH (Hube *et al.*, 1998; Husselstein *et al.*, 1999) and phenotypically switched cells predominantly express Sap1 and Sap3. The expression of *SAP8* has been detected in yeast cells grown at 25°C in a defined medium and *SAP9* is preferentially expressed in later growth phases (Borg-Von Zepelin *et al.*, 1998). Using a human epidermis model *SAP1-2* are expressed first for early invasion, then *SAP8* for extensive penetration, and finally *SAP6* for extensive hyphal growth (Schaller *et al.*, 2000). The major isoenzyme of the Sap family Sap2, has been shown to degrade mucins that play a role in protection against invasion by pathogens that colonise mucousal surfaces (Colina *et al.*, 1996; de Repentigny *et al.*, 2000). Gilfillan *et al.*, have suggested that *C. dubliniensis* has only 9 SAP genes in comparison with *C. albicans* 10 genes (Gilfillan *et al.*, 1998). Interestingly, another study detected only one gene in *C. dubliniensis* (*CdSAP4*) with similarity to *CaSAP4-6* (Moran *et al.*, 2004). The lack of the two other proteinases which are involved in the yeast to hyphae transition may be a reason for the lower virulence of *C. dubliniensis* in comparison with *C. albicans*. Conflicting results in the literature have compared the differences in extracellular enzymatic activity between *C. albicans* and *C. dubliniensis*. Some studies have reported that *C. dubliniensis* expressed higher levels of proteinase than *C. albicans* however, other studies have reported the opposite (Fotedar & Al-Hedaithy, 2005; Hannula *et al.*, 2000; McCullough *et al.*, 1995; Slifkin, 2000).

1.4.3.2 Phospholipases (PLs)

Phospholipases hydrolyze one or more ester linkages of glycerophosphopholipids and could potentially facilitate increased penetration of fungal hyphal elements by directly damaging host cell membranes. Four phospholipase subclasses have been identified so far (PLA, PLB, PLC and PLD), only PLB1 has been shown to be required for virulence in an animal model of candidiasis (Banno *et al.*, 1985; Barret-Bee *et al.*, 1985; Ghannoum, 2000; Hoover *et al.*, 1998; Hube *et al.*, 2001). To date only two extracellular PLB genes, named PLB1 and PLB2, three intracellular PL genes coding for PLC1 and for PLD have been well characterised (Bennett *et al.*, 1998; Hoover *et al.*, 1998; Hube *et al.*, 2001; Leidich *et al.*, 1998; Sugiyama *et al.*, 1999). Phospholipase B has both hydrolase (fatty acid release) and lysophospholipase-transacylase activities (Kuhn *et al.*, 2002). Phospholipase B expression

has been shown to be regulated by nutritional supplementation, environmental factors including temperature and pH and the growth phase of *C. albicans* cells (Mukherjee *et al.*, 2003). It has also been found that *C. albicans* strains isolated from blood produce higher levels of phospholipases then commensal strains and cells producing less phospholipases are less virulent than strains producing higher levels of phospholipases in a murine model suggesting that phospholipases may be virulence factors (Ghannoum, 2000). The production of phospholipases are believed to enhance the ability of the organism to colonise and evade the immune system (Borg & Ruchel, 1988), to adhere to epithelial cells and invasion of reconstituted human oral epithelium (Jayatilake *et al.*, 2005). Interestingly analysis of phospholipase production in the two species suggests that *C. dubliniensis* may produce lower levels than *C. albicans* (Hannula *et al.*, 2000; Slifkin, 2000). However, Fotedar *et al.* have reported that in a study using eighty seven *C. dubliniensis* isolates no phospholipase activity was detected (Fotedar & Al-Hedaithy, 2005). *Candida dubliniensis* has sequence homologues to the *C. albicans* phospholipase C gene *CaPLC1* (Bennett *et al.*, 1998).

1.4.3.3 Lipase encodong genes (LP)

Candida albicans possesses a large family of lipase encoding genes whose extracelllular activity may be important for colonisation and subsequent infection. Lipases hydrolyse ester bonds at the interface between the insoluble triacylglycerol phase and the aqueous phase in which the enzyme is dissolved (Schaller *et al.*, 2005). Each deduced lipase sequence has conserved lipase motifs consisting of four conserved cysteine residues, conserved putative *N*-glycosylation sites and similar hydrophobicity profiles (Schaller *et al.*, 2005). Little is known about the family of lipase genes which were recently identified in *C. albicans* (Hube *et al.*, 2000). Schofield *et al.* performed a study examining the expression of the ten lipase genes using a mouse model of mucousal candidiasis and found that *LIPS4-8* were expressed during both colonisation and infection and that *LIP1, 3* and *9* were expressed preferentially during gastric candidiasis compared to oral candidiasis (Schofield *et al.*, 2005).

1.4.4 Phenotypic switching

Phenotypic switching leads to antigenic variability as it is accompanied by changes in antigen expression, colony morphology and tissue affinity (Calderone & Fonzi, 2001).

Studies on phenotypic switching have focused on one switch system in particular, the white-opaque switch, which refers to the switch of smooth, white colonies to flat, grey colonies (opaque) found in one strain (WO-1). Several differences exist between these two colony types, including cell shape (white cells are round-ovoid and opaque cells are elongated or bean shaped), cell surface structure (pimples are found on opaque cells only) and germination at 37°C, pH 6.7 by white cells but not opaque (Anderson *et al.*, 1990; Slutsky *et al.*, 1987).

Phenotypic switching has been demonstrated to affect a variety of virulence traits, including the bud-hypha transition (Anderson *et al.*, 1989), antigenicity (Anderson *et al.*, 1990), adhesion (Kennedy, 1988; Vargas *et al.*, 1994), sensitivity to neutrophils and oxidants (Kolotila & Diamond, 1990), secretion of proteinase (Morow *et al.*, 1992; Soll, 1992) and drug susceptibility (Vargas *et al.*, 2000). Switching therefore provides *C. albicans* and related species with an extraordinary level of phenotypic variability so that every colonising population includes phenotypic variants poised for enrichment in response to selective pressures in the process of rapid adaptation. It has been reported that *C. dubliniensis* can undergo phenotypic switching more frequently than *C. albicans* (Hannula *et al.*, 2000).

1.5 *In vivo* experimental models to examine virulence in *Candida*

To determine the role of this pathogen in human disease a number of different animal models have been developed which closely resemble the process of colonisation and invasion. In some cases models have also been developed to resemble the specific immune defects or hormonal conditions associated with infection at that particular site. Animal models represent powerful tools to elucidate the molecular and cellular pathogenesis (Allen, 1994; Samaranayake & Samaranayake, 2001). The advantages of using animal models of candidiasis is that the animal and environment can be tightly controlled (Allen, 1994). The wide range of diseases caused by *C. albicans* has necessitated the development of a similarly wide range of infection models. Animal models used to date for the analysis of host-pathogen interactions include, normal adult mice in mucousal (Kamai *et al.*, 2002; Kamai *et al.*, 2001), gastrointestinal (Mencacci *et al.*, 2001), vaginal (Fidel *et al.*, 2000; Wozniak *et al.*, 2002) and systemic candidiasis (Netea *et al.*, 1999). Transgenic mice have been used for

mucousal and gastrointestinal infections (de Repentigny *et al.*, 2002). Infant mice have been used on studies of gastrointestinal (Cole *et al.*, 1992) and systemic infections (de Repentigny *et al.*, 1992; Guentzel & Herrera, 1982). Macaques have been used to examine the host-pathogen interactions in vaginal candidiasis (Steele *et al.*, 1999). Finally normal adult rats have been used to study vaginal candidiasis (De Bernardis *et al.*, 2000) and normal adult rabbits (Letterio *et al.*, 2001) and severe combined immunodeficiency (SCID) mice have been used to examine systemic infections (Jensen *et al.*, 1993). Of the existing models, by far the most commonly used is the murine systemic infection model partly because mice are cheaper than rats, guinea pigs and rabbits and because of the similarity of the murine systemic infection to that found in humans (Cole *et al.*, 1995; Odds, 1988; Papadimitriou & Ashman, 1986).

Many models of systemic candidiasis rely on intravenous inoculation of *C. albicans* however, oral or oral-intragastric inoculation more closely resembles the dominant gastrointestinal portal of entry in humans. The ability to establish colonisation of *C. albicans* in mice before any immunocompromising procedure would more closely mimic the onset and progression of candidiasis in humans. The infant mouse model is the only animal model in which compromising agents are administered to mice persistently colonised with *C. albicans* in infancy. The infant mouse model is therefore useful to examine disease progression from gastrointestinal carriage to systemic infection in an immunocompromised host.

1.5.1 Adult mouse model

For several decades, the murine model of hematogenously disseminated candidiasis has been the standard for investigating mechanisms of candidal virulence and host defense, as well as for evaluating the efficacy of antifungal agents (Brand *et al.*, 2004; Ghannoum *et al.*, 1995; Lo *et al.*, 1997; Navarro-Garcia *et al.*, 2001). Although intravenous (iv) challenge bypasses the need for the fungus to gain its own access to the bloodstream, as occurs in many human disseminated *C. albicans* infections, the popularity of the iv challenge model stems from its reproducibility and its technically relatively undemanding nature (MacCallum & Odds, 2005). The iv model reliably generates an acute, disseminated visceral infection involving multiple deep organs, and its effects can be monitored indirectly by means of body

temperature measurements (Warn *et al.*, 2003) as well as directly in terms of gross survival times and fungal tissue burdens. Mouse strains and sexes differ in their susceptibility to iv *C. albicans* challenge. In both mice and humans, the main focus of infection occurs in the kidneys and the brain, which are the most representative organs of the course of the disease. It has been shown by Ashman *et al.*, that two mouse proteins, Carg1 and Carg2, influence the susceptibility of individual murine tissues to invasion by *C. albicans*, and overall susceptibility is also affected by the animal's immune status (Ashman *et al.*, 1996).

For a given mouse strain, the general pattern of infection after iv *C. albicans* challenge at doses that lead to mean survival times of the order of 5-7 days involves rapid clearance of fungi from the bloodstream primarily into the lungs and liver and to a lower extent the spleen followed by an increasing population of fungi in the kidneys which leads ultimately to the death of the animal from renal failure. It has been speculated that mice with disseminated candidiasis die of renal failure, on the basis of the fact that the kidneys are, by far, the most heavily infected organ (Brieland *et al.*, 2001; Netea *et al.*, 1999).

Two types of systemic infection can be examined, lethal and sublethal depending on the challenge dose. Lethal infections involve the inoculation of high doses (i.e. 1×10^7-1×10^6 of blastospores per mouse) and lead to the establishment of acute infections, which result in high mortality rates. The outcome of the experiment is generally measured by median survival time, and the number of blastospores able to kill half the mice (LD50). In sublethal infection experiments the inoculum used is lower (i.e. 1×10^5- 5×10^4 blastospores per mouse) and results in the establishment of chronic infection. The endpoint of this experiment is measured by tissue damage and the fungal load in the most representative organs, brain and kidney. One of the problems however with the iv model is achieving a challenge dose high enough to avoid a rapidly fatal infection, which has been a common experience with many studies (Joly & Yeni, 1999). MacCallum & Odds, have demonstrated that the overall course of *C. albicans* infection after iv challenge in mice amounts to elimination of the fungus from blood followed by a sudden rapid growth of fungus in kidney and brain and progressive elimination of fungi from liver, lung, heart and spleen (MacCallum & Odds, 2005).

Two studies have demonstrated that *C. dubliniensis* is less virulent than *C. albicans* in a murine model of systemic candidiasis with inoculation iv through the tail vein (Gilfillan *et*

al., 1998; Vilela *et al.*, 2002). They found that mice infected with *C dubliniensis* survived longer than those infected with *C. albicans* thus showing that *C. albicans* was more virulent than *C. dubliniensis* (Gilfillan *et al.*, 1998; Vilela *et al.*, 2002).

1.5.2 Infant mouse model

Candida albicans frequently occurs as a member of the indigenous microflora of the gastrointestinal (GI) tract of normal humans (Odds, 1988). Individuals that harbour *C. albicans* at least in the upper third of their alimentary canal (mouth, oesophagus, stomach), are at high risk to develop oropharyngeal and gastric candidiasis under conditions which cause severe cellular immunodeficiency. In addition one of the predisposing risk factors for systemic candidiasis is colonisation of the GI tract with *Candida* species. The infant mouse model simulates the situations found in both normal humans with *C. albicans* as a component of the GI microflora and in immunocompromised patients, with resident *C. albicans* in the GI tract representing a reservoir for the pathogen for potential disseminated disease. Problems with establishing experimental GI and systemic candidiasis were encountered when using adult mice without administration of immunocompromising agents, broad spectrum antibiotic and or X-irradiation (Helstrom & Balish, 1979). The benefits of using the infant mouse model is that as the mice are only 6-9 days old when inoculated their immune system is not sufficiently developed to eliminate the yeast cells when they are oral-gastrically inoculated through the mouth using a feeding needle thus allowing *Candida* cells to colonise the GI tract (Cole *et al.*, 1990a; Cole *et al.*, 1996; Cole *et al.*, 1993). The stomach of these young mice contain lactobacilli which *C. albicans* is able to compete with and therefore colonise the GI tract.

Following colonisation the mice are then subjected to an immunocompromising treatment of cortisone acetate and cyclophosphamide which leads to a breach in the immune system, similar to that observed in human patients taking corticosteroids, broad spectrum antibiotics and cytotoxic drugs and allows dissemination to other organs including the liver, lung, spleen and kidneys via the bloodstream. The infant mouse model has been described as being useful for both exploring methods which may prevent dissemination of *C. albicans* from localised foci of colonisation in the GI tract after exposure of the host to immunocompromising drugs and also for testing the efficacy of anti-*Candida* drugs in the

clearance of the pathogen from body organs with established fungal abscesses (Cole *et al.*, 1989). On examination of the literature *C. dubliniensis* has never been examined in the infant mouse model to date.

1.6 *Ex vivo* experimental model to examine virulence in *Candida*

1.6.1 Reconstituted human oral epithelium (RHOE)

Reconstituted human oral epithelium was obtained by culturing transformed human keratinocytes of the TR146 cell line which was derived from squamous cell carcinoma cells from the buccal mucousa on an inert supporting membrane (Rupniak *et al.*, 1985). The RHE models available represent a wide variety of epithelial tissues including oral, vaginal, skin and other cell types. The use of RHE in the analysis of *C. albicans* virulence was first reported by Schaller *et al.* in 1998 (Schaller *et al.*, 1998a). Reconstituted human epithelium has been used for skin pharmacology experiments (Schaller *et al.*, 1997) and for *ex vivo* models of cutaneous candidiasis in man and has provided useful insights into the pathogenesis and treatment of cutaneous candidiasis. Due to ethical issues involved in using animal models, RHE models are being used more frequently as infection models. The advantages of using oral RHE are that the multilayer structure which can consist of up to 12 layers closely resembles the oral epithelium, which is in stark contrast to traditional single layer tissue culture systems and the internal milieu of the oral cavity within a pH range of 7.0-7.4 is closely mimicked by this model. Schaller *et al.* have also shown that histologically RHE resembles the normal human oral epithelium and the pathological changes when infected by *C. albicans* are similar to what occurs in human disease (Schaller *et al.*, 1998a).

The model is limited by the lack of a normal humoral and cell mediated immune response. However an altered or depressed immune system is a significant predisposing factor in human *Candida* infections and an important component of animal experimental studies (Odds, 1988). The role of *SAP* genes and *ALS* genes in virulence has been confirmed using RHE suggesting these genes play a role in human oral candidiais *in vivo* (Korting *et al.*, 1998; Korting *et al.*, 1999; Korting *et al.*, 2003; Schaller *et al.*, 2004; Zhao *et al.*, 2005; Zhao *et al.*, 2004). The role of the immune system has also been examined using the RHE model by supplementing the model with polymorphonuclear leukocytes (Schaller *et al.*, 2004) and

more recently an investigation of ultrastructural and histochemical features of invasive candidiasis in humans has been performed using the RHE model (Jayatilake *et al.*, 2005).

1.6.2 Reconstituted human vaginal epithelium

A model of vaginal candidiasis based on reconstituted human vaginal epithelium (RHVE) has also been used to examine the expression and role of Saps during infection and tissue damage of vaginal epithelium. It was obtained by culturing transformed human keratinocytes of the cell line A431 derived from a vulval epidermoid carcinoma (Rosdy *et al.*, 1986). Schaller *et al.* demonstrated that during late stages of *in vitro* infection *SAP1, SAP2, SAP4* to *SAP7*, *SAP9* and *SAP10* were observed. The study also showed that *SAP2* and *SAP5* were the most commonly expressed genes during vaginal infection (Schaller *et al.*, 2003).

1.7 Aims

The emerging reports highlighting the recovery of *C. dubliniensis* from different clinical specimens and causing different spectrum of diseases further necessitates the study of the virulence properties of this novel yeast species. It is clear from epidemiological and infection model data that despite the very close phylogenetic relationship between *C. albicans* and *C. dubliniensis*, the former is more pathogenic. The purpose of this study is to investigate the growth characteristics of each species *in vitro* and in two models of infection and to compare the ability of each species to grow alone and in competition in liquid culture and to compare the kinetics of hyphal growth of *C. albicans* and *C. dubliniensis.*

- The first aim of the study was to develop a method of differentiating *C. albicans* from *C. dubliniensis* due to the problems involved in phenotypically distinguishing *C. albicans* from *C. dubliniensis.* The aim was to construct a cassette containing two molecular markers encoding mycophenolic acid resistance and the green fluorescent protein, which can be inserted into strains of *C. dubliniensis* or *C. albicans* and will permit differentiation between strains of *C. albicans* and *C. dubliniensis* when grown in mixed cultures.

- The second aim of the present study was to perform competition studies between *C. albicans* and *C. dubliniensis in vitro*, *ex vivo* and *in vivo*. *Candida albicans* and *C. dubliniensis* will be grown together in mixed cultures to investigate whether one has the ability to out-compete the other. This will give an insight into the interactions between *C. albicans* and *C. dubliniensis* and may indicate the reason why *C. albicans* is found more readily in infections despite the similarities between the two. Two virulence models were used to compare the two species the infant mouse model and reconstituted human oral epithelium (RHE). This will allow a definitive comparison of virulence between the two species in model infection systems.
- The third aim of the present study was to conduct a thorough comparison of the kinetics of hyphal production in *C. albicans* and *C. dubliniensis* in both solid and liquid media, that have previously been shown to induce the yeast-to-hyphal transition.
- The fourth aim of the study was to investigate whether a homologue of Hwp1, the hyphal wall protein which has been studied intensively in *C. albicans* and has been shown to be involved in adhesion to host surfaces, exists in C. *dubliniensis* and if it is present to perform sequence analysis on it and compare it with the *C. albicans* Hwp1.

Chapter 2

Materials and Methods

2.1 General Microbiological Methods

2.1.1 Culture media and growth conditions

2.1.1.1 Candida *clinical isolates, strains and derivatives*

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

2.1.1.2 Escherichia coli *strains*

The *Escherichia coli* strain DH5α (*supE*Δ*lac*U169 [∅80 *lacZ*ΔM15] *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1 (Sambrook *et al.*, 1989)) was routinely cultured on Luria-Bertani agar (LA), pH 7.4 (Lennox, 1955), at 37°C for 18 h, and for liquid culture, in Luria-Bertani broth (LB), pH 7.4 (Lennox, 1955), at 37°C for 18 h in an orbital incubator (Gallenkamp) at 200 rpm. *Escherichia coli* DH5α as used as the host strain for plasmid pBluescript II KS (-) (Stratagene, La Jolla, California, USA) and its recombinant derivatives and was maintained on LA containing 100 µg ampicillin/ml.

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. (Tallaght, Dublin, Ireland), BDH (Poole, Dorset, U.K.) or from Roche Diagnostics Ltd. (Lewes, East Sussex, UK). Enzymes were purchased from the Promega Corporation (Madison, Wisconsin, USA) and from New England Biolabs (Beverley, Massachusetts, USA) and used according to the manufacturer's instructions. DNA molecular weight markers I kb and 100 bp were purchased from Promega. Zymolyase 20T (21,600 U/g) was purchased from the Seikagaku Corporation

(Tokyo, Japan). [α-^{32}P]dATP (6,000 Ci/mmol; 222 TBq/mmol) 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.

Phosphate-buffered saline (PBS) was prepared at 10x concentration, which consisted of 1.45 M NaCl, 0.33 M NaH_2PO_4, 0.95 M Na_2HPO_4, pH 7.5. PBS was diluted to 1x concentration in ultra pure water (Milli-Q® ultrapure water purification systems, Millipore Ireland B.V., Carrigtwohill, Co. Cork, Ireland) before use.

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 ultra pure water to 0.5x concentration and was used as the buffer for agarose gel electrophoresis.

DNA loading dye was also prepared at 10x concentration and consisted of 30% (v/v) glycerol, 0.25% (w/v) bromophenol blue and 3.8% (w/v) EDTA.

SSC was prepared at 20x concentration, which consisted of 3.0 M NaCl, 0.3 M tri-sodium citrate, pH 7.0.

MOPS buffer, as used for RNA electrophoresis, was prepared at 10x concentration which 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 UK Ltd. (Loughborough, Leicestershire, 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.

RNase solutions were prepared by dissolving pancreatic RNase (RNase A, Sigma-Aldrich) at a concentration of 10 mg/ml in 10 mM Tris-HCL (pH7.5), 15 mM NaCl. This solution was stored in aliquots at -20°C.

Proteinase K (Roche Diagnostics Ltd.) solutions were prepared in ultrapure water at a concentration of 20 mg/ml and also stored at -20°C.

2.1.4 Storage of *Candida* isolates

Candida cells and derivatives were stored at -70°C in cryo-vials (Protect, Protect-Technical Service Consultants, Lancashire, UK). Cells were activated by sub-culture onto PDA plates and incubated at 48 h at 37°C.

2.2. Identification of *Candida* species

2.2.1 Biotyping (Carbohydrate assimilation profile)

Biotyping was carried out using the API ID 32C yeast identification system (bioMerieux) which can identify *Candida* isolates to the species level using a series of carbohydrate-source and nitrogen-source assimilation tests contained in 32 separate cupules on a plastic strip. Tests were carried out according to the manufacturer's instructions. For each test isolate, an inoculum was prepared from 24-48 h old colonies cultured on PDA medium. Four colonies of 2-4 mm in diameter were resuspended in sterile saline to the turbidity of a 2 McFarland standard. This suspension was then used to inoculate an aliquot of C medium which was supplied by the manufacturer. Each of the cupules in the strip was then inoculated with 250 µl of the C medium suspension and incubated for 48 h at 30°C. Readings were made at 24 h and 48h by visually assessing the growth of the test isolate in each of the cupules. The presence or absence of growth was recorded for each cupule on a result sheet supplied by the manufacturer and the substrate assimilation profile of the isolate was converted into an eight-digit numerical profile. These profiles were then cross-referenced in the ATB 32C analytical profile index database. In the database, 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 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 good identification. *Candida dubliniensis* isolates yield substrate assimilation profiles which are distinct from *C. albicans* profiles. Lactate, D-trehalose, D-xylose, and methyl-α-D-glucoside are discriminatory carbohydrates for the differentiation of *C. dubliniensis* from *C. albicans* with the API ID 32C kit (Pincus *et al.*, 1999). The *C. albicans* oral reference strain 132A and the *C. dubliniensis* type strain CD36 were used as positive controls for identification of *C. albicans* and *C. dubliniensis* respectively.

2.2.2 Growth at 42°C

All isolates were tested for the ability to grow on PDA at 37°C and 42°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 poorly or not at all at 42°C.

2.2.3 Growth on CHROMagar® Candida medium

CHROMagar Candida (CHROMagar® Candida, Paris, France) is a commercially available agar medium containing chromogenic substrates which allow colonies of different *Candida* species to be tentatively identified on the basis of colony colour. 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 & Bernaerts, 1994). All putative clinical *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.

2.2.4 PCR identification of *C. dubliniensis*

PCR identification of *C. dubliniensis* was performed using the *C. dubliniensis* specific DUBF(5'-GTATTTGTCGTTCCCCTTTC-3')/DUBR(5'-GTGTTGTGTGCACTAACGTC-3') primer pair, which amplify a 300 bp portion of the *C. dubliniensis ACT1* associated intron (Gee *et al.*, 2002). Reactions were 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 of template DNA containing-cell supernatant (prepared as described below) (Donnelly *et al.*, 1999). Each reaction mixture also contained 10 pmol each of the universal fungal primers RNAF (5'-GCATATCAATAAGCGGAGGAAAAG-3')/RNAR (5'-GGTCCGTGTTTCAA-GACG-3'), which amplify approximately 610 bp from all fungal large subunit rDNA genes and were used as an internal positive control (Fell, 1993). Cycling conditions consisted of 6 min at 95°C followed by 30 s at 94°C, 30 s at 58°C, 30 s at 72°C, followed by 72°C for 10 min. Amplification reactions were carried out in a Thermo Hybaid Multiblock system (MBS) thermal cycler (Thermo hybaid, Ashford, Middlesex, UK). Amplification products were separated by electrophoresis through 2.0% (w/v) agarose gels containing 0.5 µg ethidium bromide/ml and were visualised on a UV transilluminator.

The rapid isolation of *C. dubliniensis* template DNA for use in PCR identification experiments involved placing a single colony from a culture grown for 48 h at 37°C on CHROMagar Candida media or PDA into 50 µl of sterile distilled water. The cell suspensions were boiled for 10 min and the lysed cells were subjected to a clearing spin for 5 min at 2,500 x *g* in an Eppendorf model 5804 benchtop centrifuge (Eppendorf Centrifuge 5804, Eppendorf AG, Hamburg, Germany). A 25 µl aliquot of the supernatant was used in PCR reactions.

2.2.5 PCR identification of *C. dubliniensis* genotypes

PCR identification of isolates belonging to *C. dubliniensis* genotypes 1,2,3 and 4 was performed using G1F(5'-TTGGCGGTGGGCCCCTG-3')/G1R(5'-AGCATCTCCGCCTT-

ATA-3'), G2F(5'-CGGTGGGCCTCTACC-3')/G2R(5'-CATCTCCGCCTTACC-3'), G3F(5'-TTGGTGGTGGGCTTCTG-3')/G3R(5'-GCAATCTCCGCCTTACC-3') and G4F(5'-GGCCTCTGCCTGCCGCCAGAGGATG-3')/G4R(5'-AGCAATCTCCGCCTTACT-3'), primer pairs, respectively (Gee *et al.*, 2002). These primers were designed based on observed differences in the nucleotide sequence of the ITS1 and ITS2 regions of isolates belonging to each of the four *C. dubliniensis* genotypes. Each PCR reaction was carried out with one pair of these genotype specific primers and the universal fungal primers RNAF/RNAR (Fell, 1993), which amplify approximately 610 bp from all fungal large-subunit rDNA genes and were used as an internal positive control. PCR was carried out using *Taq* DNA polymerase (Promega). Each 100 µl PCR reaction for genotypes 1,2 and 3, respectively, contained 10 mM KCl, 0.1% (v/v) Triton X-100, dATP, 10 mM Tris/HCl (pH 9.0 at 25°C); dTTP, dCTP and dGTP at 200 µM each; 2.5 U of *Taq* polymerase; 300 nm of each primer, 1.5 mM $MgCl_2$ and either 100 ng of purified template DNA or rapidly prepared template DNA obtained by boiling yeast cells. The PCR cycling conditions were carried out in a Thermo Hybaid MBS thermal cycler with initial denaturation for 3 min at 95°C, followed by 30 cycles of 1 min at 95°C, 1 min at 50°C, 20 sec at 72°C and a final extension for 10 min at 72°C. Optimum conditions for genotype 4 consisted of using 20 µM dNTPs and an annealing temperature of 55°C.

2.3 Analysis of genomic DNA

2.3.1 Extraction of genomic DNA from *Candida* species

Genomic DNA was prepared from cells grown in 50 ml of YPD broth in a 250 ml conical flask (Erlenmeyer) at 37°C in an orbital incubator at 200 rpm for 18 h. Cultures were decanted into 50 ml Falcon tubes (Beckton Dickinson, New Jersey, USA) and harvested by centrifugation at 2,500 x *g* for 5 min in an Eppendorf model 5804 benchtop centrifuge. The supernatant was decanted and the pellet was resuspended in 5 ml of 20 mM citrate phosphate buffer, 40 mM EDTA, 1.2 M sorbitol, pH 5.6. Cell walls were digested by adding 15 mg

Zymolyase 20T and incubating at 37°C for 30 to 90 min. The protoplasts obtained were harvested by centrifugation at 2,500 x *g* for 5 min and resuspended in 7.5 ml 10x TE buffer. The protoplasts were lysed by adding 0.75 ml 10% (w/v) sodium dodecyl sulphate (SDS) and proteins were precipitated by adding 2.5 ml 5 M potassium acetate and incubating on ice for 30 min. The lysed protoplasts were decanted into 50 ml Sorvall Oak Ridge tubes (Dupont Co., Denver, Texas, USA) and subjected to a cleaning spin at 8,500 x *g* at 4°C for 5 min in a Sorvall 5C Plus centrifuge (Dupont Co., Denver, Colorado, USA). The supernatant was then decanted into a fresh 50 ml Falcon tube and precipitated by mixing gently with 10 ml ice-cold isopropanol and incubating on ice for 5 min. The resulting precipitate was pelleted in a benchtop centrifuge at 2,500 x *g* for 5 min. The pellet was dried at 37°C to remove any remaining isopropanol, and resuspended in 1 ml TE buffer. The solution was then incubated with 0.1 ml of a 10 mg/ml RNase A solution for 1 h at 37°C and then incubated with 0.1 ml of a 20 mg/ml proteinase K solution for 1 h at 37°C. The DNA solution was then extracted 3 times using an equal volume of a mixture of phenol:chloroform (1:1) and precipitated with the addition of 2 volumes of ice-cold ethanol. The resulting DNA precipitate was transferred to a fresh tube, washed in 70% (v/v) ethanol, dried briefly at 37°C, resuspended in 150 µl TE buffer and stored at 4°C.

The concentrations of DNA samples were assessed by measuring their absorbance at 260 nm using a Genesys 2 spectrophotometer (ThermoSpectronic, Thermo Electron, Boston, MA, USA) and calculating the concentration using the following formula: 1 unit of A_{260}=50 µg DNA.

2.3.2 Restriction endonuclease digestion of genomic DNA and agarose gel electrophoresis

Large scale restriction endonuclease digestions of genomic DNA for Southern analysis were carried out with 10 µg of genomic DNA in a 50 µl volume containing 20 U of restriction enzyme and the appropriate restriction enzyme buffer according to the manufacturer's instructions. Horizontal 0.7% (w/v) agarose gels made up in 0.5x TBE buffer

containing 0.5 μg/ml ethidium bromide per ml were cast into horizontal gel trays. DNA loading dye was added to the restriction enzyme-digested DNA samples at a final concentration of 1x and the samples were loaded into the gel wells. A 1 kb DNA size standard was loaded on each gel. Electrophoresis was carried out at 50 V/cm with constant current until the bromophenol blue tracking dye had reached the end of the gel. Gels were visualised on a UV transilluminator (wavelength 345_{nm}) and photographed using an ImageStore 7500 Version 7.22 Gel Documentation System (Ultra-Violet products Ltd. (UVP), Cambridge, UK).

2.3.3 Southern transfer of DNA from agarose gels

Following the separation of restriction endonuclease-digested DNA fragments by agarose gel electrophoresis, DNA was transferred to nylon membrans by capillary action using the method of Southern (Southern, 1975). To depurinate DNA gels were soaked in 0.02 M HCL with gentle agitation for 10 min. Following depurination DNA was denatured by soaking the gel in denaturing solution (5 M NaCl, 0.5 M NaOH) for 45 min with gentle agitation, after which time the gel was placed in a neutralisation solution (1 M Tris-HCl, pH 7.5, 1.5 M NaCl) for a further 45 mins with shaking.

DNA fragments were transferred to BioBond^{TM}-Plus nylon membranes (Sigma-Aldrich) using 10x SSC as the transfer buffer. Following transfer, membranes were baked at 80°C for 2 h and exposed to UV light using a crosslinker (CL-508, UVI tec, Cambridge, UK) set at 0.120 J/cm^2 to fix the DNA to the membranes.

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

For all hybridisation experiments, including Southern and Northern hybridisation, DNA fragments were labelled with [α-^{32}P]dATP by random primer labeling using the Prime-A-Gene kit purchased from Promega. Probes were routinely labelled to a specific activity of >10^6 dpm per g of DNA. Purified DNA fragments (10-200 ng in a 30 μl volume) were denatured by boiling for 2-3 min. Denatured DNA was added to a reaction mixture containing 1x labelling buffer, which was supplied by the manufacturer and contained a random mixture of hexanucleotides, dNTPs (dCTP, dGTP and dTTP) and bovine serum

albumin (BSA). Five microliters of [α-^{32}P]dATP (6,000 Ci/mmol; 222 TBq/mmol) were added to the reaction mixture before adding 5 U of Klenow DNA polymerase. The reaction was left to incubate at room temperature for 1 h. Unincorporated nucleotides were removed prior to hybridisation by passing the reaction mixture through a column containing a size exclusion matrix (SigmaSpin^{TM} post-reaction columns, Sigma-Aldrich) following the protocol detailed by the manufacturer.

2.3.5 Southern hybridisation

Hybridisation reactions were carried out in a rotary hybridisation oven (Hybaid, Teddington, Middlesex, UK) in 25 x 3.5 cm bottles (Hybaid) according to the method of (Sambrook & Russell, 2001). Nylon membranes containing bound DNA were rinsed in 6x SSC prior to hybridisation to remove excess salt. Membranes were then incubated in the oven at 65°C in 15 ml of the prehybridisation solution containing 1x Denhardt's solution [1% (w/v) Ficoll, 1% (w/v) polyvinylpyrrolidone, 1% (w/v) BSA], 6x SSC, 100 μg/ml denatured salmon sperm DNA and 0.5 % (w/v) SDS for a minimum of 2 h. The radiolabeled probe (≈ 2 x 10^6 d.p.m.) was denatured by boiling for 5 min followed by incubation on ice and then added to the prehybridisation solution and incubated with the membrane at 65°C for 18 h. Following hybridisation, unbound probe was removed from the membrane by washing at room temperature for 15 min in a solution consisting of 2x SSC, 0.1% (w/v) SDS. This was followed by a second wash at 37°C in 0.1x SSC, 0.5% (w/v) SDS for 30 min and a final wash at 65°C in 0.1x SSC, 0.5% (w/v) SDS for 30 min. The membrane was then wrapped in Saran wrap (Georgia Pacific Ireland Ltd., Finglas, Dublin 11) and placed in an autoradiography cassette with a Kodak BioMax intensifying screen (Eastman Kodak Company, Rochester, New York, USA) and exposed to Kodak Biomax MS-1 X-ray film for 24 to 72 h at -70°C. Autoradiograms were developed using Kodak GBX developer and fixed in Kodak GBX fixer according to protocols supplied by the manufacturer. In order to reuse membranes in subsequent hybridisation experiments, bound probe was removed from membranes by immersing them in boiling ultrapure water, followed by a brief rinse in 2x SSC.

2.4 Recombinant DNA techniques

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

Small scale preparations of plasmid DNA from *E. coli* were prepared by the method of (Sambrook & Russell, 2001). Briefly, *E. coli* cultures were grown overnight in LB medium in the presence of selective antibiotic (100 µg ampicillin/ml 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 an Eppendorf model 5417C microfuge and resuspended in 0.1 ml ice cold solution 1 (50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA, pH 8.0). Cell were lysed by the addition of 0.2 ml 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 0.15 ml solution 3 (5 M potassium acetate, 11.3% (v/v) acetic acid). The mixture was vortexed for 1 min 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 50 µl ultrapure water. RNA was removed by incubating the DNA solution with 0.1 mg/ml RNase A for 30 min.

Small scale preparations of plasmid DNA for DNA sequencing were prepared using the GenElute™ plasmid miniprep kit (Sigma-Aldrich) according to the manufacturer's instructions.

2.4.2 Polymerase chain reaction (PCR)

Specific sequences of *C. dubliniensis* genomic DNA were amplified by PCR and cloned into pBluescript. Oligonucleotide primers were synthesised by Sigma-Genosys Ltd. (Cambridge, UK) and stored at a stock concentration of 1 mM in ultra pure water at -20°C. Amplification reactions were carried out in 0.5 ml microfuge tubes (Eppendorf) in a Thermo Hybaid MBS thermal cycler in 50 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). Alternatively, if the PCR product was to be sequenced or cloned into an expression vector, the reactions were carried out using the high fidelity proof reading enzyme mix Expand High Fidelity PCR System purchased from Roche Diagnostics Ltd., and the buffers provided by the manufacturer. Amplification conditions and specific primers are described in the relevant sections. PCR products were purified using the GenElute PCR DNA purification kit (Sigma-Aldrich) according to the manufacturer's instructions.

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

Restriction endonuclease-generated DNA fragments were purified from agarose gels using NA45 DEAE membranes (Schleicher and Schuell, Dassel, Germany). Fragments were electrophoresed as described in section 2.3.2 in agarose gels and viewed on a UV transilluminator (UV wavelength 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 adjacent to it. The excised fragment of gel was replaced and electrophoresis was allowed to continue until the fragment had run onto the paper, was 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 three times 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 ultrapure water.

2.4.4 Ligation of DNA fragments

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 via restriction sites, which had been designed within the oligonucleotide primers used in the amplification reactions. Purified DNA fragments were ligated directly into the appropriate restriction endonuclease generated site in the cloning vector. 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 25°C.

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 & Russell, 2001). *Escherichia coli* DH5α was inoculated from an overnight broth culture into 100 ml LB and grown at 200 rpm in an orbital incubator at 37°C for 3 h to an A_{600} of ~0.5. The culture was then decanted into ice-cold 50 ml Falcon tubes and chilled on ice for 10 min. Cells were then pelleted by centrifugation at 5,000 x *g* in a Sorvall 5C Plus Centrifuge at 4°C for 10 min. Each pellet was resuspended in 10 ml of ice-cold 0.1 M $CaCl_2$, and centrifuged 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 DNA 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. 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 0.8 ml 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 LA plates containing antibiotic (100 µg ampicillin/ml in the case of pBluescript II KS [-]), 1 mM isopropyl-D-thiogalactopyranoside (IPTG, Roche Diagnostics Ltd.) and 100 µg (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal, Roche Diagnostics Ltd.) and incubated for 20 h at 37°C. Recombinants were identified using blue-white selection as described by (Sambrook & Russell, 2001).

2.4.6 Transformation of yeast cells by electroporation

Expression plamids were transformed into *C. dubliniensis* and *C. albicans*, using the electroporation method of (Fonzi & Irwin, 1993). Briefly, cells grown overnight in YPD broth at 30°C were inoculated in 50 ml YPD at a density of 1 x 10^4 cfu/ml and grown for approximately 2 h until the culture reached an O.D. $_{600}$ of between 1.6 and 2 (approximately 5 to 1.0 x 10^7 cfu/ml). The cells were decanted into a Falcon tube, harvested by centrifugation at 1,000 x *g* for 10 min and resuspended in 8 ml sterile water, 1 ml 10x TE and I ml 1 M LiAc. The cells were then incubated at 30°C at 150 rpm for 1 h. Following incubation 250 µl of 1 M DTT was added and the cells were reincubated for 30 min. The cells were then washed with 40 ml of water and then pelleted by centrifugation at 1,000 x *g* for 5 min and washed sequentially in 50 ml of ice cold water and 10 ml of ice cold 1 M sorbitol and the supernatant was discarded. The pellet was then resuspended in 50 µl of 1 M sorbitol and kept on ice. From this cell suspension 40 µl was used for each transformation. Carrier DNA in a volume of 5 µl was added to the cell suspension and mixed gently with a pipette tip and transferred to 0.2 cm electroporation cuvette (Bio-Rad Labs, Hercules, CA, USA). Electroporation was carried out in Bio-Rad Gene Pulser at 1.6 kV, 200Ω, 25 F (Bio-Rad). Then 450 µl of 1 M Sorbitol was added and 50 µl of this suspension were spread on selective medium and incubated at 30°C for 2 to 7 days.

2.5 DNA Sequence analysis

2.5.1 DNA sequence analysis

DNA sequencing was performed commercially by Lark (Saffron Walden, Essex, United Kingdom) using the dideoxy chain termination method of (Sanger *et al.*, 1977), and an automated Applied Biosystems 373A DNA sequencer (Foster City, California, USA) and dye-labelled terminators. Chromatograms were analysed using the 373A Data Analysis program version 1.2.0 (Applied Biosystems). Sequence analysis was carried out using the DNA Strider™ 1.3f11 software for DNA and protein analysis (CEA/Saclay, Gif-sur-Yvette,

France). Searches of the EMBL and GenBank databases for nucleotide and amino acid sequence similarities were performed using the BLAST family of computer programmes (Altschul *et al.*, 1990). Alignments of nucleotide and amino acid sequences were carried out using the CLUSTAL W sequence alignment computer program (Higgins & Sharp, 1988).

2.5.2 Annotation of the *C. dubliniensis* genome sequence

The computer programme Artemis was used in the annotation of *C. dubliniensis* genes from the unannotated *C. dubliniensis* genome sequence. Artemis is a DNA sequence viewer programme and annotation tool that allows visualisation of sequence features and the results of analyses within the context of the sequence, and its six-frame translation (Rutherford *et al.*, 2000).

2.6 Analysis of RNA

2.6.1 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-Aldrich). DEPC was dispersed in all solutions, which were then left to incubate at room temperature for 3-4 h, before autoclaving to inactivate 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-Aldrich) used in RNA extractions were 450-600 microns in diameter and were treated in nitrous acid, washed in distilled water, and baked overnight.

2.6.2 Total RNA extraction from *Candida* isolates

Candida cells were grown overnight in 50 ml of YPD medium and washed three times in ultra pure water and then inoculated at a concentration of 2×10^6 cfu/ml into 200 ml of hyphal inducing media and harvested at t = 0,1,2,3,4,5 and 6 h. Cells were harvested by vacuum filtration with Supor®-450 membrane filters with a pore size of 0.45 µm (Pall

Gelman laboratory, Ann Arbor, MI, USA), and a diaphragm vacuum pump (Vacuubrand GMBH Co., Wertham, Germany). The cells were scraped from the filter using a scalpel and placed in a 1.5 ml screw capped tube with 1 ml of TRIreagent and 0.1 g of acid washed glass beads. The cells were then homogenised and lysed in a fastprep FP120 bead beater (Anachem, Luton, UK) at top speed for 30 s. The cells were then incubated at 15-30°C for 5-10 min before addition of 200 µl of chloroform and vortexed briefly (15 s). The cells were pelleted by centrifugation at 12,000 g for 15 min at 2 to 8°C in a Biofuge (Biofuge Fresco, Heraeus Instruments, Kendro. Laboratory Products GmbH, Hanau, Germany). The upper aqueous phase, which contains the RNA was then transferred to a fresh 1.5 ml microfuge tube. This was followed by addition of 500 µl isopropyl alcohol and incubation for 10 min at at 15-30°C. The samples were pelleted by centrifugation again at top speed for 10 min at 2 to 8°C. The supernatant was removed and the pellet was washed once with 70% (v/v) ethanol to remove salts and then centrifuged again at 7,500 g for 5 min at 2 to 8°C. The pellet was briefly air-dried and the RNA was dissolved in 35 µl of DEPC-treated water. Samples were stored at -70°C.

2.6.3 RNA electrophoresis and transfer to nylon membranes

The concentration of each RNA sample was assessed by measuring the A_{260} (1 unit of A_{260} = 40 µg RNA) and 15 µg were loaded on each gel. A test 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 ethidium bromide/ml, 1x MOPS buffer). The samples were heated at 70°C for 10 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 80 V/cm in 1x MOPS buffer. Fractionated RNA was transferred to BioBond™-Plus Nylon membranes (Sigma-Aldrich) by capillary transfer. Gels were rinsed briefly in DEPC-treated water to remove excess formaldehyde and then soaked in 0.05 M 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 Bio-Rad UV crosslinker at 125 mJ (Bio-Rad, Hercules, CA USA).

2.6.4 Northern Hybridisation

Hybridisations were carried out in 10 ml hybridisation buffer (4x SSC, 1% (w/v) SDS, 10% (w/v) dextrin sulphate, 50% (v/v) formamide, 100 µg/ml denatured salmon sperm DNA). Membranes were prehybridised for 4 h at 42°C. The radiolabeled 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 at -70°C with an intensifying screen for 24-72 h. All membranes were hybridised with a probe homologous to the *C. dubliniensis TEF3* gene (Moran *et al.*, 2002). Relative expression levels of mRNA were measured using an imaging densitometer (GelWorks 1D Intermediate, UVP) to scan hybridisation signal intensity on autoradiograms, using the signal intensity of *CdTEF3* mRNA as a loading control.

Chapter 3

Comparison of growth of *C. albicans* and *C. dubliniensis in vitro*

3.1 Introduction

3.1.1 Comparison of the kinetics of growth of *C. albicans* and *C. dubliniensis*

The virulence of *C. albicans* can be attributed to its ability to survive and thrive in multiple organs, the mucousa, and the bloodstream of the host and to virulence factors that aid in adherence to epithelial and endothelial cell types and invasion of tissue. An important microbial virulence attribute, which is often overlooked, is growth rate. Kirkpatrick *et al.* suggested that while *C. dubliniensis* grows well at 37°C, *C. albicans* is able to out-compete *C. dubliniensis* when both species are co-inoculated in liquid media and in biofilm inducing conditions (Kirkpatrick *et al.*, 2000). In their study the proportions of each species at each time point was determined by diluting the cultures every 24 h and plating aliquots of culture onto CHROMagar Candida medium plates. The results of their study suggested that *C. albicans* has a competitive advantage over *C. dubliniensis* under both conditions tested. However, it should be noted that this medium is not a definitive method for differentiating between the two species. *Candida dubliniensis* may lose its ability to produce its characteristic dark green colour upon storage and subculture. Therefore, in order to confirm and extend the studies of Kirkpatrick *et al.* as part of this study it was decided to perform an in-depth investigation of the growth of *C. albicans* and *C. dubliniensis* alone and in co-culture using a more accurate species discriminating method based on the use of a molecular cassete containing genes encoding mycophenolic acid resistance (*IMH3*) and green fluorescent protein (*GFP*).

Molecular analysis of *Candida* species has been hampered in the past by the absence of positive selection markers. However, the identification of a mycophenolic acid resistance marker (MPA^R) by Kohler *et al.* has led to the use of this marker in gene disruption studies in *C. albicans* (Kohler *et al.*, 1997). Mycophenolic acid resistance is encoded by inosine monophosphate dehydrogenase, a key enzyme in purine biosynthesis which catalyses the NAD-dependant oxidation of inosine monophosphate to xanthine 5'- monophosphate (XMP), the first specific step in the guanine nucleotide pathway. It can be specifically inhibited by mycophenolic acid (MPA), an antibiotic produced by several species in the genus *Penicillium*, leading to a block of DNA and RNA synthesis in susceptible cells (Franklin &

Cook, 1969). In *C. albicans* wild-type strains, growth inhibition readily occurs at low MPA-concentrations (1 µg/ml) in guanine-deficient medium, although no growth occurs at 5-10 µg/ml. Plasmid derived over-expression of the *IMH3* gene confers resistance to increased levels of MPA (tested up to 40 µg/ml) allowing positive selection of transformants (Kohler *et al.*, 1997). The isolation of a mutated *IMH3* allele, which provides MPA-resistance even as a single copy gene after genomic integration enabled the development of the dominant MPA^R marker system, which has been successfully used in *C. albicans* and *C. dubliniensis* (Staib *et al.*, 2000; Wirsching *et al.*, 2000).

The green fluorescent protein (GFP) of *Aequorea victoria* has been developed as a reporter for gene expression and protein localisation in *C. albicans*. Cormack *et al.* constructed a yeast-enhanced GFP gene (yEGFP) whereby all codons of the original gene were exchanged by optimal codons for *C. albicans* (Cormack *et al.*, 1997). All of the 239 amino acids are encoded by optimal codons for *C. albicans*. In addition 2 amino acid changes were incorporated near the chromophore that had been shown to increase GFP fluorescence. *GFP* gene expression and the resulting fluorescence signal can be detected in living organisms even at the level of single cells. Optimised GFPs have been used as reporters in *C. albicans* and in *C. dubliniensis* to characterise regulatable promoters and to detect gene activation (Cormack *et al.*, 1997; Barrelle, *et al.*, 2004; Staib *et al.*, 2000; Wirsching *et al.*, 2000)

The aim of this part of the study was to compare the growth kinetics of *C. albicans* and *C. dubliniensis* when grown alone and in co-culture to identify if the reasons for the higher virulence of *C. albicans* may be at least partly due to a higher growth rate in comparison with that of *C. dubliniensis.*

3.2 Materials and Methods

3.2.1 *Candida* isolates

The *Candida* strains used in experiments involving comparative growth between *C. albicans* and *C. dubliniensis* are listed in Table 3.1. Two strains of *C. albicans* (SC5314 and 132A) and five strains of *C. dubliniensis* (CD36, CD57, H004, P6265 and CS1) were examined.

3.2.2 Phenotypic methods to differentiate *C. dubliniensis* from *C. albicans*

Agar containing 2,3,5-triphenyltetrazolium chloride (TTC) was used in an attempt to differentiate between *C. albicans* and *C. dubliniensis*. A stock solution was prepared by dissolving 1 g of 2,3,5-triphenyltetrazolium chloride in 10 ml of ultra pure water. The solution was filter sterilised and stored at 4°C in the dark until added to 1 litre sterilised basal agar medium cooled to 50°C and containing 10 g mycological peptone (Oxoid,), 10 g of glucose and 15 g agar (Oxoid). The final concentration of the stock TTC solution in the medium was 100 mg/ml and the final pH of the medium was 7.0 (Velegraki & Logotheti, 1998). Pal's and Staib agar were prepared as outlined by Al Mosaid *et al.* (Al Mosaid *et al.*, 2003; Al Mosaid *et al.*, 2001). CHROMagar Candida medium was also used to differentiate between the two species. Overnight cultures of the two species grown in YNB were counted using a haemocytometer (Neubauer) and an equal inoculum of each species was added to fresh YNB and at various timepoints aliquots were removed and plated onto each of the discriminatory media.

3.2.3 Construction of a molecular marker cassette to allow accurate differentiation between *C. albicans* and *C. dubliniensis* in mixed culture

The construction of the cassette is illustrated in Fig. 3.1. The cassette was based on plasmid CdIp (Gary Moran unpublished) which is a derivative of the *Candida* integrating plasmid CIp10 (Murad *et al.*, 2000) (GenBank Accession No. AF181970), in which the MPA^R gene has been substituted for the *URA3* marker and the *CdRP10* gene has been substituted for the *CaRP10* gene. To construct the cassette the *CdRP10* gene was substituted

Table 3.1. *Candida* isolates used in this study.

Species	Strain	Country of origin	Body site	Type of patient	Reference
C. albicans	SC5314	N/A[a]	Systemic	N/A[a]	Gillum *et al.* (1984)
	132A	Ireland	Oral	HIV+	Gallagher *et al.* (1992)
	CA411	Ireland	Oral	HIV+	Pinjon *et al.* (1998)
	52.1	Ireland	Oral	HIV+	Pinjon *et al.* (1998)
	103	Ireland	Oral	HIV+	Pinjon *et al.* (1998)
	JP14CA	Ireland	Oral	HIV+	Pinjon *et al.* (1998)
C. dubliniensis	CD33	Ireland	Oral	HIV+	Sullivan *et al.* (1995)
	CD36	Ireland	Oral	HIV+	Sullivan *et al.* (1995)
	CD506	Ireland	Oral	AIDS	Gee *et al.* (2002)
	CD57	Ireland	Vagina	HIV-	Moran *et al.* (1997)
	CD514	Ireland	Oral	HIV-	Gee *et al.* (2002)
	CD519	Ireland	Oral	AIDS	Gee *et al.* (2002)
	CD539	England	Oral	AIDS	Pinjon *et al.* (1998)
	CD541	United Kingdom	Blood	HIV-	Pinjon *et al.* (1998)
	CD96.34	Germany	Oral	HIV+	Pinjon *et al.* (1998)
	CS1	Ireland	Oral	HIV+	This study
	H004	Ireland	Oral	AIDS	Gee *et al.* (2002)
	P6265	Israel	Sputum	HIV-	Polacheck *et al.* (2000)
	P6785	Israel	Urine	HIV-	Polacheck *et al.* (2000)
	P7718	Israel	Wound	HIV-	Gee *et al.* (2002)

[a] N/A Not available

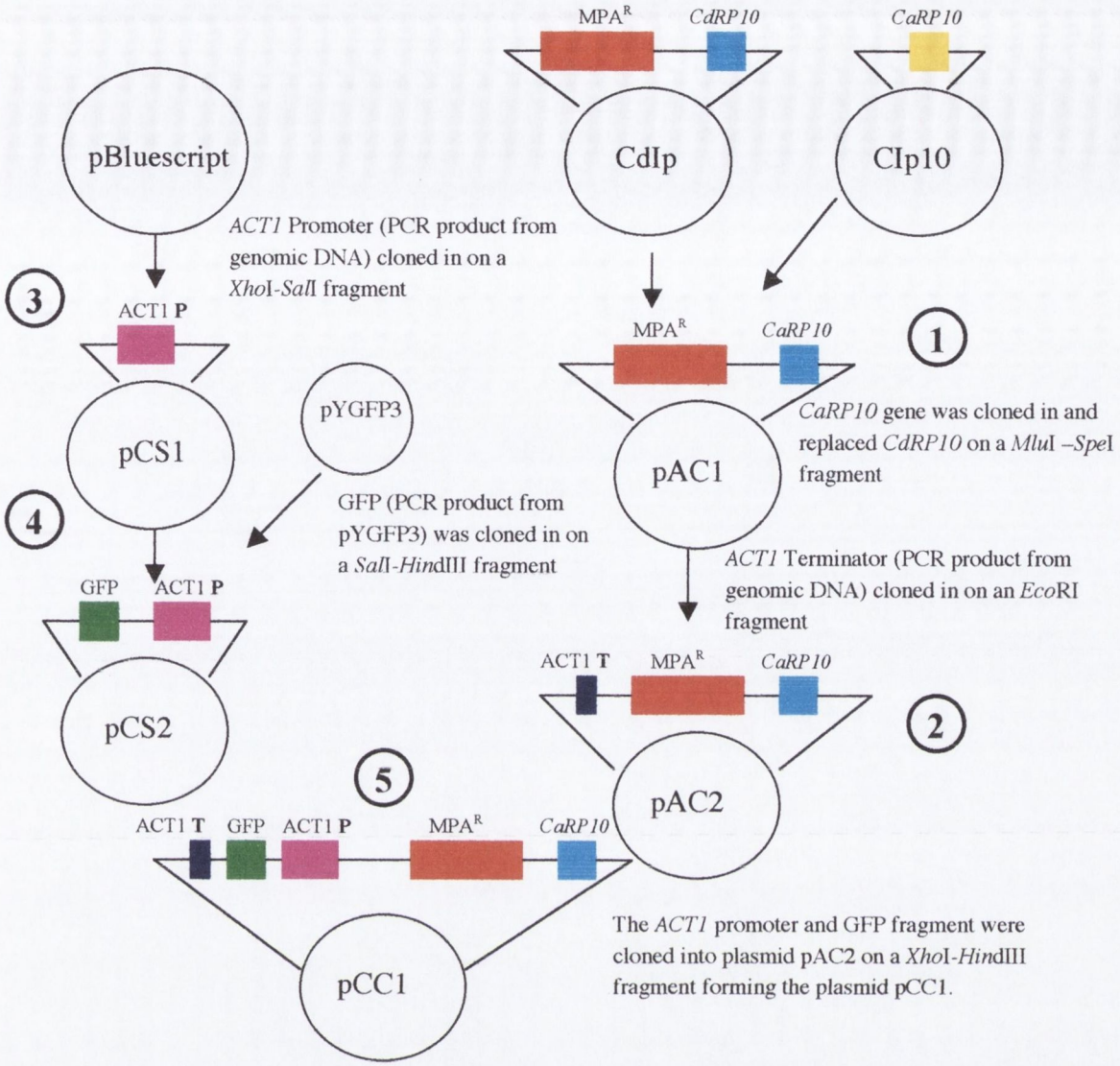


Figure 3.1. Flow diagram showing construction of the molecular marker cassette containing the *MPA*^{R} and *GFP* genes. The cassette was based on plasmid CdIp (Gary Moran unpublished) which is a derivative of the *Candida* integrating plasmid CIp10 (Murad *et al.*, 2000) (GenBank Accession No. AF181970). (1) To construct the cassette the *CdRP10* gene was substituted with the *CaRP10* gene thus generating pAC1. (2) A *CaACT1* downstream fragment was obtained by PCR amplification of genomic DNA of *C. albicans* strain 132A with primers ACTTF/ACTTR (Table 3.2), thereby introducing *Eco*RI restriction sites, and cloned into *Eco*RI digested plasmid pAC1 generating plasmid pAC2. (3) A *CaACT1* promoter fragment (positions -1019 to - 7) was obtained by PCR amplification of strain 132A genomic DNA of with primers ACTPF/ACTPR (Table 3.2), thereby introducing *Xho*I and *Sal*I restriction sites, and cloned into the *Xho*I /*Sal*I-digested vector pBluescript to generate plasmid pCS1. (4) The yeast optimised *GFP* gene was PCR amplified from plasmid pYGFP3 (Cormack *et al.*, 1997) with the primer pair GFPF/GFPR (Table 3.2) thereby introducing *Sal*I and *Hin*dIII restriction sites respectively. Following restriction enzyme digestion with *Sal*I /*Hin*dIII, the amplimers were cloned into pCS1 generating pCS2. (5) Plasmid pCS2 was digested with *Xho*I/*Hin*dIII to release the actin promoter-GFP fragment which was cloned into plasmid pAC2 thereby generating plasmid pCC1 which contained the molecular markers GFP and MPA^R.

with the *CaRP10* gene using the restriction enzymes *Mlu*I-*Spe*I thus generating pAC1. In order to obtain constitutive expression of the *GFP* gene, it was necessary to place it under the control of the *C. albicans ACT1* gene promoter and terminator regions. A *CaACT1* downstream fragment (positions +1789 to + 2182 with respect to the *CaACT1* start codon) was obtained by PCR amplification of genomic DNA of *C. albicans* strain 132A with primers ACTTF/ACTTR (Table 3.2), thereby introducing *Eco*RI restriction sites, and cloned into *Eco*RI digested plasmid pAC1 generating plasmid pAC2. A *CaACT1* promoter fragment (positions -1019 to -7) was obtained by PCR amplification of strain 132A genomic DNA with primers ACTPF/ACTPR (Table 3.2), thereby introducing *Xho*I and *Sal*I restriction sites, and cloned into the *Xho*I/*Sal*I-digested vector pBluescript to generate plasmid pCS1. The yeast optimised *GFP* gene was PCR amplified from plasmid pYGFP3 (Cormack *et al.*, 1997) with the primer pair GFPF/GFPR (Table 3.2) thereby introducing *Sal*I and *Hin*dIII restriction sites respectively. Following restriction enzyme digestion with *Sal*I/*Hin*dIII, the amplimers were cloned into pCS1 generating pCS2. Plasmid pCS2 was digested with *Xho*I/*Hin*dIII to release the actin promoter-GFP fragment, which was cloned into plasmid pAC2 thereby generating plasmid pCC1, which contained the molecular markers GFP and MPA^R. Recombinant plasmids used in the construction of the cassette are listed in Table 3.3. The position of each gene in the cassette is detailed in Fig. 3.2.

3.2.4 Transformation of *Candida* strains

Transformation of *Candida* strains was performed by electroporation as outlined in chapter 2 section 2.4.6. Transformation into the *RP10* locus was confirmed by PCR. Amplification reactions were carried out in 0.5 ml microfuge tubes (Eppendorf) in a Thermo Hybaid MBS thermal cycler in 50 µl volumes containing 1x *Taq* reaction buffer, 2.5 mM $MgCl_2$, 250 M (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 PCR cycling conditions used were 1 cycle for 1 min at 94°C followed by 35 cycles of denaturation for 20 s at 94°C, primer annealing for 20 s at 56°C, and extension for 2 min at 72°C; this was followed by a final incubation at 72°C for 10 min. The primers used were designed using the *IMH3* gene (*MPA*) sequence which is contained in the cassette and

Table 3.2. Nucleotide sequences of primers used in this study.

Primer	Sequence[a](5'-3')	Restriction site	Nucleotide coordinates[b]	Reference
ACTPF	GCACTCGAGATAGAGCTATTAAGATCACC	*Xho*I	-1019 to –999[c]	This study
ACTPR	GCGTCGACTGATTATATTTTTTTAATATTAATATC	*Sal*I	-7 to –33[c]	This study
ACTTF	GCGAATTCGAGTGAAATTCTGG	*Eco*RI	+1802 to +1789[c]	This study
ACTTR	GCGGAATTCAGAGTCGACATTTTATG	*Eco*RI	+2182 to +2169[c]	This study
GFPF	GCGTCGACATGTCTAAAGGTG	*Sal*I	+1 to +13	This study
GFPR	CCAAGCTTGGTTATTTGTACAATTC	*Hin*dIII	+718 to +702	This study
RP10F	AAGTACAACATGGCAGTCGGAA	-	-8 to + 13	This study
RP10R	TTAGAATCTGTTTAAGGATAATG	-	+757 to +779	This study
MPA1[d]	AAATTCAATCAAAGATCTTG	-	-521 to -501	This study
TEM1[e]	TGGTTGTTATGATCCTGTTG	-	+16 to +35	This study

[a] The restriction endonuclease recognition sites include in the primer sequences are underlined

[b] The nucleotide coordinates of the gene (where position + 1 corresponds to the first base of the ATG translational start codon)

[c]The ACT primers were designed based on the published sequence of the *C. albicans* gene encoding actin (*ACT1*) (Losberger & Ernst, 1989)

[d] The MPA1 primer was designed based on the *IMH3* gene (Genbank accession no. AY864854)

[e] The TEM1 primer was designed on the TEM1 sequence of *C. dubliniensis*

Table 3.3. Recombinant plasmids used in this study.

Plasmid Name	Vector	Cloning site	Insert	Insert source	Reference/ Source
pBII KS [-][a]	Cloning vector	Multiple	None	None	Stratagene
pCS1	pBII KS [-]	*Xho*I-*SalI*	*CaACT1* promoter region	*C. albicans* strain 132A	This study
pCS2	pCS1	*Sal*I-*Hin*dIII	GFP	Plasmid pYGFP3	(Cormack *et al.*, 1997)
pAC1	CdIp	*Mlu*I-*Spe*I	*CaRP10*	Plasmid CIp10	(Murad *et al.*, 2000)
pAC2	pAC1	*Eco*RI	*CaACT1* teminator region	*C. albicans* strain132A	This study
pCC1	pAC2	*Xho*I-*Hin*dIII	*CaACT1* promoter and GFP	Plasmid pCS2	This study

[a] pBII KS [-] : pBluescript II KS [-]

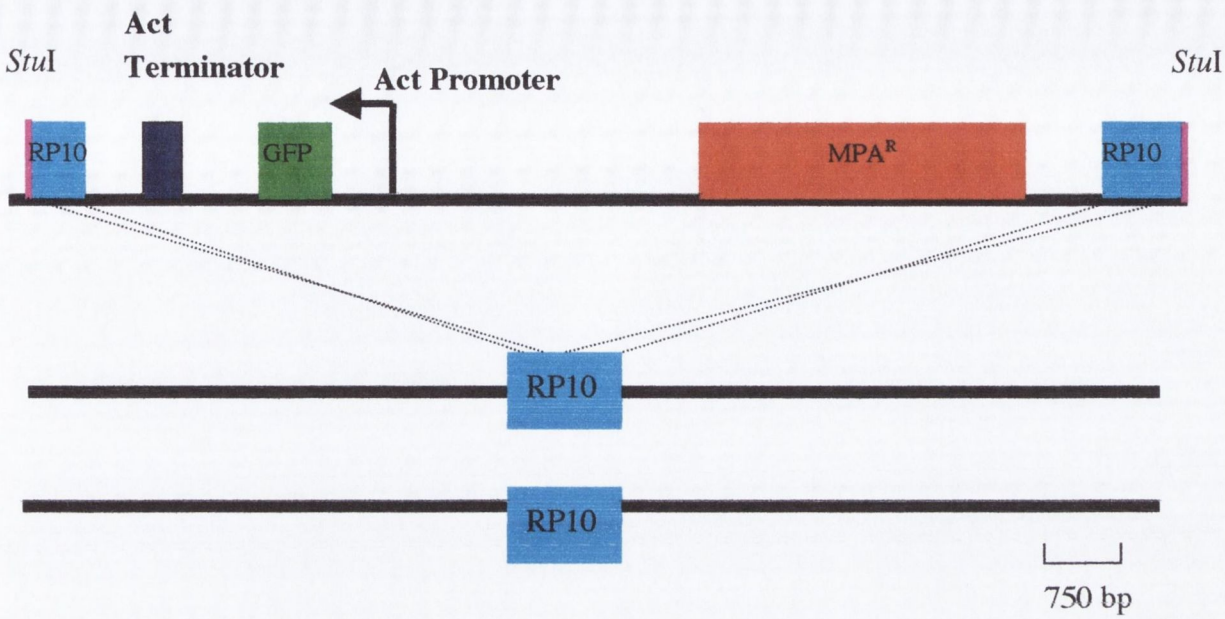


Figure 3.2. Molecular marker cassette containing the *MPA*^{R} and *GFP* genes. The position of the *CaACT1* promoter (**P***CaACT1*) and the *CaACT1* terminator (**T***CaACT1)* are highlighted in bold and the direction of transcription is indicated by the solid arrow. The solid flanking regions of the cassette are representative of the *CaRP10* gene to allow insertion into the chromosome of transformed strains by homologous recombination into a single copy of the *RP10* gene.

TEM1 1 (TEM1*)* which is the gene downstream of ribosomal protein 10 (*RP10)* in the *Candida* genome. The primers are listed in Table 3.2. An amplimer of 1500 bp was obtained if the cassette has inserted into the RP10 locus (Fig. 3.3). Southern hybridisation analysis was also carried out as outlined in chapter 2 section 2.3.5 to confirm the correct insertion of the cassette by digesting the DNA with *Hin*dIII and using the *RP10* gene which was amplified by PCR using the primers RP10F/RP10R (Table 3.2) as a probe. Derivatives which have been tagged with this cassette will contain the abbreviation (M/G) after the strain name.

3.2.5 Analysis of fluorescence of tagged strains

The intensity of fluorescence of tagged strains was measured by growing tagged strains for 18 h with shaking in YPD medium at 37°C. Cells were washed three times in ultra pure water and counted using a haemocytometer (Neubauer) to an inoculum of 2 X 10^6 cfu/ml and analysed in triplicate in a 96 well round bottomed plate (Greiner bio-one Ltd., Stonehouse, Gloucestershire, UK). The level of fluorescence was determined in a Genios plate reader (Tecan UK Ltd., Reading, UK) in triplicate using an excitation wavelength of 485_{nm} and an emission wavelength of 535_{nm}.

3.2.6 Comparative growth analysis of *C. albicans* and *C. dubliniensis*

Growth curves were performed on pairs of tagged and untagged strains to investigate if the cassette had any effect on growth. Overnight cultures from either (1) yeast nitrogen base (YNB) and (2) Sabouraud liquid broth modified antibiotic medium 13 (SDB) were counted using a haemocytometer (Neubauer) to an inoculum of 2 X 10^6 cfu/ml and incubated at 37°C in (1) YNB and (2) SDB with vigorous shaking. At specific time points (t = 0, 1, 2, 3, 4, 5, 6, 7, 8, 24 and 48 h), aliquots of each isolate were removed and their optical density at 600_{nm} ($O.D._{600}$) was also measured using a Genesys 2 spectrophotometer (Thermo Spectronic). These values were then used to plot a growth curve and determine doubling times for each isolate at 37°C. Growth curves were generated using CA-CricketGraph III (Computer Associates, Islandia, NY, USA).

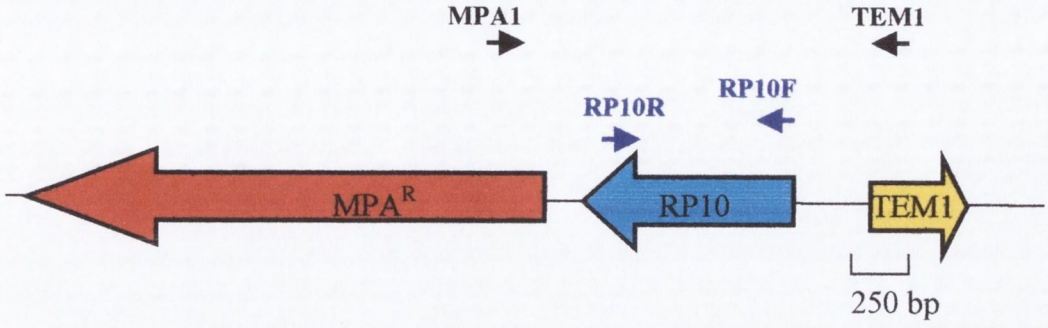


Figure 3.3. Illustration of the location and orientation of primers used to amplify the inserted molecular marker cassette from strains of *C. albicans* and *C. dubliniensis*. Large black filled arrows represent the *MPA*^{R}, *RP10* and *TEM1* genes and indicate the direction of transcription. Small bold arrows indicate the positions of primer sequences used in this study. The primer set MPA1/TEM1 highlighted in black was used to confirm the correct insertion of the cassette into *RP10*. The primer set RP10F/RP10R (Table 3.2) highlighted in blue were used to amplify by PCR the *RP10* gene which was subsequently used as a probe in Southern hybridisation analysis to also confirm the correct insertion of the cassette into the *RP10* gene on the chromosome.

Two methods were used to investigate the comparative growth of each species when grown in mixed culture.

3.2.6.1 Comparative growth and competition studies performed in Sabouraud liquid broth modified antibiotic medium 13 (SDB)

In order to repeat the experiments by Kirkpatrick *et al.*, experimental conditions used in their study were repeated in this study (Kirkpatrick *et al.*, 2000). An inoculum consisting of 5 X 10^5 cfu/ml of each strain (total of 10^6 cfu/ml) from an overnight culture grown at 37°C in SDB medium (Oxoid). The solution was dissolved, dispensed in 4 ml volumes into 18 by 150 mm culture tubes, and autoclaved. The inoculum was added into 4 ml of SDB (Oxoid) and incubated at 37°C with vigorous shaking. Controls in which each species were grown alone were inoculated with 10^6 cfu/ml. Daily transfers of 100 µl of resultant broth culture into fresh medium was performed. The number of surviving cells of each species present in the culture at 24 and 96 h were determined by serially diluted aliquots of each culture in ultra pure water, plated onto YNB agar, and incubated at 30°C for 2 days and then replica plated using velveteen squares (Bel-Art products, Pequannock, NJ, USA) onto YNB agar with MPA (10 µg/ml) and incubated at 30°C for 2 days.

3.2.6.2 Comparative growth and competition studies performed in yeast nitrogen base medium (YNB)

Competition studies were also performed in 50 ml of YNB medium consisting of 0.67% (w/v) yeast nitrogen base without amino acids and 2% (w/v) glucose. An inoculum of 1 X 10^6 cfu/ml from an overnight culture grown in YNB medium at 37°C, of each species was inoculated into YNB broth for the competition experiments, and incubated at 37°C with vigorous shaking. A larger inoculum was used than in section 3.2.6.1 as a larger volume of media was used and unlike above dilution into fresh culture every 24 h was not performed. Control experiments with each strain grown alone were performed in exactly the same manner with the exception that the inoculum used was 2 X 10^6 cfu/ml. At specific time points (t = 0, 2, 4, 6, 8, 24 and 48 h), aliquots of each isolate were removed and plated onto

YNB medium as before and their optical density at 600_{nm} (O.D. 600) was also measured using a Genesys 2 spectrophotometer (Thermo Spectronic). These values were then used to plot a growth curve and determine doubling times for each isolate at 37°C. Growth curves were generated using CA-CricketGraph III (Computer Associates). The doubling times were obtained from portions of the curves corresponding to logarithmic growth phase and were determined using the formula Log N-Log N_0/ Log 2 to determine the generation time and then converted to mins. Each experiment was performed in triplicate.

3.2.7 Fluorescence microscopy

Strains were grown overnight in either YPD liquid medium or minimal medium and 10 µl was spotted on microscope slides. Fluorescence was detected using a Nikon Eclipse 600 microscope (Nikon) fitted with a super high power mercury lamp (Nikon) and the GFP specific filter set (Endow GFP Bandpass Emission (FGP®-BP) filter combination), (Nikon).

3.2.8 Statistical analysis

Statistical analysis was carried out using the two sample Student *t*-test assuming unequal variances. *P* values of <0.05 were considered significant.

3.3 Results

3.3.1 Phenotypic methods to differentiate *C. dubliniensis* from *C. albicans*

It has previously been shown that CHROMagar Candida medium is a definitive means of differentiation of *C. albicans* and *C. dubliniensis* (Coleman *et al.*, 1997a; Schoofs *et al.*, 1997; Sullivan *et al.*, 1997). However, when this medium was examined when aliquots of a mixed culture containing *C. albicans* and *C. dubliniensis* were plated out and incubated at 37°C for 48 h, numerous white colonies were observed. DNA was extracted from these colonies and analysed by PCR using *C. albicans*-specific and *C. dubliniensis*-specific PCR primers as outlined in section 2.2.4. These colonies were deemed to be both *C. albicans* and *C. dubliniensis* and therefore CHROMagar Candida medium was not a suitable means of differentiation when the two species were grown in co-culture. Studies have also shown that both Pals's and Staib medium allow accurate species differentiation as *C. dubliniensis* unlike *C. albicans* produces abundant hyphae, pseudohyphae and chlamydospores on these media (Al Mosaid *et al.*, 2003; Al Mosaid *et al.*, 2001; Staib & Arasteh, 2001). However in this study when aliquots of a co-culture of both species were plated onto both media difficulty was incurred when colonies were growing very close together as to which colony was producing the hyphal fringe. These media provide a more accurate differentiation when strains of both species are streaked onto the media. Studies by Velegraki & Logotheti, have also shown that incorporating 2,3,5-triphenyltetrazolium chloride (TTC) into agar provides accurate discrimination between the two species as *C. albicans* produces a pale pink to whitish colour and *C. dubliniensis* produces a red to maroon colour (Velegraki & Logotheti, 1998). In the present study when a variety of strains of both species were examined it was found that strains of both species produce a variety of colours on this medium and is not as accurate a discrimination as outlined by Velegraki *et al.* (Velegraki & Logotheti, 1998). It was therefore decided to use a molecular method to accurately differentiate the two species from one another.

3.3.2 Construction of a molecular marker cassette to allow accurate differentiation between *C. albicans* and *C. dubliniensis* in mixed culture

Construction of the molecular marker cassette was performed as to provide an easy, reliable and accurate means of differentiation of *C. dubliniensis* from *C. albicans* when the two species were grown together in co-culture. Previously CHROMagar Candida medium has been used to phenotypically differentiate between the two species however, this method is not wholly accurate with colour variation occuring due to subculture and storage. In order to investigate the relationship between *C. albicans* and *C. dubliniensis* when grown together in mixed culture it was necessary to be able to accurately differentiate between the two species. Due to the close phenotypic properties shared by the two species and the lack of an accurate test to differentiate the two after growth in a mixed culture it was decided to investigate whether tagging strains with a molecular cassette could be used to discriminate between the two species. In order to maximise the efficiency of a cassette it was decided to include two genes to allow two means of differentiation.

Therefore the cassette was designed containing the gene encoding mycophenolic acid resistance (*IMH3*) and yeast optimised green fluorescent protein (*GFP*) encoding gene. Mycophenolic acid resistance (MPA^R), conferred by a mutation of the *IMH3* gene which encodes IMP dehydrogenase, which is the key enzyme in the de novo biosynthesis of GMP, is a selectable phenotype that has been successfully used in *C. albicans* for selection in spheroplast fusion (Goshorn & Scherer, 1989) and as a reporter for virulence gene activation *in vivo* (Staib *et al.*, 1999) or as a selectable marker if used in high copy number (Kohler *et al.*, 1997). Optimised GFPs have been used as reporters in *C. albicans* and in *C. dubliniensis* to characterise regulatable promoters and to detect gene activation (Barelle *et al.*, 2004; Cormack *et al.*, 1997; Staib *et al.*, 2000; Wirsching *et al.*, 2000). The *RP10* locus was chosen to target genomic integration as there are two copies of the *RP10* gene as *Candida* is a diploid organism and it has been shown in *C. albicans* that disruption of one *RP10* allele does not significantly affect the growth of transformants on rich or defined media (Murad *et al.*, 2000). The ribosomal protein 10 coding region (*RP10)*; (Swoboda *et al.*, 1995) provides homology to target chromosomal integration (Fig. 3.2).

This cassette was designed to permit differentiation between tagged and untagged strains by growth on both MPA-containing and drug-free media and also by examination of

fluorescence from GFP under a fluorescent microscope. Therefore, when strains of *C. dubliniensis* which have been tagged with MPA^R and GFP were competed with *C. albicans* strains in mixed culture the two species were readily differentiated from each other as tagged strains were able to grow on drug-containing media and tagged cells also express fluorescence as a result of (GFP) which was detected using a fluorescent microscope (Nikon).

The strains into which the cassette was transformed were *C. albicans* strain SC5314 which was chosen as it is used as a reference strain throughout the world and 132A was chosen as it is a well characterised oral reference strain. The *C. dubliniensis* strain CD36 was chosen as it is also a reference strain that is used worldwide and the other *C. dubliniensis* strains were chosen as they consist of a broad range of isolates from three of the four different *C. dubliniensis* genotypes which have previously been characterised (Gee *et al.*, 2002). Integration of the cassette into the *C. albicans* and *C. dubliniensis* strains was achieved by transformation with linearised plasmid cassette DNA as outlined in section 2.4.6. Positive transformants were detected by both growth on MPA containing media and examination of fluorescence. Confirmation of insertion of the cassette into strains of *C. dubliniensis* and *C. albicans* was confirmed by performing PCR analysis on the *IMH3* gene (MPA^R) in the cassette and the gene which lies upstream from *RP10* on the chromosome which is *TEM1* (Fig. 3.3). A product of 1.5 kb was indicative of correct insertion of the cassette into the *RP10* gene (Fig. 3.4). Southern analysis was also performed on genomic DNA from strains of *C. dubliniensis* and *C. albicans* and derivatives of each species, which contained the cassette, cut with the restriction enzyme Hind*III* and probed with a PCR amplimer generated from amplification of *C. dubliniensis* strain CD36 with the primers RP10F/RP10R listed in Table 3.2. The hybridisation bands detected resulted from insertion of the cassette into the strains at least twice due to the hybridising band detected at 8.7 kb (Figs. 3.5 and 3.6). The hybridisation bands at 11 and 12 kb for *C. albicans* and *C. dubliniensis* derivatives tagged with the molecular cassette identical to the bands in the parental strains, were derived from the homologous chromosome not affected by the integration event. The intensity level of the band located at 8.7 kb is indicative of multiple copies of the cassette having been inserted into the *RP10* locus (Fig. 3.7). *Candida dubliniensis* strain Can6 despite containing an amplimer with PCR gave an unusual

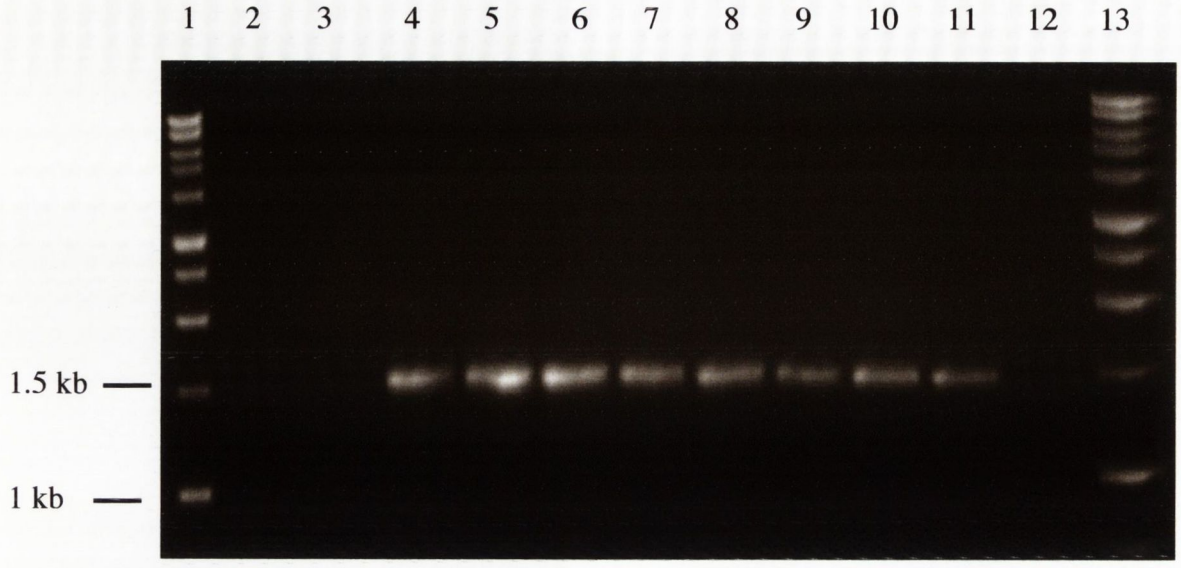


Figure 3.4. Agarose gel showing ethidium bromide-stained PCR-generated amplimers of both *C. albicans* and *C. dubliniensis* template DNA using the MPA1/TEM1 primer pair (Table 3.2). The amplimers shown in the lanes were obtained from *Candida* strains as follows: lane 2, untagged *C. albicans* SC5314; lane 3, untagged *C. dubliniensis* CD36; lane 4-5, *C. albicans* SC5314 (M/G) and 132A (M/G); lanes 6-11 CD36 (M/G), H004 (M/G), CD57 (M/G), Can6 (M/G), CS1 (M/G) and P6265 (M/G). Lane 11, no DNA control. Lane 1 and 13, 1 kb ladder.

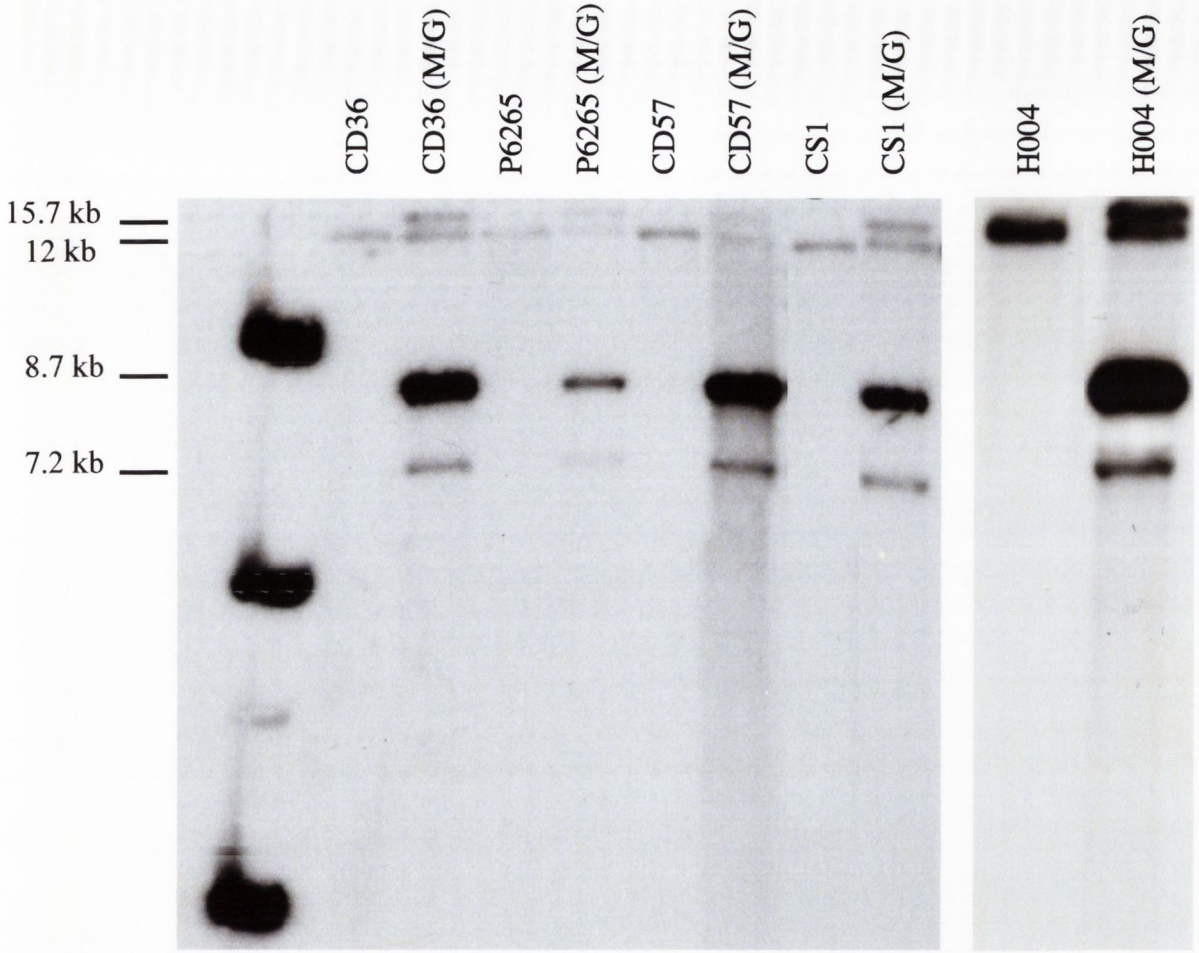


Figure 3.5. Southern blot of *Hin*dIII-digested genomic DNA from five pairs of tagged and untagged *C. dubliniensis* strains (CD36, CD33, CD514, P7718, CS1, CD539 and CD541) following hybridisation analysis with the PCR amplimer encoding *CdRP10.* The bands at 7.2 and 15.7 kb are derived from the flanking fragments. The band at 8.7 kb is diagnostic of a double integration event. The band at 12 kb in the transformant, identical to the band in the parental strain, is derived from the homologous chromosome not affected by the integration event.

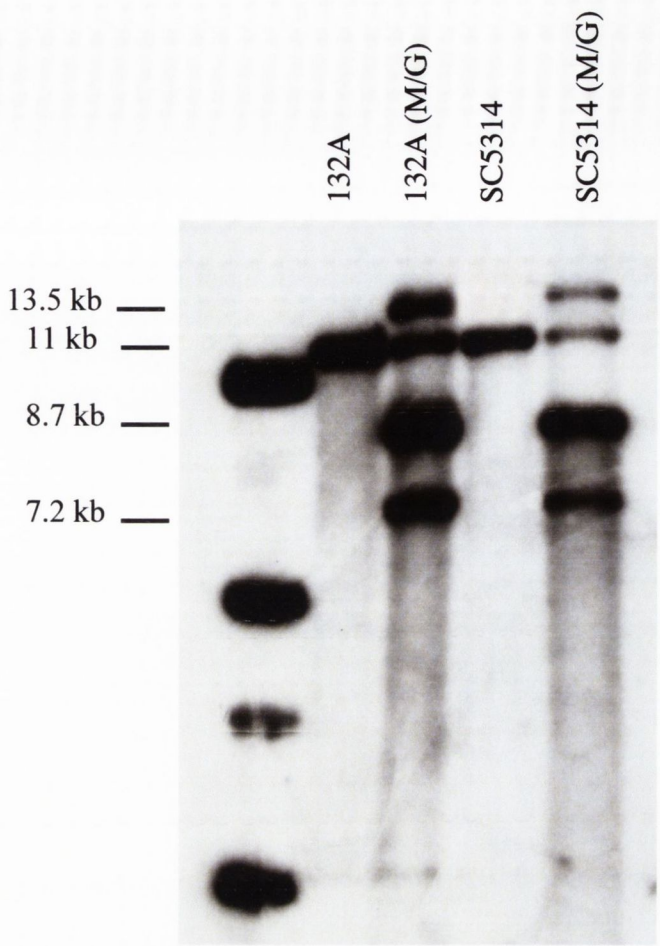


Figure 3.6. Southern blot of *Hin*dIII-digested genomic DNA from two pairs of tagged and untagged *C. albicans* strains (SC5314 and 132A) following hybridisation analysis with the PCR amplimer encoding *CaRP10.* The bands at 7.2 and 13.5 kb are derived from the flanking fragments. The band at 8.7 kb is diagnostic of a double integration event. The band at 11 kb in the transformant, identical to the band in the parental strain, is derived from the homologous chromosome not affected by the integration event.

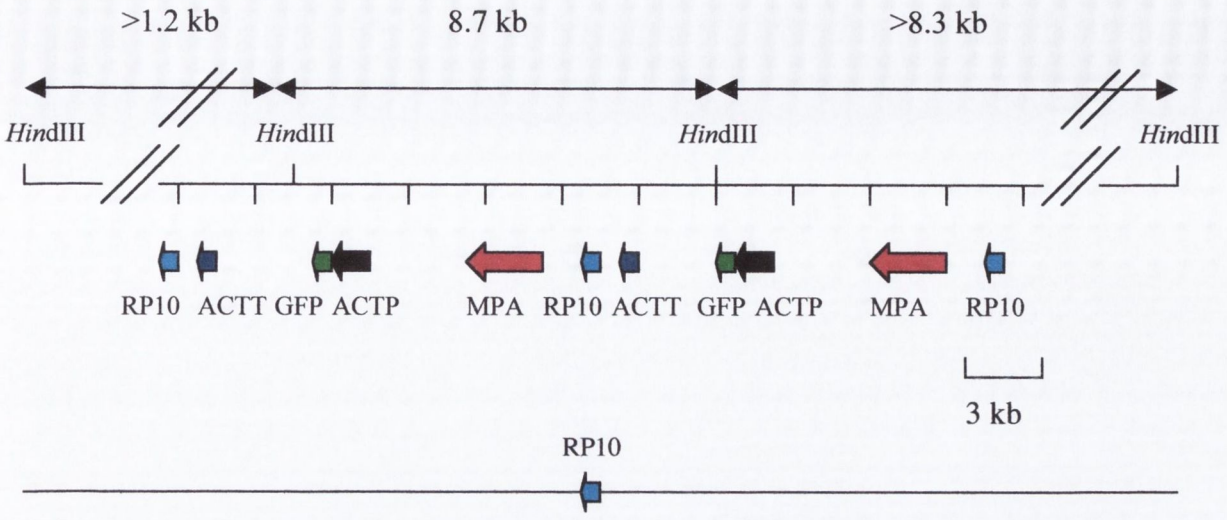


Figure 3.7. Schematic diagram of predicted chromosomal structure resulting from a double integration event. The map line shows the distances calculated from the known sequences of the *RP10* locus and pCC1. The position of the *Hin*dIII sites flanking the *RP10* locus are unknown indicated by the break in the map line.

hybridising pattern upon Southern analysis and so was disregarded at this point. To investigate if tagged derivatives had differences in fluorescence due to differences in copy number, the level of fluorescence was investigated (Figs. 3.8 and 3.9), *C. dubliniensis* derivative CD57 (M/G) was the most intense and *C. dubliniensis* derivative P6265 (M/G) was the least intense which correlates with the hybridisation band intensitites observed in the Southern blot (Fig. 3.5).

The growth of each tagged derivative and parent strain were compared to investigate if the cassette had any effect on growth (Figs. 3.10 and 3.11). No significant difference was observed. Preliminary experiments were also performed using tagged derivatives and plating 100 µl aliquots onto both MPA-containing and drug-free media. In all of the derivatives examined there was no significant difference observed between growth of the derivative on either of the media tested.

3.3.3 Examination of the comparative growth of *C. albicans* and *C. dubliniensis*

3.3.3.1 Examination of the comparative growth between the two species in SDB medium over 96 h

Kirkpatrick *et al.* previously suggested that *C. albicans* was able to outcompete *C. dubliniensis* when grown over 96 h in 4 ml SDB medium at 37°C (Kirkpatrick *et al.*, 2000). Each day 100 µl of the broth culture was transferred to fresh SDB medium and the cell density of each species was measured after 24 and 96 h. Previously Kirkpatrick *et al.* used 7 *C. albicans* strains and 7 *C. dubliniensis* clinical isolates from AIDS patients (Kirkpatrick *et al.*, 2000). In the present study the *C. albicans* strains SC5314 and 132A and *C. dubliniensis* strains CD36, CD57, H004, CS1 and P6264 (genotypes I, II and II) were compared. It is clear from the results that *C. albicans* strains when grown alone show higher cell densities after both 24 h (3.5×10^7- 5.1×10^7 cfu/ml) and 96 h (3.6×10^7- 5×10^7 cfu/ml) (Figs. 3.12 and 3.13). The results also show higher cell densities of *C. dubliniensis* strains after both 24 h (1.8×10^7- 3.5×10^7 cfu/ml) and 96 h (2.2×10^7- 4.0×10^7 cfu/ml). However, when equal inocula of *C. albicans* and *C. dubliniensis* were co-inoculated *C. albicans* was always present at significantly ($P < 0.001$) higher cell densities (1.8×10^7- 4.3×10^7 cfu/ml) than *C. dubliniensis* at both 24 h (1.0×10^6 - 6.0×10^6 cfu/ml) and ($P < 0.001$) 96 h, (2.9×10^7- 3.9 x

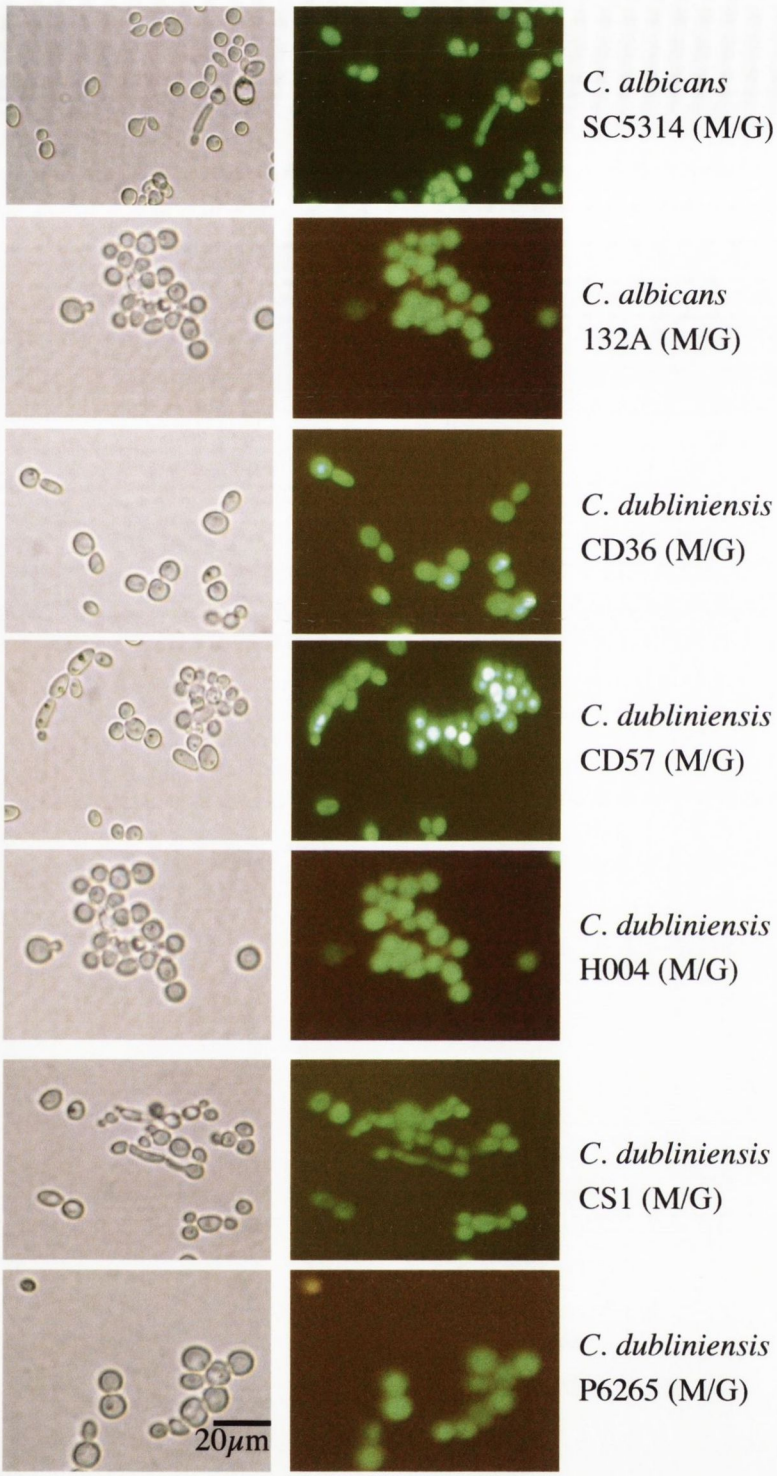


Figure 3.8. Light micrographs showing fluorescent cells from *C. albicans* derivatives SC5314 (M/G) and 132A (M/G) and *C. dubliniensis* derivatives CD36 (M/G), CD57 (M/G), H004 (M/G), CS1 (M/G) and P6265 (M/G), which had been tagged with the molecular marker cassette containing MPA^R and GFP. The scale bar is representative of each of the light micrographs.

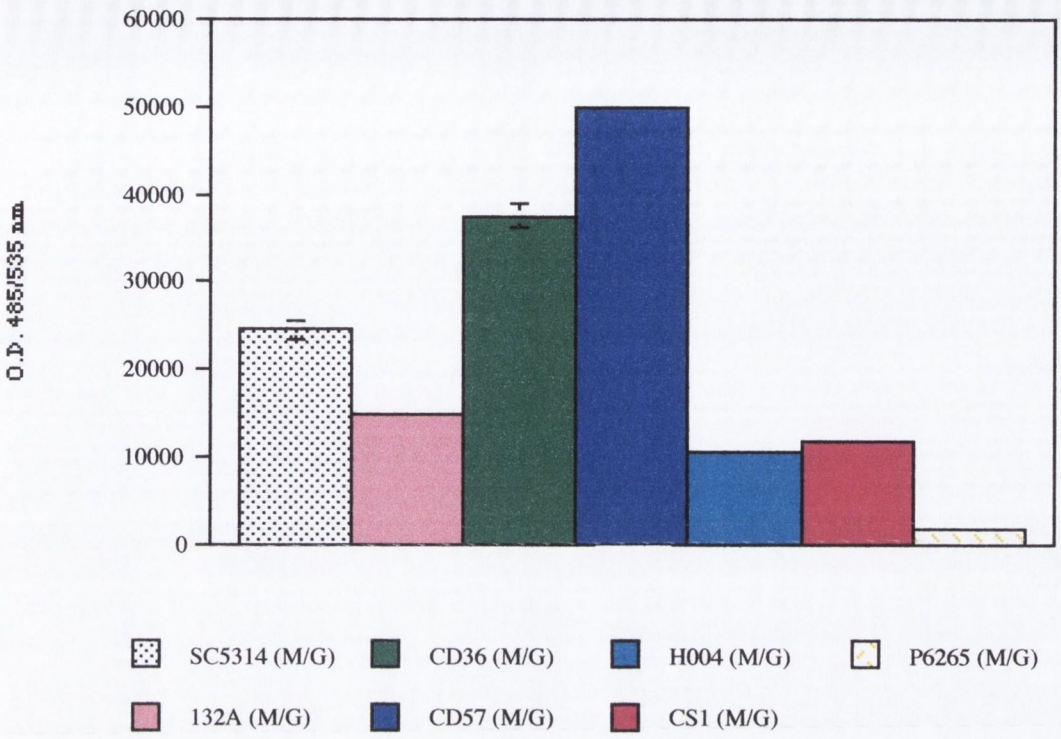


Figure 3.9. Graph showing the level of fluorescent intensity of *C. albicans* derivatives SC5314 (M/G) and 132A (M/G) and *C. dubliniensis* derivatives CD36 (M/G), CD57 (M/G), H004 (M/G), CS1 (M/G) and P6265 (M/G) which had been tagged with the molecular marker cassette containing MPA^R and GFP.

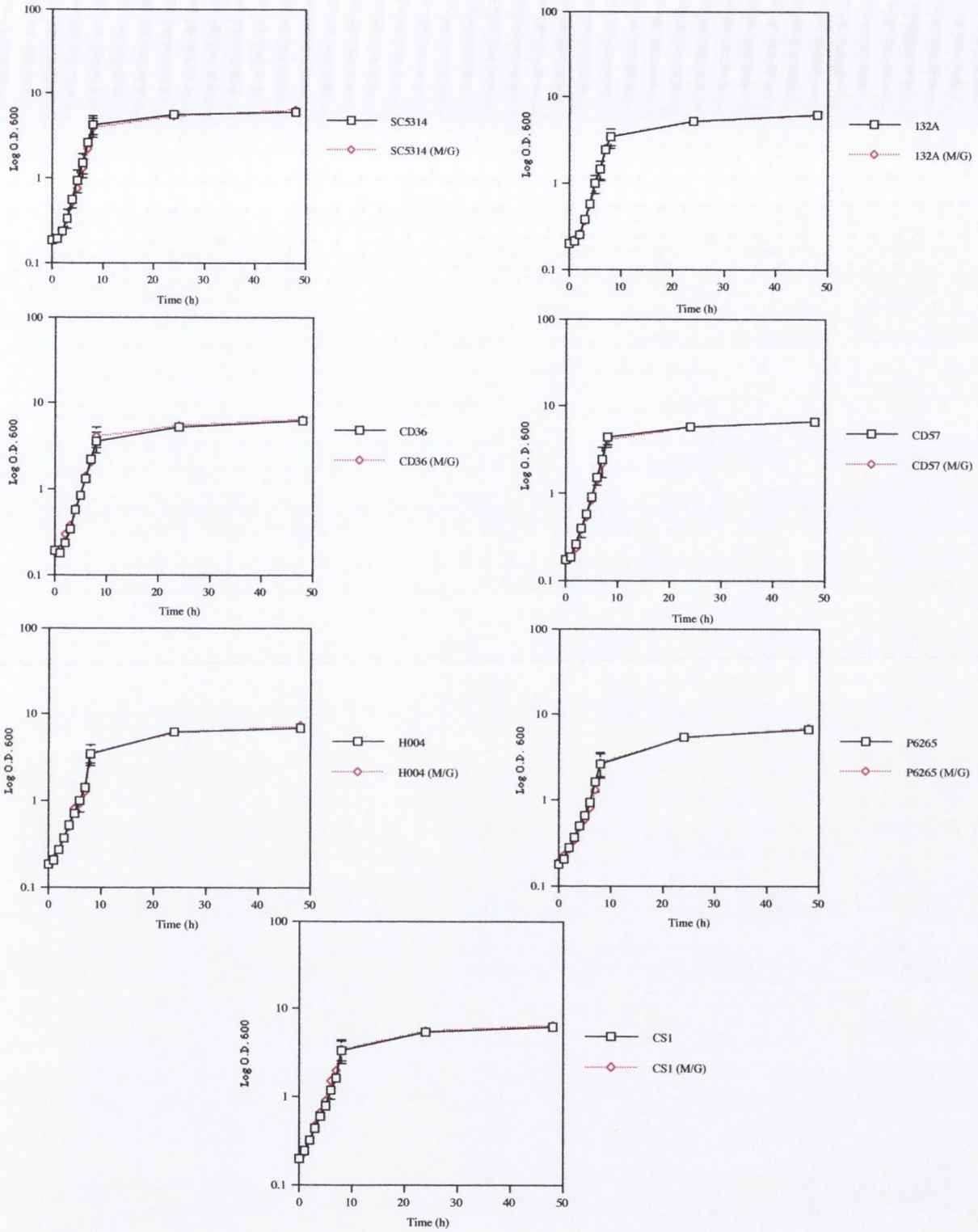


Figure 3.10. Graphs showing growth of *C. albicans* strains (SC5314 and 132A) and *C. dubliniensis* strains (CD36, CD57, H004, P6265, CS1), compared with derivatives which had been tagged with the molecular marker cassette during 48 h of growth in YNB at 37°C.

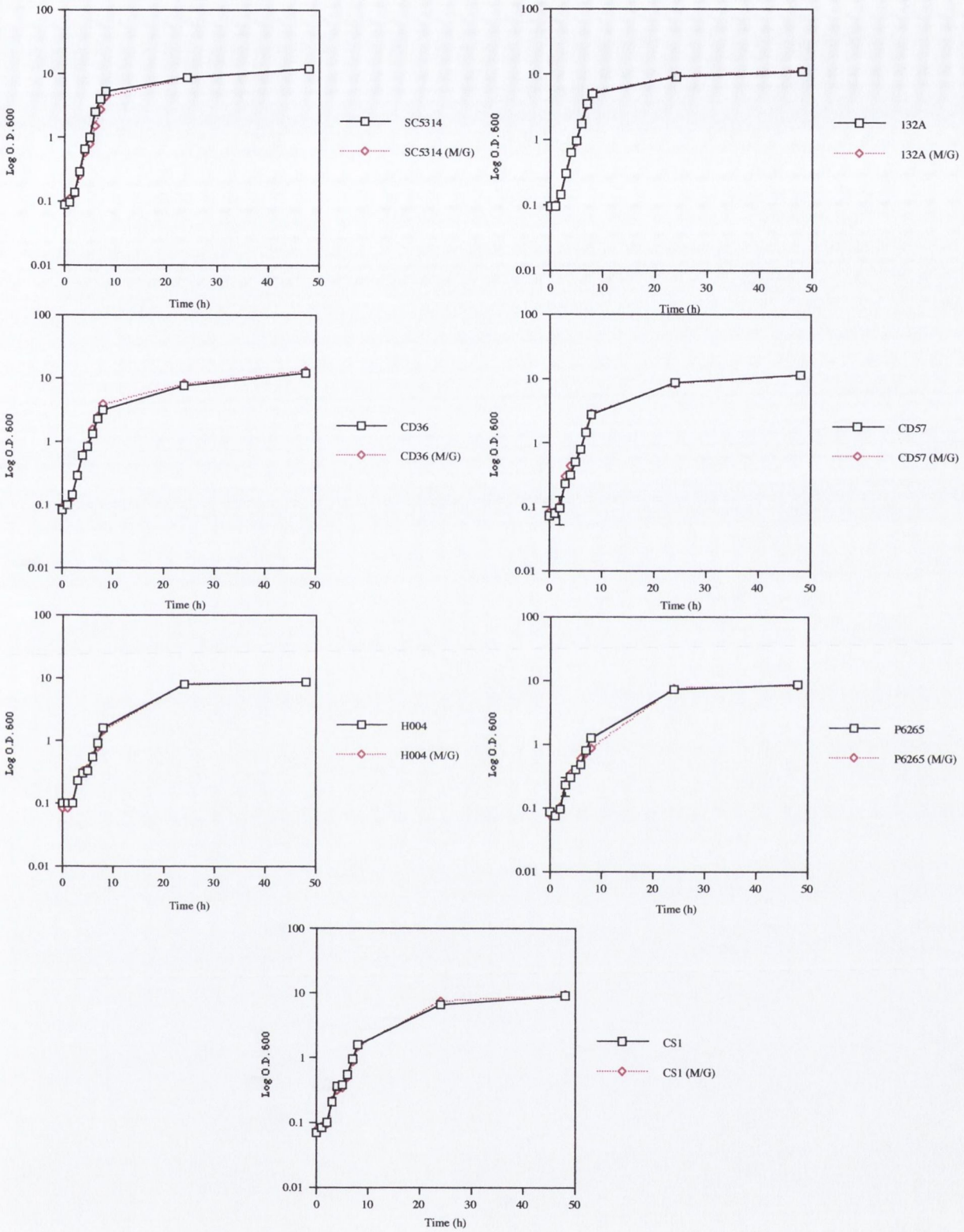


Figure 3.11. Graphs showing growth of *C. albicans* strains (SC5314 and 132A) and *C. dubliniensis* strains (CD36, CD57, H004, P6265, CS1), compared with derivatives which had been tagged with the molecular marker cassette during 48 h of growth in SDB at 37°C.

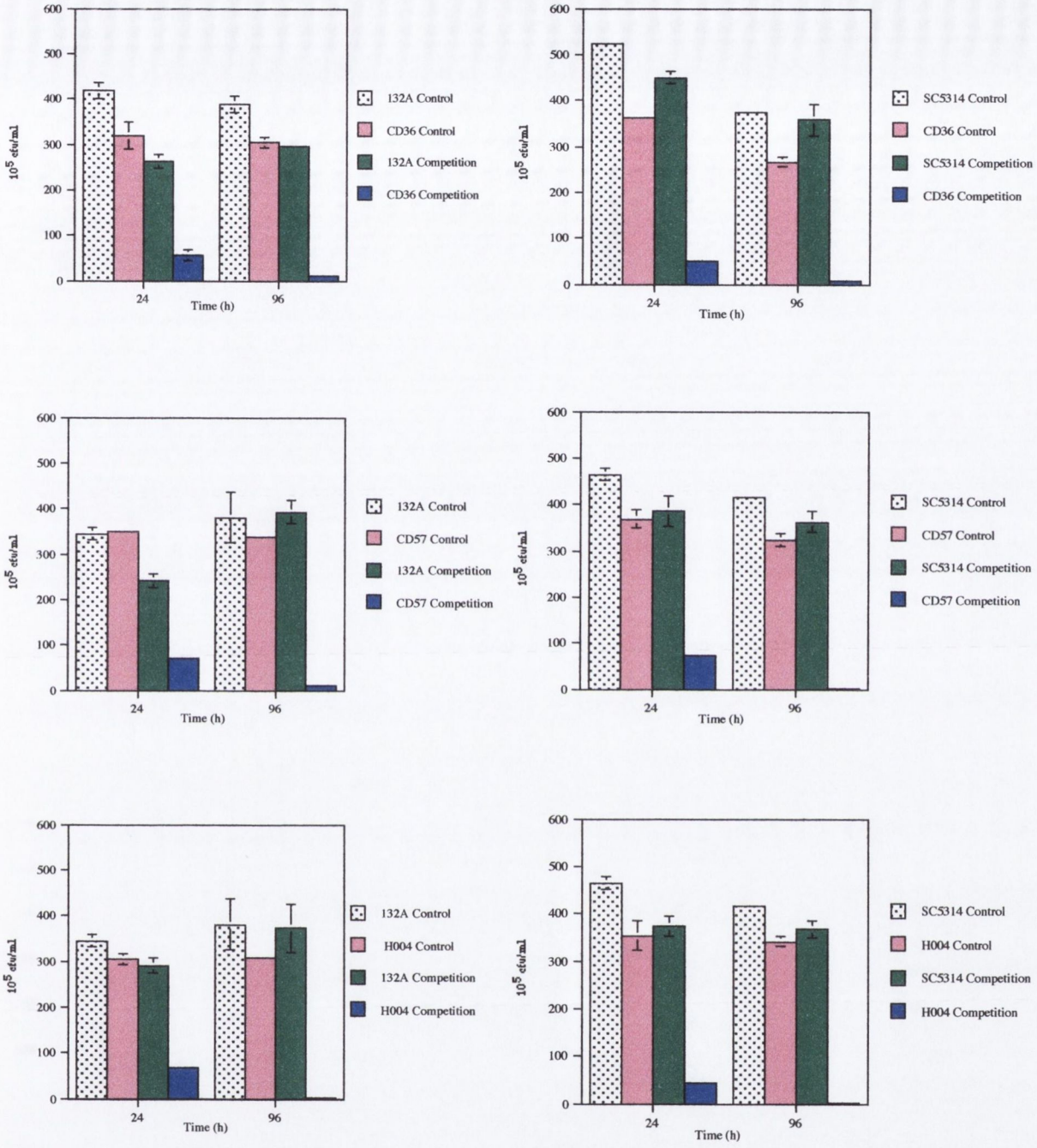


Figure 3.12. Graphs showing biomass concentrations of *C. albicans* strains (SC5314 and132A) and *C. dubliniensis* derivatives CD36 (M/G), CD57 (M/G) and H004 (M/G) when grown at 37°C in SDB medium for 96 h alone (Control) and in co-culture together (Competition). All *C. dubliniensis* derivatives were tagged with the molecular marker cassette. All experiments were performed in triplicate.

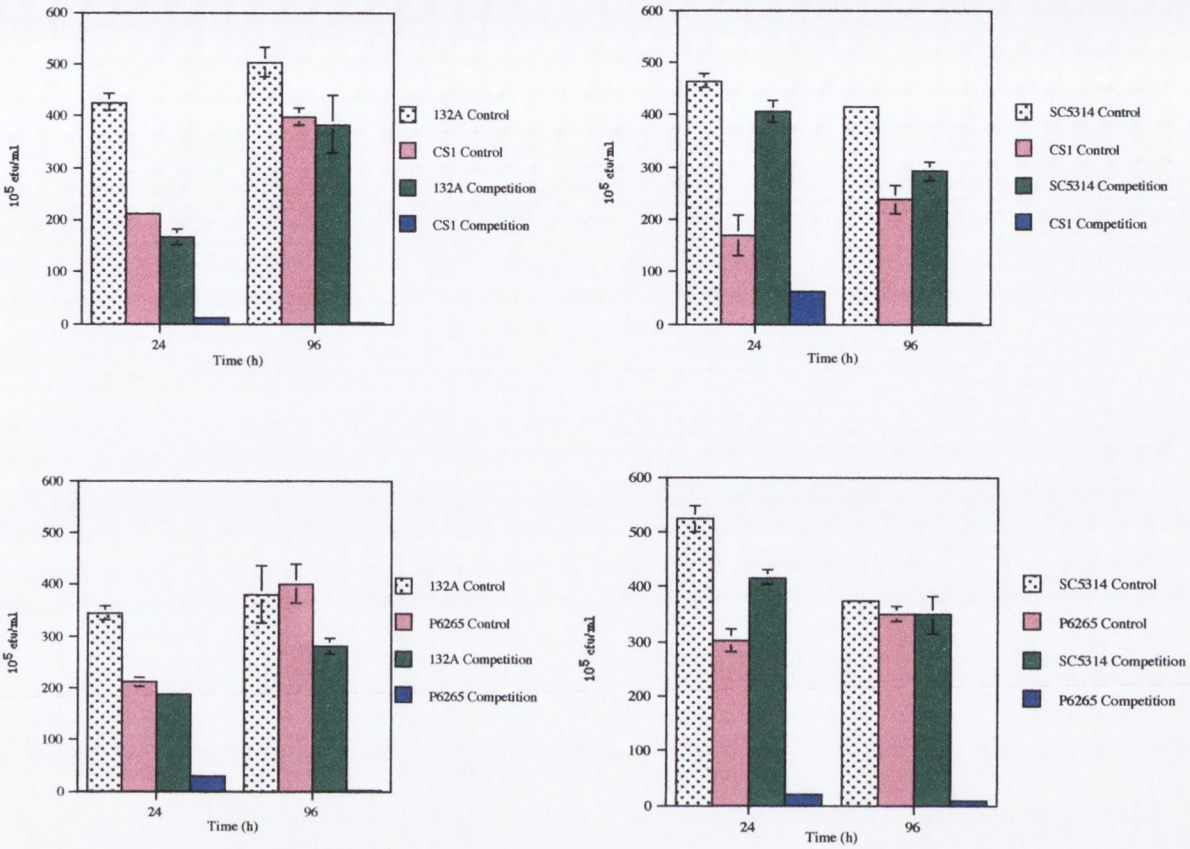


Figure 3.13. Graphs showing biomass concentrations of *C. albicans* strains (SC5314 and 132A) and *C. dubliniensis* derivatives CS1 (M/G) and P6265 (M/G) when grown at 37°C in SDB medium for 96 h alone (Control) and in co-culture together (Competition). All *C. dubliniensis* derivatives were tagged with the molecular marker cassette. All experiments were performed in triplicate.

10^7 cfu/ml) in comparison with (4×10^5 - 1.0×10^6 cfu/ml). *Candida dubliniensis* was present at very low densities after 96 h in all co-inoculation experiments which is in contrast to what Kirkpatrick *et al.* found as they detected *C. dubliniensis* in only 2 out of 11 pairings (Kirkpatrick *et al.*, 2000).

To confirm that tagged derivatives had no negative effect on the experiments a reverse experiment was performed where tagged *C. albicans* derivatives (SC5314 (M/G) and 132A (M/G)) were co-inoculated with untagged *C. dubliniensis* strain CD36 (Fig. 3.14). The results obtained were similar to those found for tagged *C. dubliniensis* derivatives when co-inoculated with *C. albicans* strains therefore showing that MPA^R was a definitive means of differentiation and that tagging the strains had no negative effect on growth. Doubling times for all strains were determined over 48 h in SDB medium with shaking at 37°C (Table 3.4). It is clear that the doubling times for *C. albicans* strains (67-70 ± 2.9 min) are significantly faster than *C. dubliniensis* strains (86-112 ± 2.5 min), ($P < 0.001$) in SDB medium.

3.3.3.2 Examination of the comparative growth between the two species in YNB medium over 48 h

The experimental design of the Kirkpatrick *et al.* study suggests that the reason for the apparent disappearance of *C. dubliniensis* in co-culture experiments may be due to the species being diluted out at each 24 h time-point (Kirkpatrick *et al.*, 2000). In order to determine that the significantly lower cell densities of *C. dubliniensis* obtained in this study when grown in co-culture with *C. albicans* was due to the fact that *C. dubliniensis* was being diluted out at every 24 h, the comparative growth of each species was investigated using a different experimental design. Competitive growth comparisons were performed in YNB medium which is a defined medium, over 48 h in the same culture flask unlike the previous experiment whereby cultures were diluted into fresh medium every 24 h. At t=0, 2, 4, 6, 8, 24 and 48 hours dilution plating onto YNB agar was performed and plates were incubated at 37°C for 48 h. Also at t= 0, 1, 2, 3, 4, 5, 6, 7, 8, 24 and 48 h an aliquot was removed and their optical density at 600_{nm} ($O.D._{600}$) was measured using a Genesys 2 spectrophotometer (Thermo Spectronic). This allowed the growth rate of *C. albicans* and *C. dubliniensis* to be compared over 48 h (Figs. 3.15 and 3.16). The doubling times were calculated and as

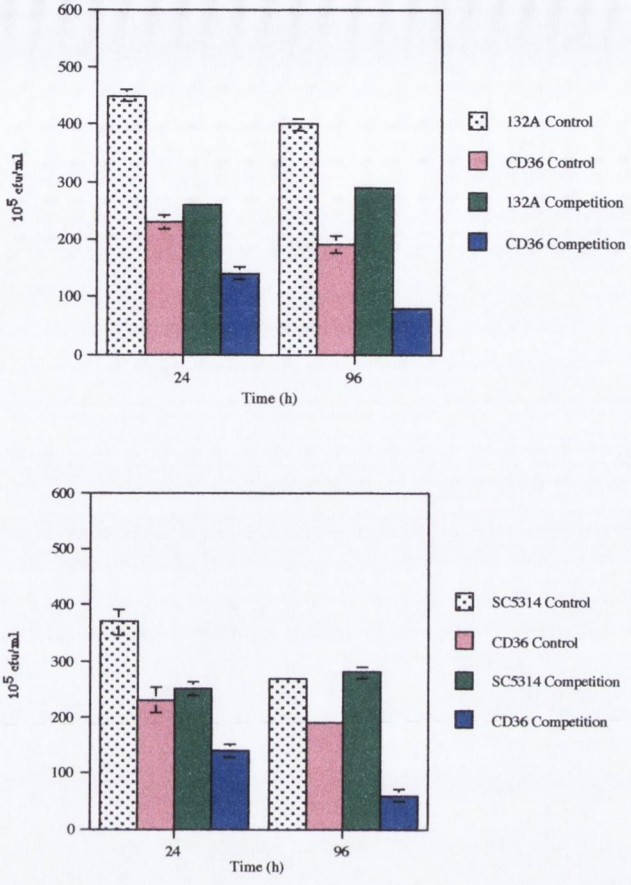


Figure 3.14. Graphs showing the biomass concentrations of derivatives of *C. albicans* strains SC5314 and 132A, which had been tagged with the molecular marker cassette when examined in co-culture with *C. dubliniensis* strain CD36 when grown in SDB medium over 96 h at 37°C.

Table 3.4. Doubling times of *C. albicans* and *C. dubliniensis* strains when grown in SDB and YNB medium at 37°C over 48 h.

	Doubling time (min)	
Strain	SDB	YNB
C. albicans		
SC5314	67 ± 2.6	83 ± 0.6
132A	70 ± 2.9	85 ± 1.2
C. dubliniensis		
CD36	86 ± 2.8	90 ± 2.1
CD57	87 ± 3.3	91 ± 2.3
H004	107 ± 1.7	110 ± 1.0
CS1	112 ± 1.7	114 ± 1.9
P6265	112 ± 2.5	117 ± 3.5

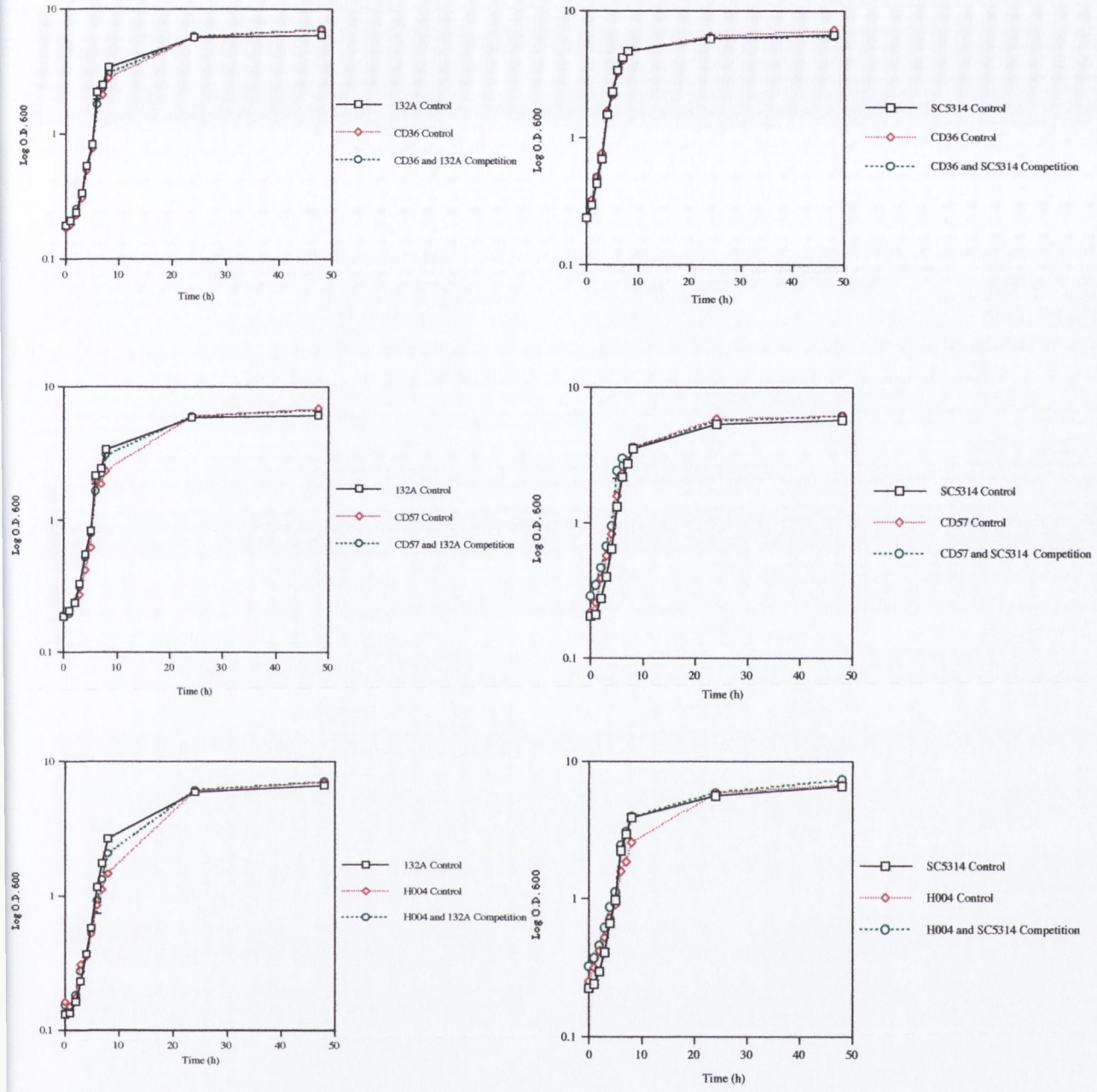


Figure 3.15. Comparison of growth rates between derivatives of *C. dubliniensis* strains CD36, CD57 and H004 (which had been tagged with the molecular cassette containing MPA^R and GFP) and *C. albicans* strains SC5314 and 132A when grown alone and in co-culture in YNB medium at 37°C over 48 h. Aliquots were taken at t = 0,1,2,3,4,5,6,7,8,24 and 48 h and measured spectrophotometrically.

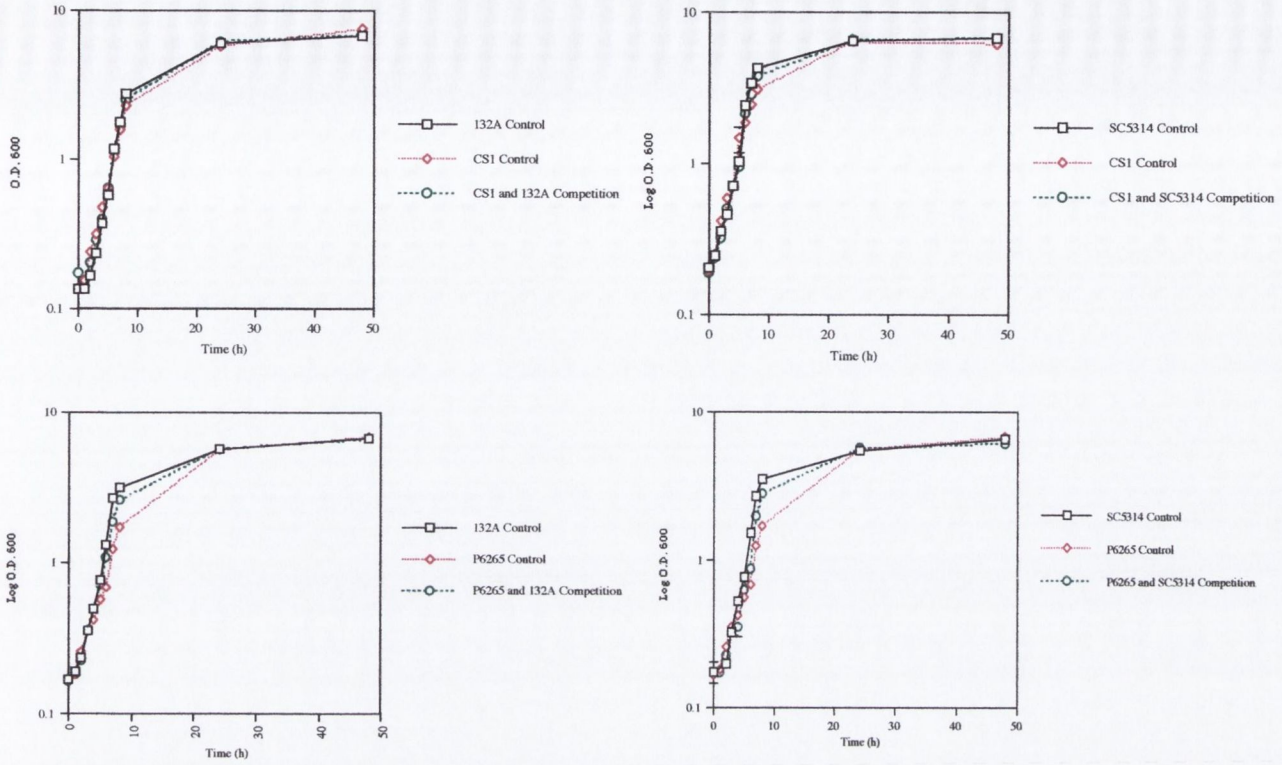


Figure 3.16. Comparison of growth rates between derivatives of *C. dubliniensis* strains CS1 and P6265 (which had been tagged with the molecular cassette containing MPA^R and GFP) and *C. albicans* strains SC5314 and 132A when grown alone and in co-culture in YNB medium at 37°C over 48 h. Aliquots were taken at t = 0,1,2,3,4,5,6,7,8,24 and 48 h and measured spectrophotometrically.

observed in Table 3.4 there is a significant difference ($P < 0.001$) in the doubling times between *C. albicans* (83-85 ± 1.2 min) and *C. dubliniensis* (90-117 ± 3.5 min) when grown in YNB medium over 48 h as was observed in SDB medium over 48 h.

In order to determine the cfu/ml of each species present at each time point a 100 µl aliquot was plated out onto YNB medium. In the case of co-cultures the proportion of each species present was determined by replica plating onto MPA containing medium. Under these experimental conditions the cell density of each species increased during the 48 h incubation period. In the majority of experiments when *C. dubliniensis* and *C. albicans* strains were grown alone in separate flasks similar levels of both species were found after 48 h. However, in all pairings examined, the proportion of *C. dubliniensis* cfu/ml present at 48 h was less than *C. albicans*. In the case of *C. dubliniensis* derivative CD36 (M/G) and *C. albicans* strain 132A the proportions of both species present at 48 h when co-cultured were found to be similar with the difference between the two species 5 x 10^6 cfu/ml. When *C. dubliniensis* derivative CD36 (M/G) was co-cultured with *C. albicans* strain SC5314 the difference between the two species was 1.5 x 10^7 cfu/ml after 48 h. The reason for this difference is most likely because *C. albicans* strain 132A (85 ± 1.2 min) has a doubling time more similar to *C. dubliniensis* derivative CD36 (M/G) (90 ± 2.1 min), than *C. albicans* strain SC5314 (83 ± 0.6 min) (Table 3.4). Over earlier timepoints namely t=2 to 8 h *C. dubliniensis* is present in lower cell densities, which is likely to be due to its slower doubling time. Strains which showed the slowest doubling times showed the lowest cell densities. The results obtained for the levels of *C. dubliniensis* when grown in the same flask for a continuous period of 48 differ from those when the culture was diluted out in SDB medium above (Figs. 3.17 and 3.18). The results in Figs. 3.17 and 3.18 show that when *C. dubliniensis* and *C. albicans* are co-inoculated and examined in the same tube over 48 h that *C. dubliniensis* is present after 48 h in relatively comparable levels to *C. albicans* for *C. dubliniensis* strain CD36 (genotype I) and which showed the fastest doubling time for *C. dubliniensis* strains tested. The other *C. dubliniensis* derivatives showed lower cell densities, with P6265 (M/G) showing the lowest cell density which correlates with it having the slowest doubling time. To confirm that tagging the strains had no negative effect on the experiments, a reverse experiment was performed as before where tagged *C. albicans* strains

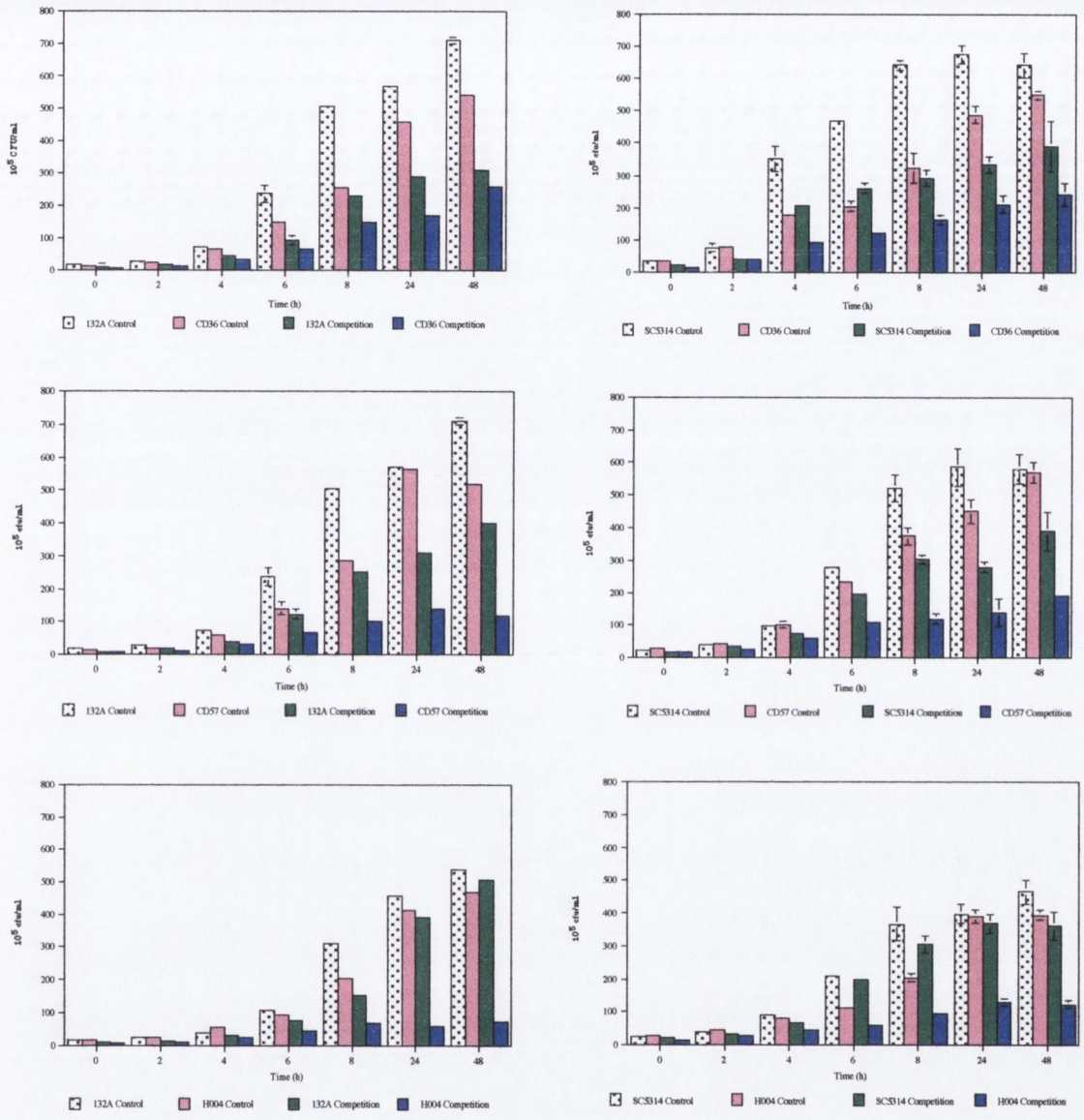


Figure 3.17. Graphs showing biomass concentrations of *C. albicans* strains (SC5314 and 132A) and *C. dubliniensis* derivatives CD36 (M/G), CD57 (M/G) and H004 (M/G) when grown at 37°C in YNB medium for 48 h alone (Control) and in co-culture (Competition) together. All *C. dubliniensis* strains were tagged with the molecular marker cassette

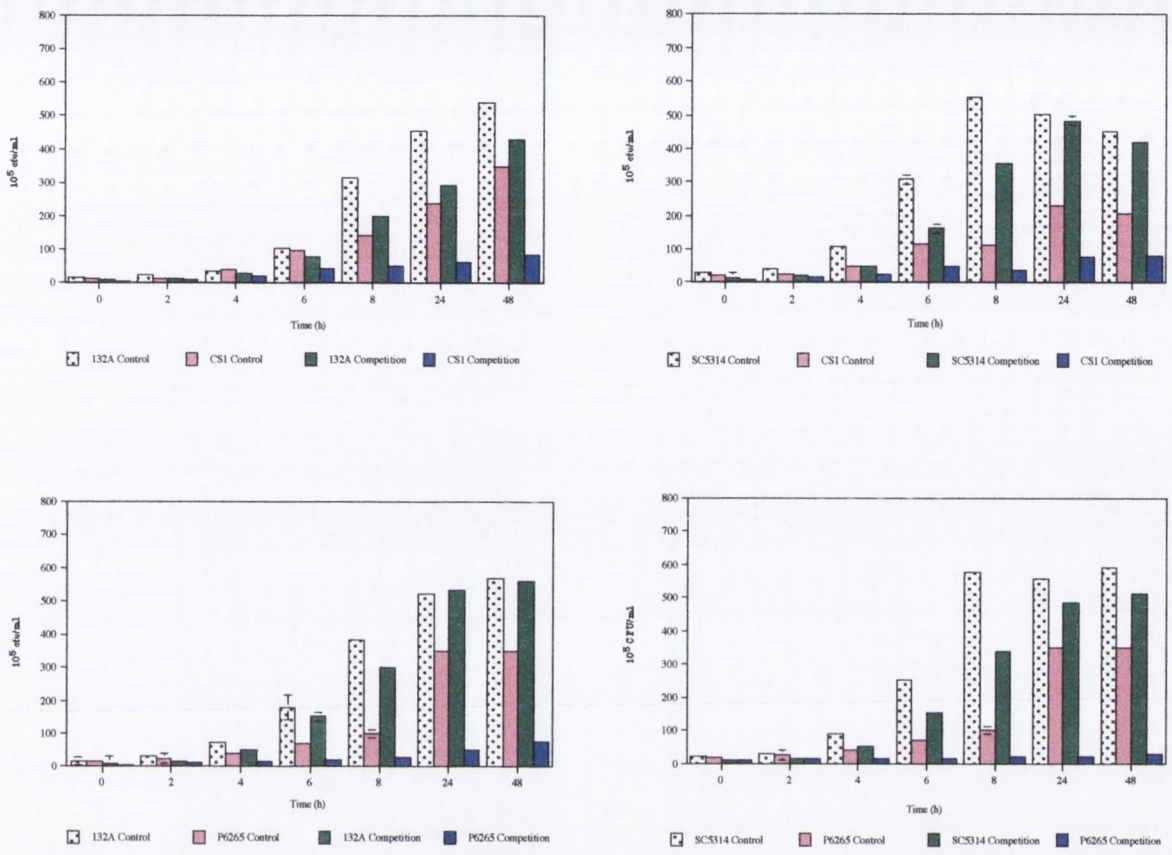
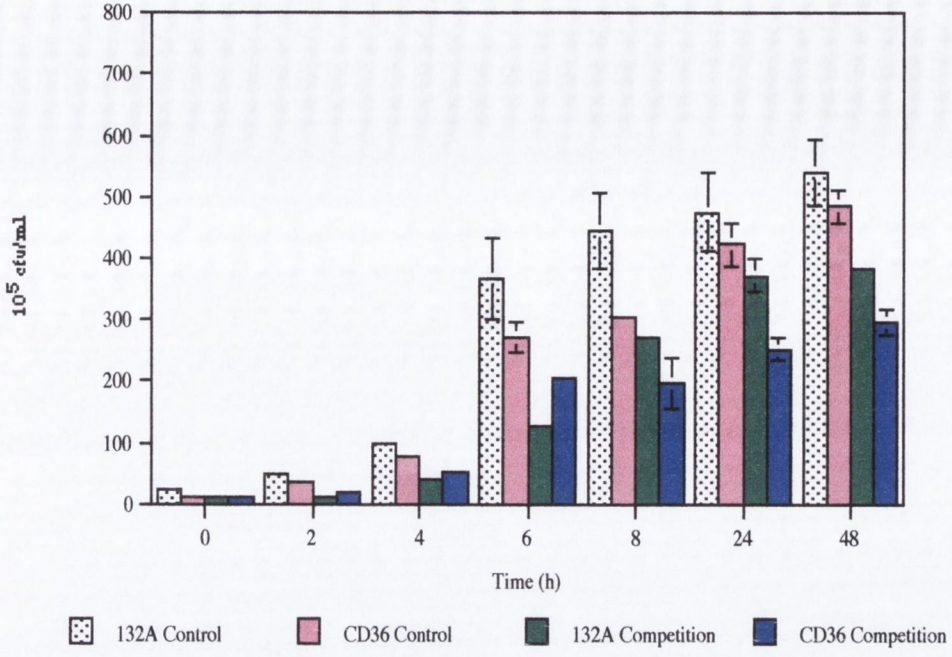


Figure 3.18. Graphs showing biomass concentrations of *C. albicans* strains (SC5314 and 132A) and *C. dubliniensis* derivatives CS1 (M/G) and P6265 (M/G) when grown at 37°C in YNB medium for 48 h alone (Control) and in co-culture (Competition) together. All *C. dubliniensis* strains were tagged with the molecular marker cassette

were co-inoculated with untagged *C. dubliniensis* strain CD36 (Fig. 3.19). The results obtained were very similar to that found for tagged *C. dubliniensis* when co-inoculated with *C. albicans* thereby confirming that MPA^R was a definitive means of differentiation and that tagging the strains had no negative effect on strains.

To confirm the results obtained in both the YNB and SDB media were due to the nature of the competition between the two species and not due to the conditions used experiments were performed using YNB medium with the lower inoculum over 96 h and diluting the culture into fresh media every day and also in SDB medium in the same flask over 48 h for *C. albicans* strain SC5314 and *C. dubliniensis* derivative CD36 (M/G) which had been tagged with the molecular marker cassette. The results obtained in Fig. 3.20 show that when the graphs are compared with the previous experiments that a similar pattern of results occurred. When grown in YNB medium over 96 h with dilution of the culture every day as was observed in previous results using SDB medium *C. dubliniensis* is present after 96 h however at lower cell densities in comparison with *C. albicans* (Fig. 3.20). In comparison with the results obtained in SDB medium over 96 h, lower cell densities of each species were obtained which is likely to be due to the difference in the content of the media as SDB medium is a nutrient rich medium in comparison with YNB medium.

Also when co-inoculating the two species in SDB medium in the same flask over 48 h a similar pattern of results was obtained when comparing Fig. 3.12 with Fig. 3.20. *Candida dubliniensis* derivative CD36 (M/G) was present at less than half the level of cell density of *C. albicans* strain SC5314 when grown in co-culture over 48 h in SDB medium. On comparison of the results when grown in YNB medium over 48 h it is clear that there is twice the level of cell density of each species when grown in SDB medium which as before is likely due to the nutrient rich nature of SDB medium and the faster doubling times in SDB medium.



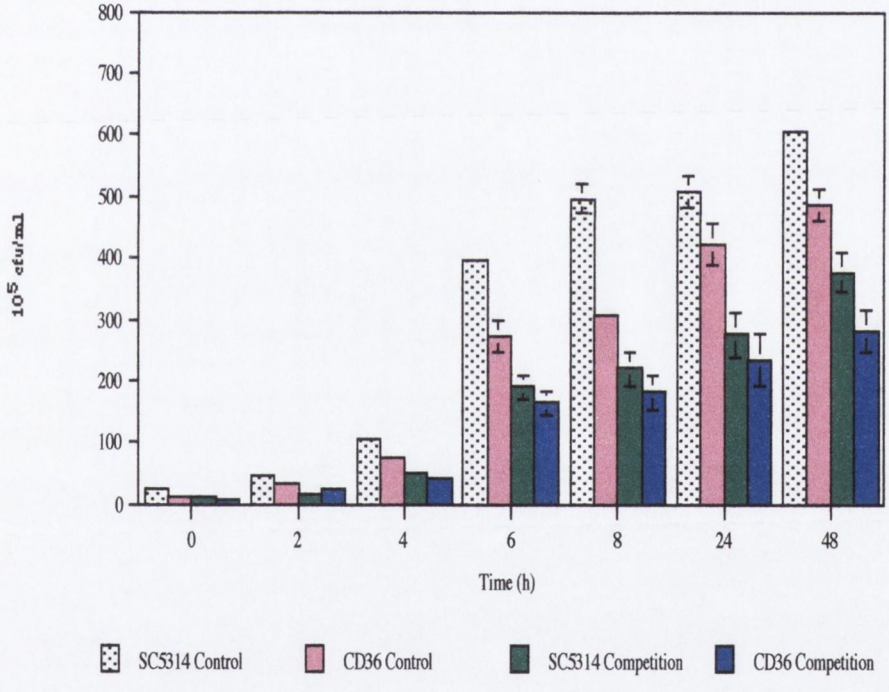


Figure 3.19. Graphs showing biomass concentrations of derivatives of *C. albicans* strains SC5314 and 132A which had been tagged with the molecular marker cassette when examined in co-culture with *C. dubliniensis* strain CD36 when grown in YNB medium over 48 h at 37°C.

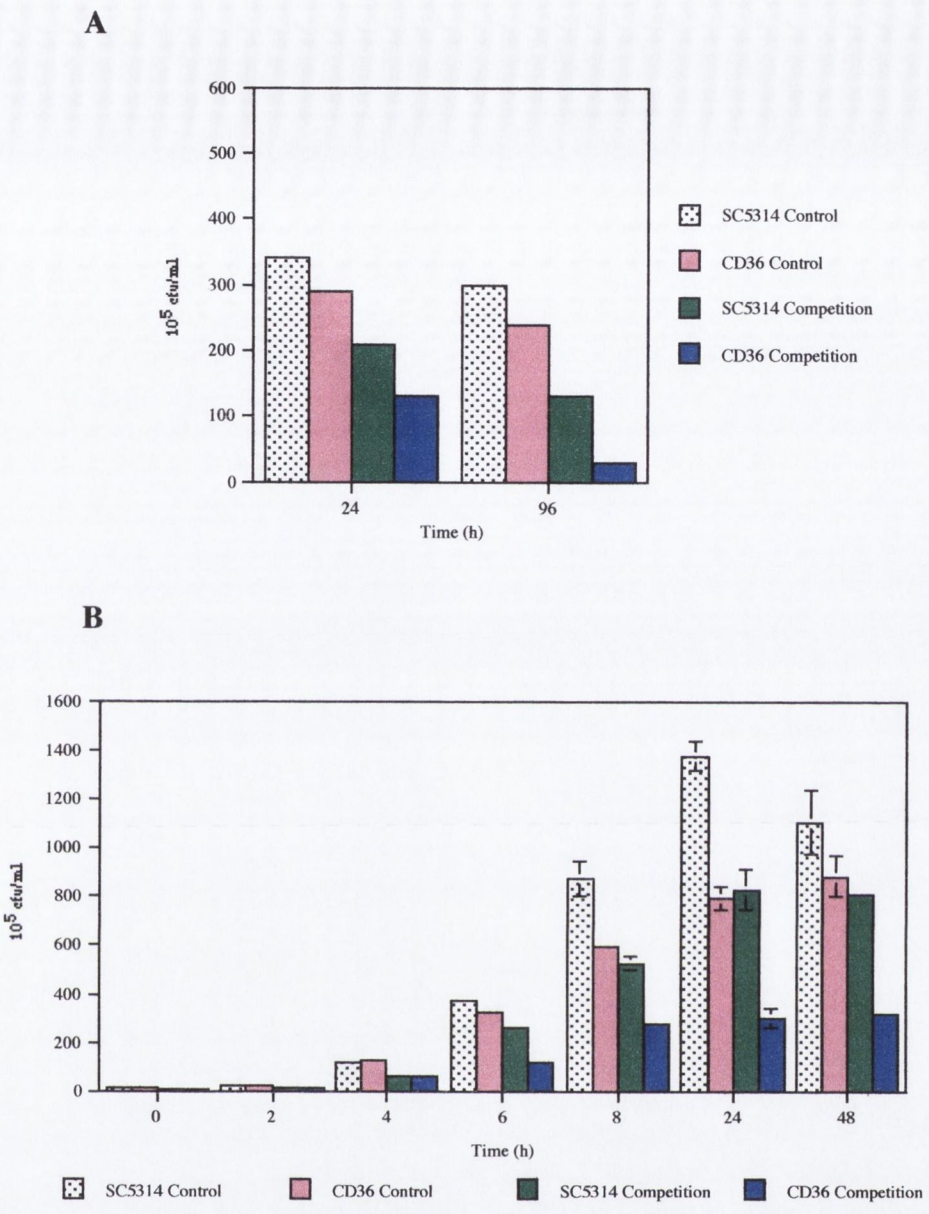


Figure 3.20. Graphs showing biomass concentrations of *C. albicans* strain SC5314 and a derivative of *C. dubliniensis* strain CD36 (which had been tagged with the molecular marker cassette) when grown in (A) YNB medium over 96 h and (B) SDB medium over 48 h.

3.4 Discussion

3.4.1 Investigation into the competitive interactions between *C. albicans* and *C. dubliniensis* in mixed cultures

Little is known about interspecies interactions between *C. albicans* and *C. dubliniensis*, yet these species have been frequently isolated concomitantly from the oral cavities of immunocompromised patients. The mouth is a diverse habitat containing soft mucousal tissues as well as hard tissue and junctures between them, as well as being warm, moist and bathed in nutrients which provides a suitable environment for the proliferation of mixed species (Gilbert *et al.*, 1997; Marcotte & Lavoie, 1998). Factors which have been reported to have an effect on the retention and distribution of oral *Candida* species are acidic pH, sucrose or glucose rich diets and cell surface hydrophobicity, all which enhance adhesion (Lopez-Ribot *et al.*, 1998; Webb *et al.*, 1998). The human oral cavity is known to be a common site from which both *C. albicans* and *C. dubliniensis* can be recovered. However, while *C. albicans* is commonly identified as a member of the human oral flora *C. dubliniensis* is only rarely found in the oral cavity of normal healthy individuals and when it is found it is usually found in combination with *C. albicans*. One possible explanation for these apparently low levels of colonisation of humans with *C. dubliniensis* is that it is out-competed by *C. albicans*.

As already described in Chapter 1, *C. albicans* and *C. dubliniensis* are very closely related and are very difficult to distinguish from one another. In the study by Kirkpatrick *et al.* the proportion of *C. albicans* and *C. dubliniensis* at specific time points in cultures containing both species was determined by plating onto CHROMagar Candida medium (Kirkpatrick *et al.*, 2000). While this medium is useful in discriminating between *C. albicans* and *C. dubliniensis* in fresh clinical samples the unique dark green colour of *C. dubliniensis* can be lost following subculture and storage of *C. dubliniensis* strains. Phenotypic switching of strains on this medium also causes problems when mixed cultures are used as difficulty is incurred in determining which species switch colonies belong to. In preliminary studies, attempts to identify *C. albicans* and *C. dubliniensis* grown in mixed culture on CHROMagar Candida medium, Pal's and Staib agar and medium containing TTC failed. Therefore in order to confirm the data from the Kirkpatrick *et al.* study (Kirkpatrick *et al.*, 2000) representative *C. albicans* and *C. dubliniensis* strains were tagged with a molecular cassette

to facilitate discrimination between the two species. In this study competitive interactions between *C. albicans* and *C. dubliniensis* were examined using strains containing two molecular markers mycophenolic acid resistance (MPA^R) and green fluorescent protein (GFP). *Candida dubliniensis* and *C. albicans* strains were tagged with the genes conferring mycophenolic acid resistance and green fluorescence, which allowed differentiation between *C. albicans* and *C. dubliniensis* when grown in competition with one another, by comparing the numbers of cfu/ml of each species when grown on MPA-containing medium and comparing the results against drug-free medium. In all strains examined the cassette was found to have inserted more than once, however this had no apparent effect on the growth of strains.

The results from this study clearly show that *C. dubliniensis* isolates grow more slowly than *C. albicans in vitro* ant that tagging strains with the molecular markers allowed definitive differentiation between *C. albicans* and *C. dubliniensis* without any adverse effect on the growth of either species.

3.4.1.1 Examination of the comparative growth of *C. albicans* and *C. dubliniensis* in Sabouraud liquid broth modified antibiotic medium 13 (SDB) at 37°C over 96 h

In experiments replicating the conditions used by Kirkpatrick *et al.*, (i.e. 37°C in SDB medium over 96 h), tagged strains of *C. dubliniensis* were always present in co-culture experiments with *C. albicans* after 96 h, however, they were always present at lower cell densities (Figs. 3.12 and 3.13) (Kirkpatrick *et al.*, 2000). These data while similar to those of Kirkpatrick *et al.* differ in that *C. dubliniensis* was not completely out-competed in any of the experiment performed, while in the study by Kirkpatrick *et al.* nine out of eleven *C. dubliniensis* strains disappeared completely from the culture by 96 h of growth (Kirkpatrick *et al.*, 2000). The reason for this discrepancy in the data is possibly due to an inability to effectively discriminate between *C. albicans* and *C dubliniensis* on CHROMagar Candida medium due to overcrowding of colonies on plates. When the growth rates of representative strains belonging to both species were compared the results clearly show that the five *C. dubliniensis* isolates examined have significantly longer mean doubling times than *C. albicans* (Table 3.4). Vilela *et al.* have also shown that *C. dubliniensis* has a lower growth

rate than *C. albicans* when examined in four commercially available culture media at 37°C over 48 h (Vilela *et al.*, 2002).

3.4.1.2 Examination of the comparative growth of *C. albicans* and *C. dubliniensis* in Yeast Nitrogen Base medium (YNB) at 37°C over 48 h

In experiments performed using a more defined medium (YNB) in which the growth of each strain was determined at t= 0, 2, 4, 6, 8, 24 and 48 h intervals without dilution, it was confirmed that *C. albicans* can outgrow *C. dubliniensis* although the levels of cell density of *C. dubliniensis* strains continue to increase during growth. The results from this part of the present study show higher levels of cell density of *C. dubliniensis* in comparison with growth in SDB medium. This is likely to be due to the fact that this experiment was performed in the same flask and was not diluted into fresh medium every 24 h. In YNB medium a difference in the level of cell density of the two species was observed and for *C. dubliniensis* derivative CD36 (M/G) half the level of *C. dubliniensis* was detected compared to *C. albicans*. However, strain CD36 (genotype I) has a doubling time of 90 min which is close to what was observed for *C. albicans* strains SC5314 (83 min) and 132A (85 min). The other *C. dubliniensis* strains (genotype II and III) had slower doubling times and therefore lower cell densities were present after 48 h in YNB medium (Figs. 3.16 and 3.17).

The results from this part of the present study showed that *C. dubliniensis* strains have a slower growth rate than *C. albicans.* Also *C. dubliniensis* genotype II (H004) and III (CS1 and P6265) isolates had slower doubling times in comparison with the *C. dubliniensis* genotype I isolate (CD36). The results also contradict what Kirkpatrick *et al.*, found as *C. dubliniensis* was detected after both 96 h in SDB medium at 37°C and also after 48 h in the same flask in YNB medium (Kirkpatrick *et al.*, 2000). The results therefore show that when grown in competition with one another that *C. albicans* does not completely out compete *C. dubliniensis* as *C. dubliniensis* was always present at the end of each experiment.

Chapter 4

Examination of the kinetics of hyphal production in *C. albicans* and *C. dubliniensis*

4.1 Introduction

4.1.1 Morphology

Candida albicans and *C. dubliniensis* are the only *Candida* species that can produce germ tubes, however, many *Candida* species can exist in both yeast, pseudohyphal and hyphal forms. Changes in environmental conditions such as pH and temperature, the presence of serum, CO_2 concentration, carbohydrates, amino acids, salts, growth within a matrix and starvation induce the transition from yeast to hyphae *in vitro* (Ernst, 2000). This pleiomorphism is thought to contribute to colonisation and dissemination within host tissues and thereby to promote infection and has been considered a major requirement for virulence (Leberer *et al.*, 1997; Lo *et al.*, 1997; Saville *et al.*, 2003). It has been proposed that the yeast form is likely to play a major role in dissemination in the bloodstream and early colonisation but that the hyphal form is required for tissue penetration during the early stages of infection, as hyphae grow by apical tip extension and can generate significant pressure at the tip, which allows penetration (Gow *et al.*, 2002).

Hyphae have been shown to induce phagocytosis by endothelial cells which has been suggested as a potential strategy of *C. albicans* to escape from the bloodstream (Phan *et al.*, 2000; Zink *et al.*, 1996). Hyphal cells have also been shown to adhere more strongly than yeast cells to endothelial cells possibly due to the expression of specific *ALS* adhesins (Hoyer *et al.*, 2001) furthermore, when *C. albicans* cells are phagocytosed by macrophages, hyphae are produced which penetrate the surrounding membrane resulting in death of the macrophage and escape of the fungus (Borg-Von Zepelin *et al.*, 1998). The transition from a yeast to a hyphal form may also alter the host inflammatory response as the higher expression of superoxide dismutases which convert damaging superoxide radicals, a key form of reactive oxygen species, to less damaging hydrogen peroxide that can be converted into water by catalase action may counteract the phagocytic burst of phagocytic cells (Nantel *et al.*, 2002). It has been shown that *Candida* organisms locked into a single morphology uniformly have reduced virulence in animal models of candidiasis (Bahn & Sundstrom, 2001; Lo *et al.*, 1997; Murad *et al.*, 2001), suggesting that the ability to produce hyphae is a major contributor to the success of *C. albicans* as a pathogen in comparison with other yeast species. It is important to emphasise that it is not just the ability of cells to switch between a

yeast and hyphal phase that is responsible for pathogenesis but rather the entire transcriptional programme associated with the transition (Odds *et al.*, 2003).

Preliminary analysis of the kinetics of hyphal production by *C. albicans* and *C. dubliniensis* under a limited set of induction conditions *in vitro* has been examined and suggests that hyphal production is less efficient in *C. dubliniensis* in comparison with *C. albicans* (Gilfillan *et al.*, 1998). Vilela *et al.* have demonstrated that mice injected with *C. dubliniensis* intravenously have a higher survival rate than mice injected with *C. albicans* and also that *C. dubliniensis* remains mostly in the yeast form in infected organs whereas in contrast, *C. albicans* changed to the mycelial form (Vilela *et al.*, 2002).

4.1.2 Adhesion

One of the most important virulence factors of *C. albicans* is its ability to adhere to a wide range of human tissues which also suggests the presence of a wide range of surface adhesins. *Candida albicans* is unique, as unlike other oral pathogens it possesses the ability to invade cornified layers of stratified squamous epithelium of the tongue, buccal surfaces, hard and soft palate, and oesophagus (Mandell *et al.*, 2000). Adhesion is a critical step for colonisation and establishment of infection. *Candida albicans* adheres to epithelial cells, endothelial cells, soluble factors, extracellular matrix, and inert materials implanted in the body of the host (Chaffin *et al.*, 1998). However, the molecular basis of the adhesion of *C. albicans* to these surface is poorly understood. A limited number of putative adhesins have been identified and one of the most interesting of these is Hyphal Wall Protein 1 (Hwp1) (Staab *et al.*, 1996).

4.1.2.1 Hyphal wall protein (Hwp1)

The hyphal wall protein was discovered in a genetic screen for developmentally regulated genes at the mRNA level and in an immunological screen for hyphal and germ tube-specific proteins. This protein is expressed solely on the surface of *C. albicans* hyphae and germ tubes and mediates attachment to host epithelial cells via a transglutamination reaction catalysed by host transglutaminase enzymes. Unlike other adhesins such as Als1, Als5 and Epa1, Hwp1 contains a Kex2 endoproteinase cleavage site after the signal peptide (Sundstrom, 2002). The NH_2-terminal domain of the protein that attaches to buccal epithelial

cells is rich in proline and glutamine and shows similarity with mammalian transglutaminase substrates present on epithelial cells (Staab *et al.*, 1996). In stratified squamous epithelium, transglutaminases mediate the formation of an innate host barrier defense by cross-linking substrate proteins such as small proline rich proteins, involucrin, loricrin and keratins, thereby producing an insoluble cornified envelope (Steinert *et al.*, 1998). Transglutaminases (Tgases) cross-link proteins through the formation of isodipeptide bonds between glutamine and lysine residues on substrate proteins. Due to the apparent similarity between Hwp1 and these host Tgases, it has been proposed that the Tgases covalently cross link the Hwp1 on the candidal hyphal surface with host substrate proteins resulting in stable attachment between *C. albicans* hyphae and the host epithelial tissues. This novel method of attachment is a highly stable one unaffected by treatment with heat and sodium dodecyl sulfate and is therefore unlike most other microbial adhesins that operate through weaker lectin-like or hydrophobic interactions (Sundstrom, 1999).

4.1.2.2 Sequence analysis and expression of Hwp1

The hyphal wall protein is a member of a class of proteins termed glycosylphosphatidylinositol-dependent cell wall proteins (GPI-CWP). These proteins share many common features including an N-terminal signal peptide and C-terminal features that mediate glycosylphosphatidylinositol (GPI) membrane anchor addition (Sundstrom, 2002).

Gene disruption of *HWP1* in *C. albicans* reduces virulence, indicating that Hwp1 is an important virulence factor in *C. albicans* (Sundstrom, 2002). Surface localisation, expression in host tissues and importance in systemic candidiasis have been rigorously demonstrated for Hwp1 (Staab *et al.*, 1996; Sundstrom, 2002). This suggests that Hwp1 is likely to contribute to the efficient colonisation and establishment of infection and possibly prevent dislodgement of *C. albicans* from the cornified surface layer of the stratified squamous epithelium by mucousal secretion of saliva. The similarity of Hwp1 to small proline-rich proteins that are expressed only in stratified squamous epithelium suggests that Hwp1 may also be more important for mucousal than systemic candidiasis. Consistent with this idea, deletion mutants lacking Hwp1 were no longer a substrate for human transglutaminase, were less adherent to human buccal epithelial cells and were attenuated in virulence in a mouse systemic model of candidiasis (Sundstrom, 2002).

The purpose of this part of the present study was to examine the dynamics of hyphal induction in both species under a wide range of conditions previously shown to induce the production of hyphae in *C. albicans*. This was undertaken as previous studies have suggested that *C. dubliniensis* may not produce hyphae as efficiently as *C. albicans in vitro* or *in vivo* using a limited number of strains and conditions (Gilfillan *et al.*, 1998; Vilela *et al.*, 2002). A comparison was also performed between both the sequences and the expression levels of *HWP1* in *C. albicans* and *C. dubliniensis* under a wide range of media which are known to induce hyphae and which are examined in section 4.1.1. Finally, since the *C. dubliniensis* genome project was commenced during this project a survey of the *C. dubliniensis* genome was performed to determine the presence in the genome of genes implicated in the induction of hyphae in *C. albicans*.

4.2 Materials and Methods

4.2.1 *Candida* isolates

The *Candida* strains used in experiments involving the induction of germ tubes and hyphae are listed in Table 3.1. Four strains of *C. albicans* (SC5314, 132A, JP14CA and 52.1) and 11 strains of *C. dubliniensis* (CD36, CD96.34, CD541, CD539, CD57, H004, CD519, CD514, P6265, CS1 and P7718) belonging to the four genotypes were examined.

4.2.2 Induction of germ tubes and hyphae

Production of hyphae in *Candida* isolates was induced by inoculation of cells in the yeast phase (grown for 18 h at 37°C in YPD) into media that promote the yeast-hypha transition (Table 4.1). Cells were washed twice in ultra pure water and inoculated to a cell density of 2 x 10^6 cfu/ml in 20 ml of each medium and incubated at 37°C. In the case of *N*-acetylglucosamine-yeast-nitrogen base-proline medium (NYP), the cells were incubated in the presence of 5% (v/v) CO_2. The pH/temperature shift induction of hyphae (Buffo *et al.*, 1984) was performed by inoculating 25 ml of supplemented Lee's medium, pH 6.5, 37°C with 0.5 ml of cells grown to stationary phase in supplemented Lee's medium, pH 4.5, 25°C (Lee *et al.*, 1975). The percentage of cells which were in a germ tube or hyphal phase were determined every hour from t=0 h to t=6 h and then again at t=24 h by placing 10 µl of cell suspension onto a microscope slide (Sigma-Aldrich) and counting cells using a Nikon Eclipse 600 microscope (Nikon Europe B.V., Badhoevedorp, Holland). One hundred cells were counted in triplicate and the percentage of cells, which were hyphal, were determined from this.

The induction of hyphae on solid medium was determined by streaking the *Candida* strains out on Spider medium (1% (w/v) nutrient broth, 1% (w/v) mannitol, 0.2% (w/v) K_2HPO_4; 1.35% (w/v) agar) and incubating at 37°C for 5 days. Strains were monitored for the production of hyphae every day for each of the 5 days as hyphae have previously been shown to develop after 5 days (Liu *et al.*, 1994). *Candida* were also streaked out onto Pal's and Staib agar and incubated at 30°C for 3 days. Strains were monitored for the production

Table 4.1. Media used that induce hyphal production.

Type of Medium	Medium	Supplements	Temperature and conditions
Liquid	RPMI-1640[a]	None	37°C
Liquid	RPMI-1640[a]	10% (v/v) newborn calf serum	37°C
Liquid	YNB[b]	2% (w/v) glucose	37°C
Liquid	YNB[b]	2% glucose and10% (v/v) newborn calf serum	37°C
Liquid	NYP[c]	None	37°C
Liquid	Water	10% (v/v) newborn calf serum	37°C
Liquid	Lee's Medium	Arginine, biotin and trace metals[d]	pH/Temperature shift[e]
Liquid	M-199[f]	Hanks salts, 25 mM HEPES buffer and L-glutamine	37°C
Liquid	M-199	Hanks salts, 25 mM HEPES buffer and L-glutamine and 10% (v/v) newborn calf serum	37°C
Solid	Spider medium	None	37°C
Solid	Pal's	None	30°C
Solis	Staib	None	30°C
Solid	YPS[g]	None	25°C

[a]RPMI-1640 medium (Sigma-Aldrich) was buffered with 0.165 M Morpholinepropanesulphonic acid (MOPS) buffer (Sigma-Aldrich) which was adjusted to pH 7.0 using 10 N NaOH

[b]Yeast Nitrogen Base (Sigma-Aldrich)

[c]*N*-acetylglucosamine-yeast-nitrogen base-proline medium (Moran *et al.*, 1998)

[d]400 mM arginine, 0.001% (w/v) biotin and trace metals (0.2 mM $ZnSO_4$, 0.25 mM $CuSO_4$, 1 mM $FeCL_3$, 1 mM $MgCl_2$, 1 mM $CaCl_2$)

[e] The pH/temperature shift induction of hyphae (Buffo *et al.*, 1984) was performed by inoculating 25 ml of supplemented Lee's medium, pH 6.5, 37°C with 0.5 ml of cells grown to stationary phase in supplemented Lee's medium, pH 4.5, 25°C (Lee *et al.*, 1975).

[f]M-199 medium with Hanks salts, 25 mM HEPES buffer and L-glutamine (Gibco BRL Life Technologies)

[g]Yeast extract-peptone-sucrose

of hyphae every day for each of the 3 days, as previous studies have shown that after 3 days it is more difficult to discriminate the differences between the two species (Al Mosaid *et al.*, 2003; Al Mosaid *et al.*, 2001). Induction of hyphae was also examined on Yeast extract-peptone-sucrose (YPS) medium by plating a suspension of approximately 100 cells from a culture grown overnight for 18 h, onto the surface of this medium and incubated at 25°C for five days. Production of hyphae was also examined using embedded conditions using a sandwich of YPS medium. Firstly 15 ml of agar was poured into a petri dish and left to solidify. Then a suspension of approximately 100 cells from a culture grown overnight for 18 h was plated on top of this and left to dry for 30 min. Finally another 15 ml of YPS agar was poured on top of this and incubated at 25°C for five days, resulting in a layer of cells sandwiched between two solidified layers of agar (Douglas *et al.*, 1999). Five days was chosen as previous studies by Douglas *et al.*, have shown that there was a significant difference in hyphal production between strains grown on the surface and sandwiched between YPS agar after 5 days (Douglas *et al.*, 1999).

4.2.3 PCR amplification of *C. dubliniensis HWP1*

For the amplification of *CdHWP1* the primer set HWP1F/HWP1R (with *Eco*R1 restriction sites included at the respective 5' ends; Table 4.2) was designed using the nucleotide sequence of the *CaHWP1* (Genbank accession no. U64206). Reactions were 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 of template DNA. The PCR cycling conditions were carried out in a Thermo Hybaid MBS thermal cycler with 30 cycles of denaturation for 1 min at 94°C, primer annealing for 1 min at 50°C, and extension for 2 min at 72°C; this was followed by a final incubation at 72°C for 10 min. To examine the upstream region of *CdHWP1,* primers were designed based on the gene upstream (5') from *HWP1* in *C. albicans,* namely *CaAPL6,* and the intervening region between the two genes.

Table 4.2. Nucleotide sequences of primers used in this study.

Primer	Sequence[a](5'-3')	Restriction site	Nucleotide coordinates[b]	Reference
HWP1F	CGGAATTCCGATGAGATTATCAACTGCT	*Eco*RI	+1 to +18	This study
HWP1R	CGGAATTCCGAATTAGATCAAGAATGCAG	*Eco*RI	+1908 to +1889	This study
APL6(1)F	CGGAATTCCGAATACAAGATGTTTC	*Eco*RI	+1678 to +1693	This study
APL6(2)F	CGGAATTCCATCCTCAACATCAAG	*Eco*RI	+1988 to +2003	This study
IV1F	CGGAATTCCGGATCAAGATTTATG	*Eco*RI	475 to 489[c]	This study
IV2F	CGGAATTCCAACCATTGACTTG	*Eco*RI	1423 to 1436[c]	This study
HWP1(2)R	GGAATTCTAGGATTGTCACAAGG	*Eco*RI	+223 to +210[d]	This study

[a] The restriction endonuclease recognition sites included in primer sequences are underlined.

[b] Position + 1 corresponds to the first base of the ATG translational start codon in each case.

[c] The nucleotide coordinates of the intervening region between two genes (where position 1 corresponds to the first base after the TAA stop codon of the first gene).

[d] The HWP1(2)R primer was designed on the deduced nucleotide sequence of *CdHWP1*.

The primer sets APL6(1)F/HWPI(2)R, APL6(2)/HWP1(2)R, IV1F/HWP1(2)R and IV2F/HWP1(2)R were used to identify the upstream region of *HWP1* (Table 4.2).

4.2.4 Cloning of *HWP1*

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

4.2.5 Statistical analysis

Statistical analysis was carried out using the two sample *t*-test assuming unequal variances. *P* values of <0.05 were considered significant.

4.3 Results

4.3.1 Induction of hyphae under selected environmental conditions

The ability to exist in both a yeast and a hyphal phase is thought to be crucial for the virulence of *C. albicans* as it enables it to colonise and disseminate within host tissues thereby promoting infection. Gilfillan *et al.* previously compared hyphal production between *C. albicans* and *C. dubliniensis* under a limited set of induction conditions and found that hyphal production is less efficient in *C. dubliniensis* (Gilfillan *et al.*, 1998). In order to thoroughly investigate the production of hyphae in both *C. albicans* and *C. dubliniensis*, the effects of a number of media known to induce hyphae in *C. albicans* were examined with representative strains of the two species. The media tested induce hyphae production in *C. albicans* as a result of nitrogen starvation, pH and temperature shift, growth within a matrix, presence of serum and CO_2. Several strains of both species were examined (*C. albicans*; SC5314, 132A, 52.1, JP14CA and *C. dubliniensis*; CD36, CD96.34, CD539, CD57, CD541, CS1, CD519, CD514, H004, P6265 and P7718), which are described in Table 3.1. The *C. albicans* strain SC5314 was chosen as it is used as a reference strain throughout the world and 132A was chosen as it is a well characterised oral reference strain. The other two *C. albicans* strains are oral clinical isolates which have not been well characterised. The *C. dubliniensis* strain CD36 was chosen as it is also a reference strain that is used worldwide and the other *C. dubliniensis* strains were chosen as they consist of a broad range of isolates from the four different *C. dubliniensis* genotypes which have previously been characterised (Gee *et al.*, 2002). Production of hyphae was taken to be significant if greater than 50% of cells were observed to be in a germ tube or hyphal phase under each set of conditions tested (Table 4.4). Cells which were in a pseudohyphal phase were not counted.

4.3.1.1 Induction of hyphae in liquid media

Hyphae were induced in each strain by inoculating the hypha-inducing media listed in Table 4.1 with an inoculum of 2×10^6 cfu/ml of yeast cells, which had been grown overnight in YPD medium and washed twice in ultra pure water. The results showed a clearly noticeable difference in the ability of the two species to produce hyphae as shown by the hyphal production time course with representative strains in water with 10% (v/v) serum and

RPMI-1640 medium (Figs. 4.1 and 4.2). In all of the media examined hyphal production was less efficient in *C. dubliniensis* than in *C. albicans* and for some strains of *C. dubliniensis* no significant hyphal production (<10%) was detected at all (Figs. 4.3 and 4.4). Hyphal production following incubation was found to be rapid for *C. albicans* and within 1-2 h 50% or more of cells were in the hyphal phase in water containing 10% (v/v) serum. In RPMI-1640 medium containing 10% (v/v) serum three out of four *C. albicans* strains tested were in a hyphal phase, with strain JP14CA taking 2-3 h before 50% or more cells were in a hyphal phase. In YNB with and without 10% (v/v) serum, Lee's medium and M199 medium containing 10% (v/v) serum it took 2-3 h for greater than 50% of *C. albicans* strains to be in a hyphal phase, whereas the induction was slower in M199 medium without serum and NYP medium 3-4 h, and even slower in RPMI-1640 medium 4-5 h. In all of the media tested strains SC5314 and 132A produced hyphae faster than the two clinical isolates 52.1 and JP14CA.

Eight out of the eleven (73%) *C. dubliniensis* strains tested produced significant amounts of hyphae and 50% or more of cells were in the hyphal phase in water containing 10% (v/v) serum. However, this occurred between 0-1 h for 5 of these strains, which is similar to *C. albicans,* and between 4-5 h for the other 3 strains. In NYP medium induction of hyphae was slower in *C. dubliniensis* than that found for water containing 10% (v/v) serum. Interestingly, strain P7718 and CD57 showed significant amounts of hyphal production in NYP medium (i.e. > 50% between 2-3 h and 3-4 h, respectively). Strain CD96.34 also showed induction of hyphae in NYP medium, however, this only approached 50% after 6 h. In RPMI-1640 medium production of hyphae was only observed for two strains of *C. dubliniensis* (P7718 and CS1). However, the percentage of cells, which were in the hyphal phase was less than 20% after 6 h compared to strains of *C. albicans,* which approached 95% after 6 h (Table 4.3).

In RPMI-1640 medium supplemented with 10% (v/v) serum 7/11 (64%) *C. dubliniensis* strains tested (CS1, CD514, CD57, CD96.34, CD541, H004 and P7718) produced hyphae, however, the amount of hyphal cells present was less than 30%, except for strain CS1 which showed greater than 50% of cells in a hyphal mode of growth after 3 h. This represented an increase in the production of hyphae in comparison with RPMI-1640 medium without serum in which only two out of eleven (18%) strains tested (CS1 and

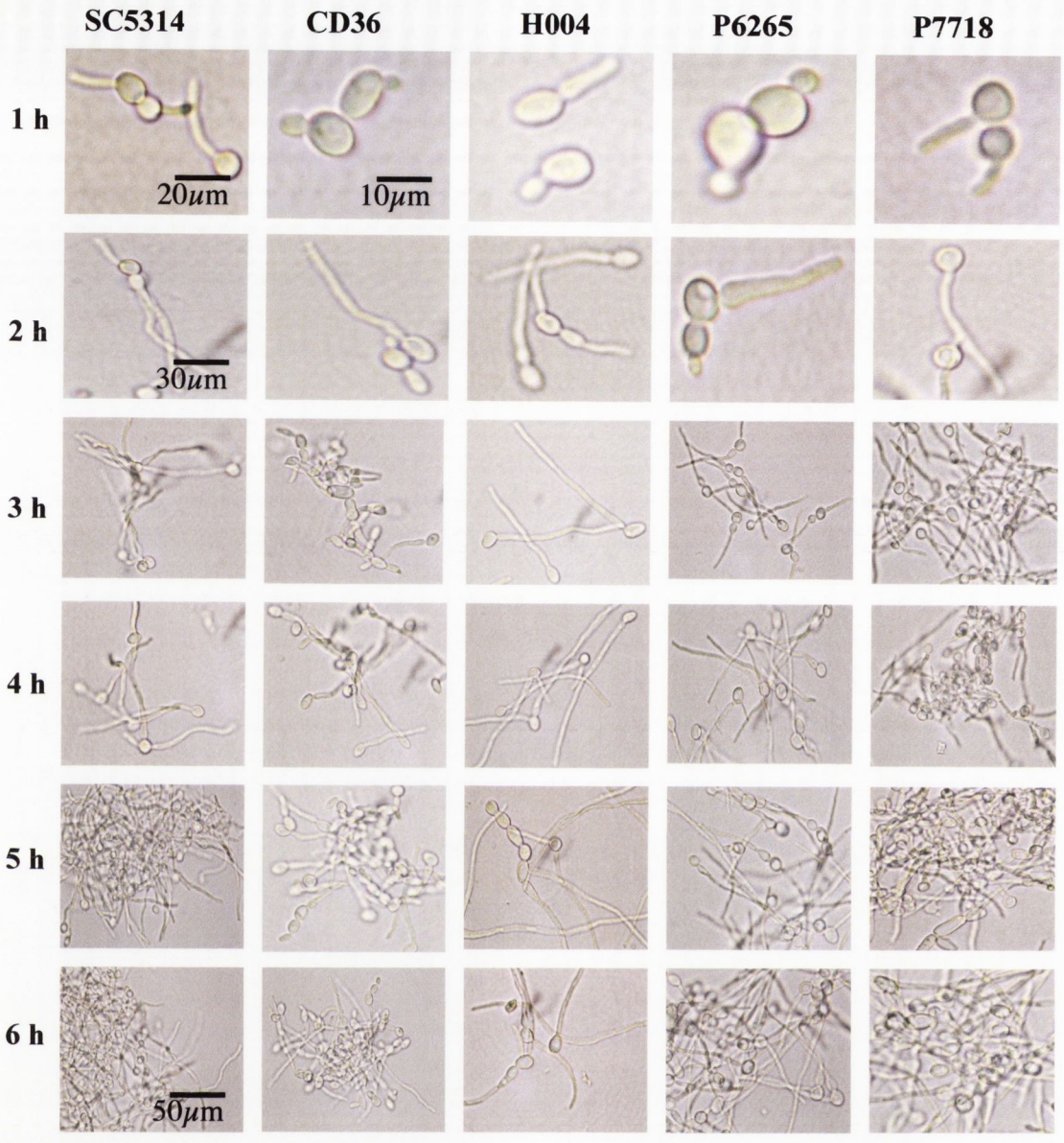


Figure 4.1. Light micrographs of cells of *C. albicans* strain (SC5314) and *C. dubliniensis* strains (CD36, H004, P6265 and P7718) taken over a time course of 6 h at 37°C in water with 10% serum (v/v). The scale bar of 10μm is representative of all light micrographs taken at 1 h for strains CD36, H004, P6265 and P7718, the scale bar of 30μm is representative of light micrographs taken at 2 h for all strains and the scale bar of 50μm is representative of light micrographs taken at 3,4,5 and 6 h for all strains.

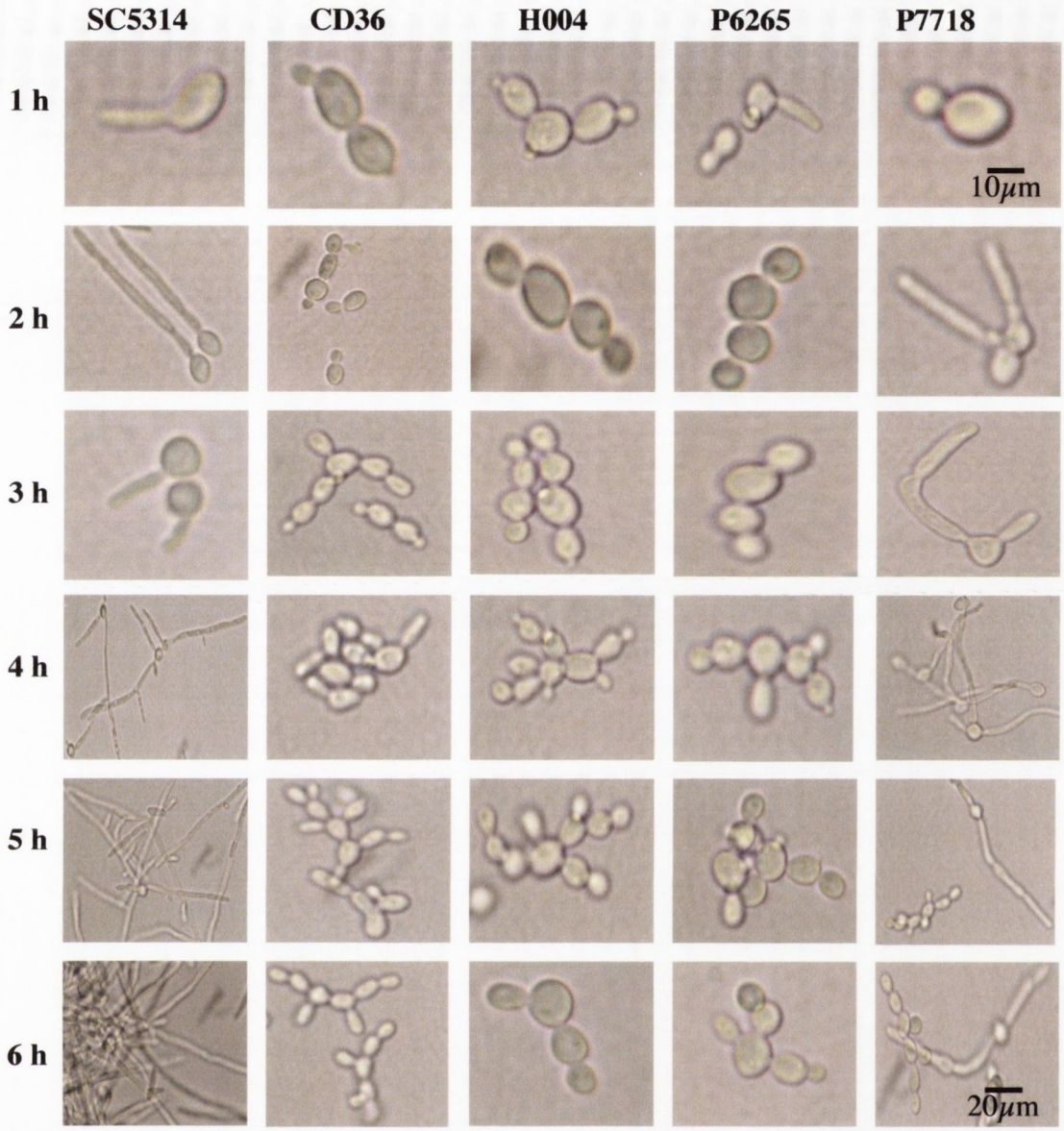


Figure 4.2. Light micrographs of cells of *C. albicans* strain (SC5314) and *C. dubliniensis* strains (CD36, H004, P6265 and P7718) taken over a time course of 6 h at 37°C in RPMI-1640 medium. The scale bar of 10μm is representative of all light micrographs for strains CD36, H004 and P6265 and time 1 h for strains SC5314 and P7718. The scale bar of 20μm is representative of all light micrographs taken at 2-6 h for strains SC5314 and P7718.

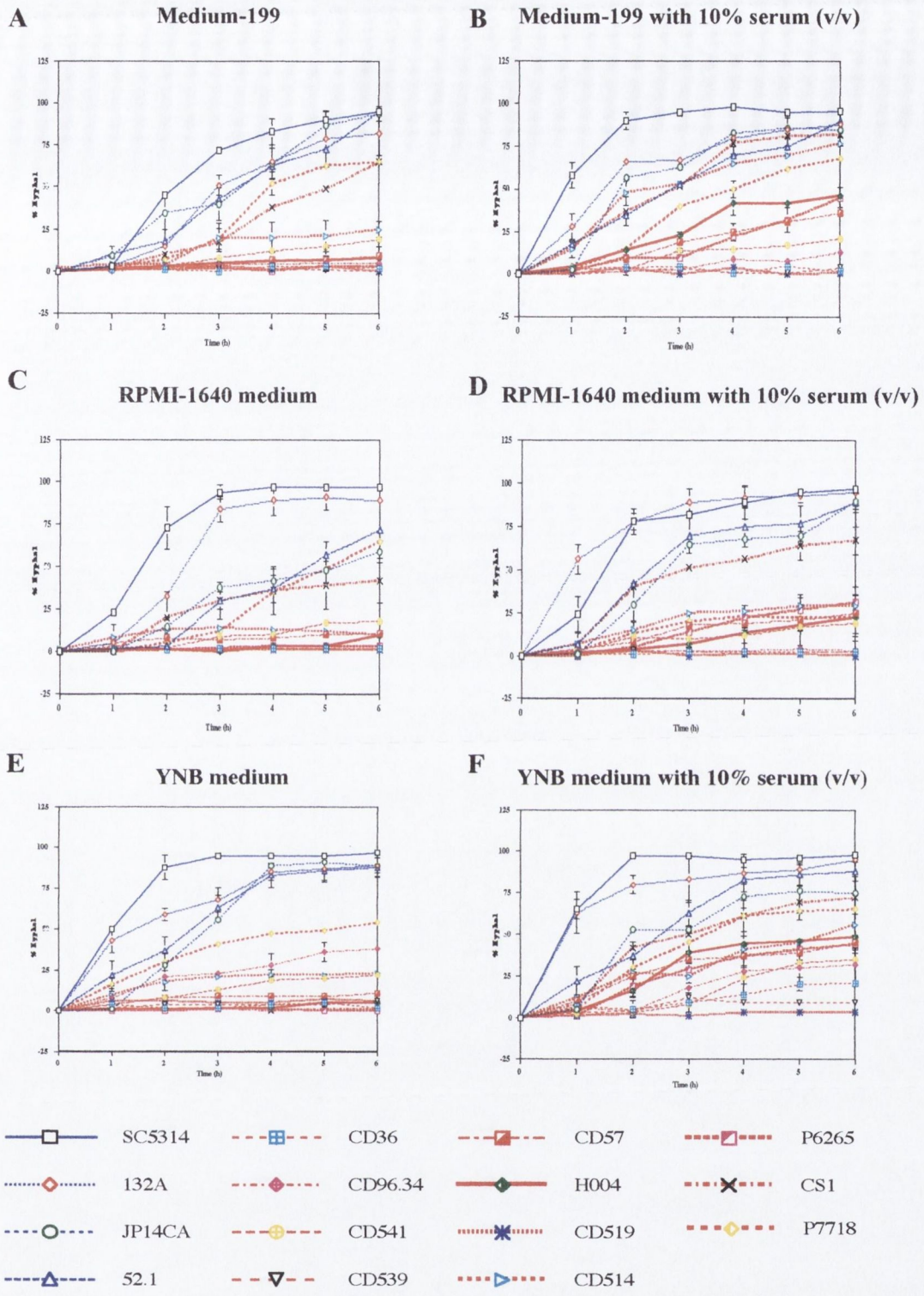


Figure 4.3. Graphs showing the percentage hyphal induction of representative strains of *C. albicans* in blue and *C. dubliniensis* in red over 6 h in hyphal inducing media. (A) M-199 with and (B) without 10% (v/v) newborn calf serum, (C) RPMI-1640 medium with and (D) without 10% (v/v) serum and (E) YNB medium with and (F) without 10% (v/v) serum, respectively.

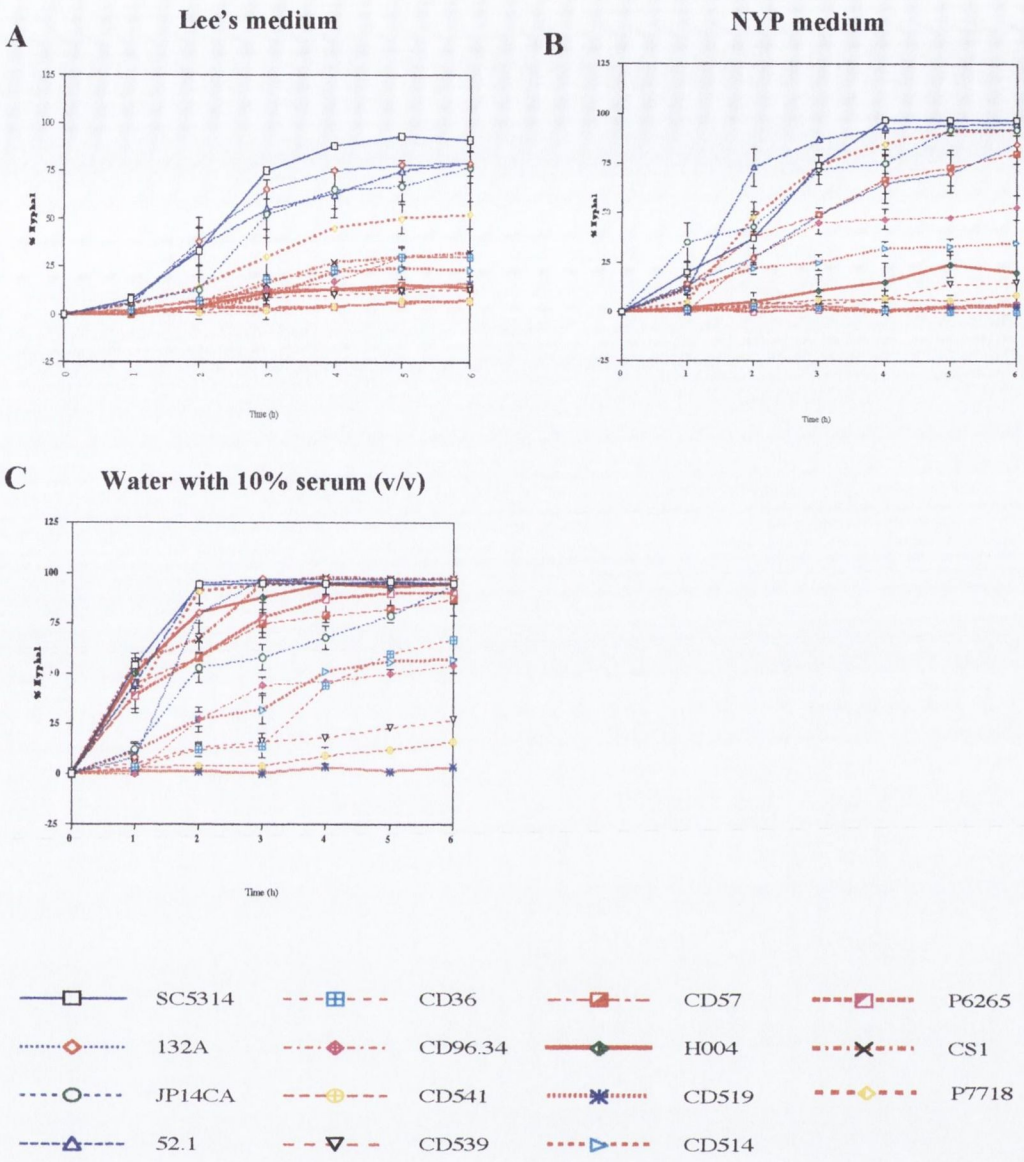


Figure 4.4. Graphs showing the percentage hyphal induction of representative strains of *C. albicans* in blue and *C. dubliniensis* in red over 6 h in hyphal inducing media. (A) Lee's medium under a temperature and pH shift, (B) NYP medium and (C) water with 10% newborn calf serum (v/v), respectively.

Table 4.3. Percentage of *C. albicans* and *C. dubliniensis* strains in which 50% or more of cells were in the hyphal phase following 6 h growth in each of the liquid media examined.

Media	*C. albicans*[a]	*C. dubliniensis*[b]
	% of strains which were ≥ 50% in the hyphal phase of growth after 6 h	
Medium-199	100	18
Medium-199 with 10% newborn calf serum (v/v)	100	27
RPMI-1640 medium	100	9
RPMI-1640 with 10% newborn calf serum (v/v)	100	9
YNB medium	100	9
YNB with 10% newborn calf serum (v/v)	100	27
Water with 10% newborn calf serum (v/v)	100	73
Lee's medium	100	9
NYP medium	100	27

[a] Four strains of *C. albicans* were used in this part of the present study.

[b] Eleven strains of *C. dubliniensis* were used in this part of the present study.

P7718) produced hyphae, and in this case the amount of hyphal cells present only approached greater then 50% for strain P7718 after 5 h and only approached approximately 30% for strain CS1 after 6 h. These results indicate that serum has a significant effect on the induction of hyphae, however, the amount of hyphal production by *C. dubliniensis* clearly does not approach that of *C. albicans*. A similar situation occurred when comparing YNB medium and YNB supplemented with 10% (v/v) serum where five out of eleven *C. dubliniensis* strains tested (P7718, CD96.34, CD514, CD57 and CD541) produced hyphae, with only one strain (P7718) yielding greater than 50% after 5 h. However, when serum was added to the medium ten out of eleven strains tested (CD514, CD57, H004, CD519, P6265, CD36, CD539, CS1, P7718, CD541 and CD96.34) showed hyphal production but only three strains P7718, CD514 and CS1, yielded greater than 50%. Similar results were also found in M199 medium and M199 medium supplemented with 10% (v/v) serum.

Comparing the mean values for all *C. dubliniensis* strains tested in each of the media against the mean values for all strains of *C. albicans* tested in each of the media after 2 h and 6 h, showed a significant difference between the species ($P < 0.05$) at both time points for all of the media examined (Table 4.4). The only exception was RPMI-1640 medium after 2 h where the difference was not significant ($P > 0.05$). Clearly hyphal production is less efficient in *C. dubliniensis* than *C. albicans* (Figs. 4.3 and 4.4). Hyphal induction is also much slower in *C. dubliniensis* (Figs. 4.3 and 4.4). It is also apparent that hyphal production in *C. dubliniensis* is both strain-specific and media-dependent. However, despite this there is a clear difference between the levels of hyphal production between the two species.

4.3.1.2 Induction of hyphae on solid media

4.3.1.2.1 Spider medium

On the solid media tested, (i.e. Spider, YPS, Staib and Pal's media), several strains of *C. albicans* and *C. dubliniensis* tested produced hyphae when examined over a period of five days (Table 4.5). The induction of hyphae on solid media was examined on Spider medium (Liu *et al.*, 1994) by streaking the *Candida* strains out on this medium and incubating at 37°C for 5 days. Spider medium has previously been used in *C. albicans* for investigating the contribution of individual genes to hyphal formation. A gene is deemed to be required for dimorphism if a null mutant shows a defect in the induction of hyphae (Liu *et al.*, 1994). All

Table 4.4. Levels of hyphae produced in *C. albicans* and *C. dubliniensis* after 2 and 6 h at 37°C in all of the hyphal induction media examined.

	C. albicans [a]		***C. dubliniensis*** [b]		***P* value** [c]	
Media	2 h	6 h	2 h	6 h	2 h	6 h
	(%) [d]					
YNB	53	91	9	15	0.02	< 0.001
YNB with 10% (v/v) serum	67	89	16	39	0.01	< 0.001
RPMI-1640	31	79	5	14	0.09	< 0.001
RPMI-1640 with 10% (v/v) serum	57	93	10	20	0.02	< 0.001
M-199	28	92	3	13	0.02	< 0.001
M-199 with 10% (v/v) serum	62	90	15	43	0.01	< 0.001
NYP	45	92	14	29	0.02	< 0.001
Lee s medium	30	82	5	22	0.01	< 0.001
Water with 10% (v/v) serum	81	95	40	62	0.01	0.01

[a] Four strains of *C. albicans* were used in this part of the present study.

[b] Eleven strains of *C. dubliniensis* were used in this part of the present study.

[c] Statistical analysis to compare the results for *C. albicans* and *C. dubliniensis* was carried out using the two sample *t*-test assuming unequal variances. *P* values of <0.05 were considered significant.

[d] Percentage of cells in the hyphal phase following incubation. The data shown are mean values for all strains examined in triplicate in each of the hyphal induction media.

Table 4.5. Percentage of *C. albicans* and *C. dubliniensis* strains which produced a hyphal fringe following growth on the solid media examined in this study.

		C. albicans[a]	*C. dubliniensis*[b]
Medium	Temperature	% of strains which are in the hyphal phase	
Spider	37°C	50	45
YPS surface	25°C	0	0
YPS embedded	25°C	100	73
Pal's	30°C	0	100
Staib	30°C	0	100

[a] Four strains of *C. albicans* were used in this part of the present study.

[b] Eleven strains of *C. dubliniensis* were used in this part of the present study.

of the strains listed in section 4.2.1 were streaked onto Spider medium and examined every day over a period of 5 days (Fig. 4.5). Five out of the eleven *C. dubliniensis* strains tested (CD541, P7718, CS1, CD539 and CD514) produced hyphae on Spider medium. *Candida dubliniensis* strains P7718, CD541 and CD514 produced hyphae after two days, CS1 after 3 days and CD539 produced hyphae after 5 days. Two out of four *C. albicans* strains tested (SC5314 and 132A) produced hyphae on Spider medium. *Candida albicans* strain SC5314 produced hyphae after 3 days whereas 132A produced hyphae after 5 days.

4.3.1.2.2 Yeast extract-peptone-sucrose (YPS) medium

Typically, the change from the yeast form to the filamentous form is induced by incubation of cells at elevated temperature (37°C or higher) or in medium containing special components, such as serum or amino acids in the case of *C. albicans* (Gow, 1997). In contrast, in this part of the present study the incubation temperature was low (25°C) and the medium used was similar to standard rich medium. It was found that when cells were grown on the surface of Yeast extract-peptone-sucrose (YPS) medium no induction of hyphae was observed in any of the strains described above after 5 days, however, hyphae were observed when *Candida* were embedded between two solidified layers of YPS medium (Fig. 4.6). Previously, these conditions have been used for *C. albicans* and filamentous growth was found after 3-4 days when cells were sandwiched. However, when cells were grown on the surface of YPS medium under these conditions no hyphae were detected after 4 days and when the cells were embedded into molten agar hyphae were produced within 2 days (Douglas *et al.*, 1999). Embedded conditions were examined using a sandwich of YPS medium by plating a suspension of 100 cells between two solidified layers of YPS agar (sandwiched) as outlined in section 4.2.2. The chemical environment of cells when plated onto the surface of the agar is the same as the embedded colony as YPS medium was used for both, however, the physical environment is different. The chemical environment of a sandwiched colony having no air interface would be expected to be similar to that of an embedded colony however the physical environment would be different (Douglas *et al.*, 1999). After five days eight out of eleven (73%) of *C. dubliniensis* strains tested (CS1, CD36 CD519, P6265, CD57 CD514, CD96.34 and P7718) produced hyphae and all (100%) *C.*

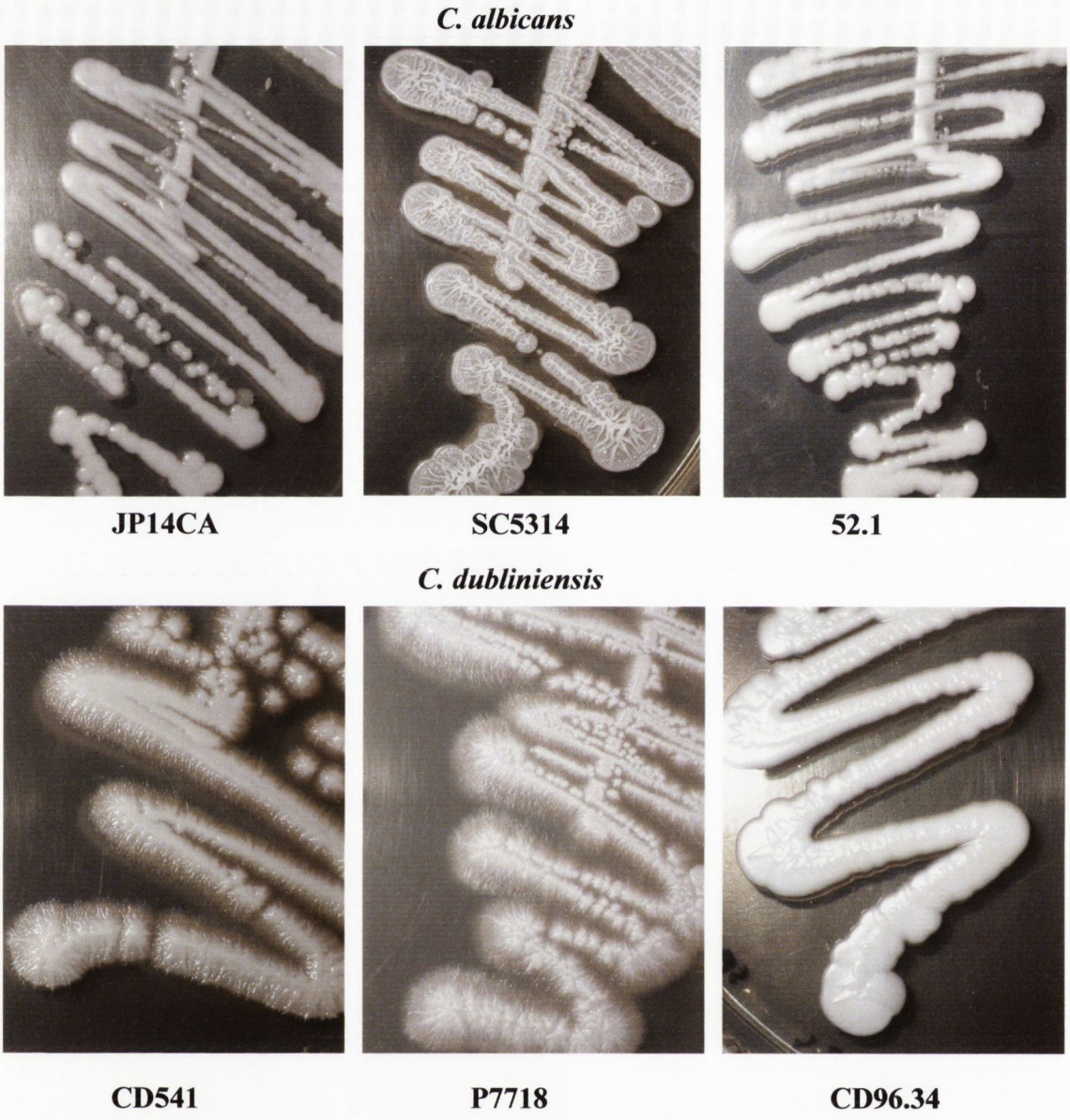


Figure 4.5. Appearance of culture growth of *C. albicans* strains (JP14CA, SC5314 and 52.1) and *C. dubliniensis* strains (CD541, P7718 and 52.1) following growth on Spider agar after incubation at 37°C for 5 days.

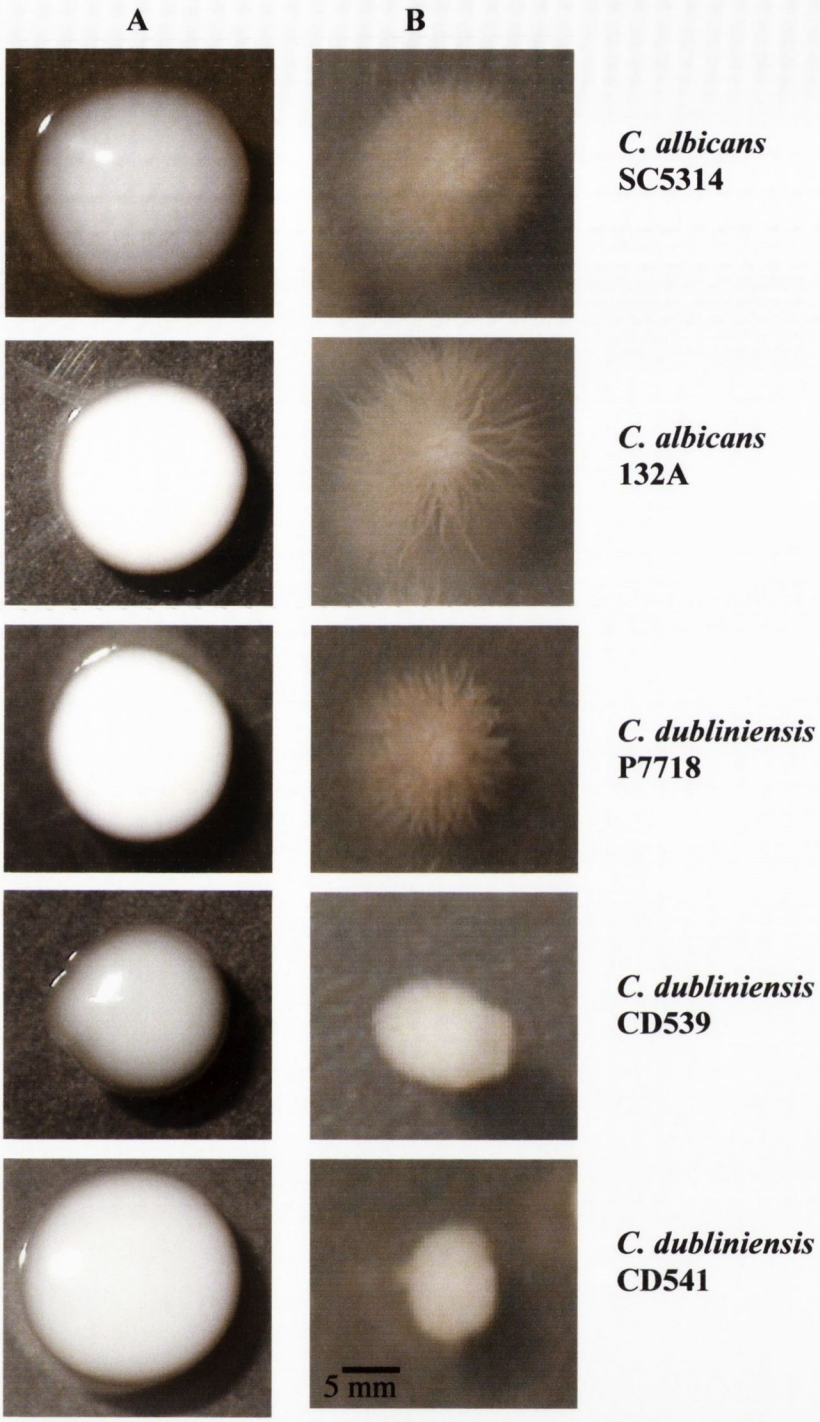


Figure 4.6. Appearance of culture growth of *C. albicans* strains (SC5314 and 132A) and *C. dubliniensis* strains (P7718, CD539 and CD541) following growth on (A) the surface of YPS agar and (B) embedded between two slabs of YPS agar. The strains were incubated at 25°C and examined for the presence of a hyphal fringe each day for 5 days. The scale bar is representative of each of the photographs.

albicans strains (SC5314, 132A, 52.1 and JP14CA) produced hyphae on YPS medium (Fig. 4.6).

4.3.1.2.3 Staib medium

Staib agar is a medium, which was originally used in the identification of *Crytococcus neoformans* based on the ability of this organism to produce melanin-like pigment on this agar (Staib *et al.*, 1987). However, it has since been discovered that this medium is also very useful for the differentiation of *C. dubliniensis* from *C. albicans* due to colony morphology and chlamydospore production (Al Mosaid *et al.*, 2001; Staib & Morschhauser, 1999). *Candida dubliniensis* colonies are generally rough and also a high percentage produce a hyphal halo or fringe around the colonies (64.6% was reported by Al-Mosaid *et al.*) (Al Mosaid *et al.*, 2001). In contrast, *C. albicans* produce shiny colonies after 48 h incubation with no hyphal fringe. All of the strains listed in Table 3.1 were streaked onto this agar and examined after 48 h at 30°C. All of the *C. dubliniensis* strains examined produced hyphae, pseudohyphae and chlamydospores on this medium in contrast to *C. albicans* whereby no hyphae were observed (Fig. 4.7).

4.3.1.2.4 Pal's medium

Pal's agar, which contains sunflower seed extract is a medium, which was also originally developed for the identification of *Cryptococcus neoformans* (Staib *et al.*, 1987). This medium was also examined for its ability to differentiate between *C. albicans* and *C. dubliniensis* based on the success of Staib agar and it was found that all *C. dubliniensis* isolates tested produced a hyphal fringe whereby *C. albicans* isolates grew as smooth creamy-grey colonies following 48 to 72 h incubation at 30°C (Al Mosaid *et al.*, 2003). In the present study a similar result was found as all *C. dubliniensis* isolates exhibited a hyphal fringe, pseudohyphae and chlamydospores after 72 h incubation whereas no *C. albicans* isolates exhibited these forms (Fig. 4.8).

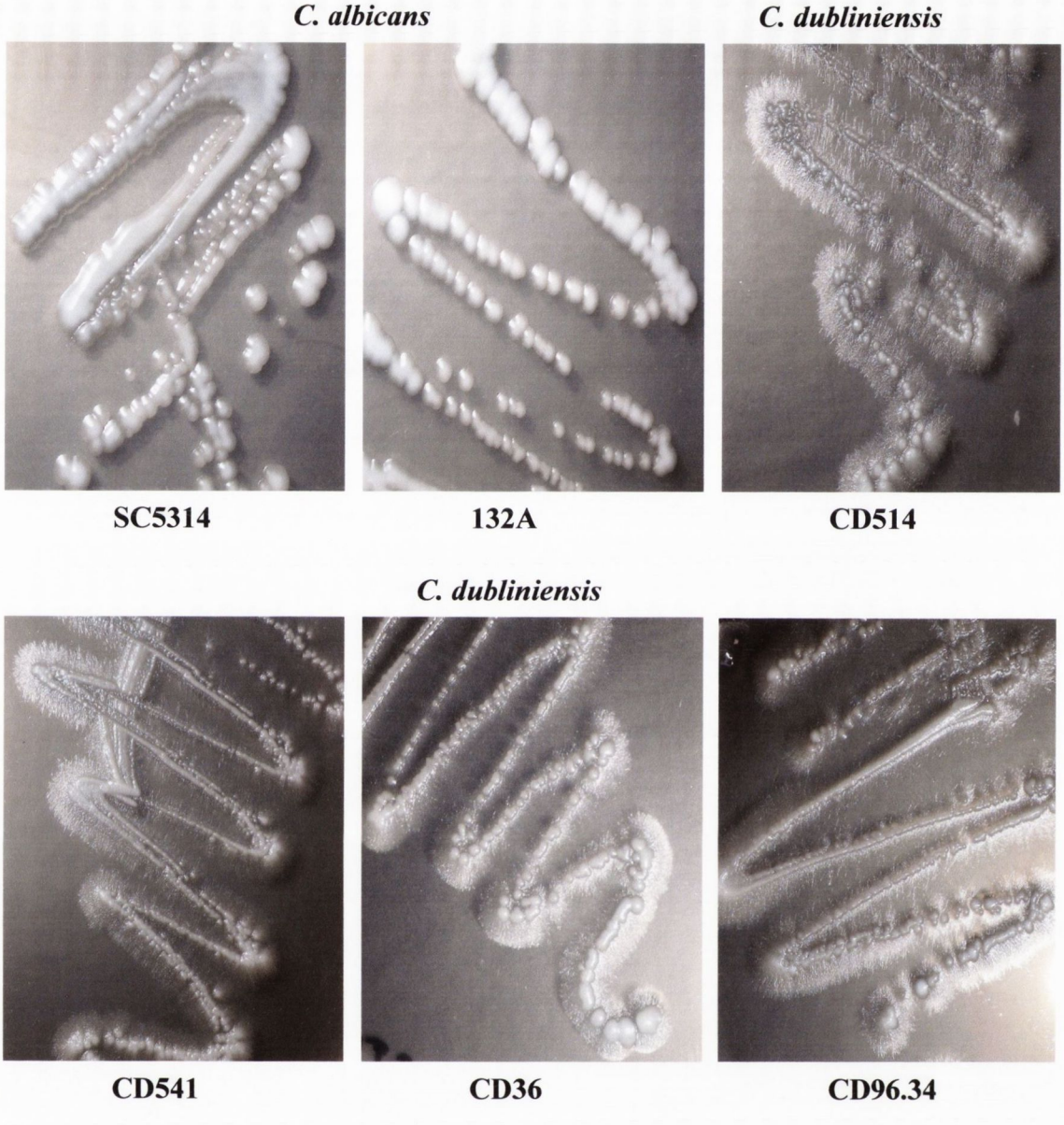


Figure 4.7. Appearance of culture growth of *C. albicans* strains (SC5314 and 132A) and *C. dubliniensis* strains (CD514, CD541, CD36 and CD96.34) following growth on Staib agar after incubation at 30°C for 72 h.

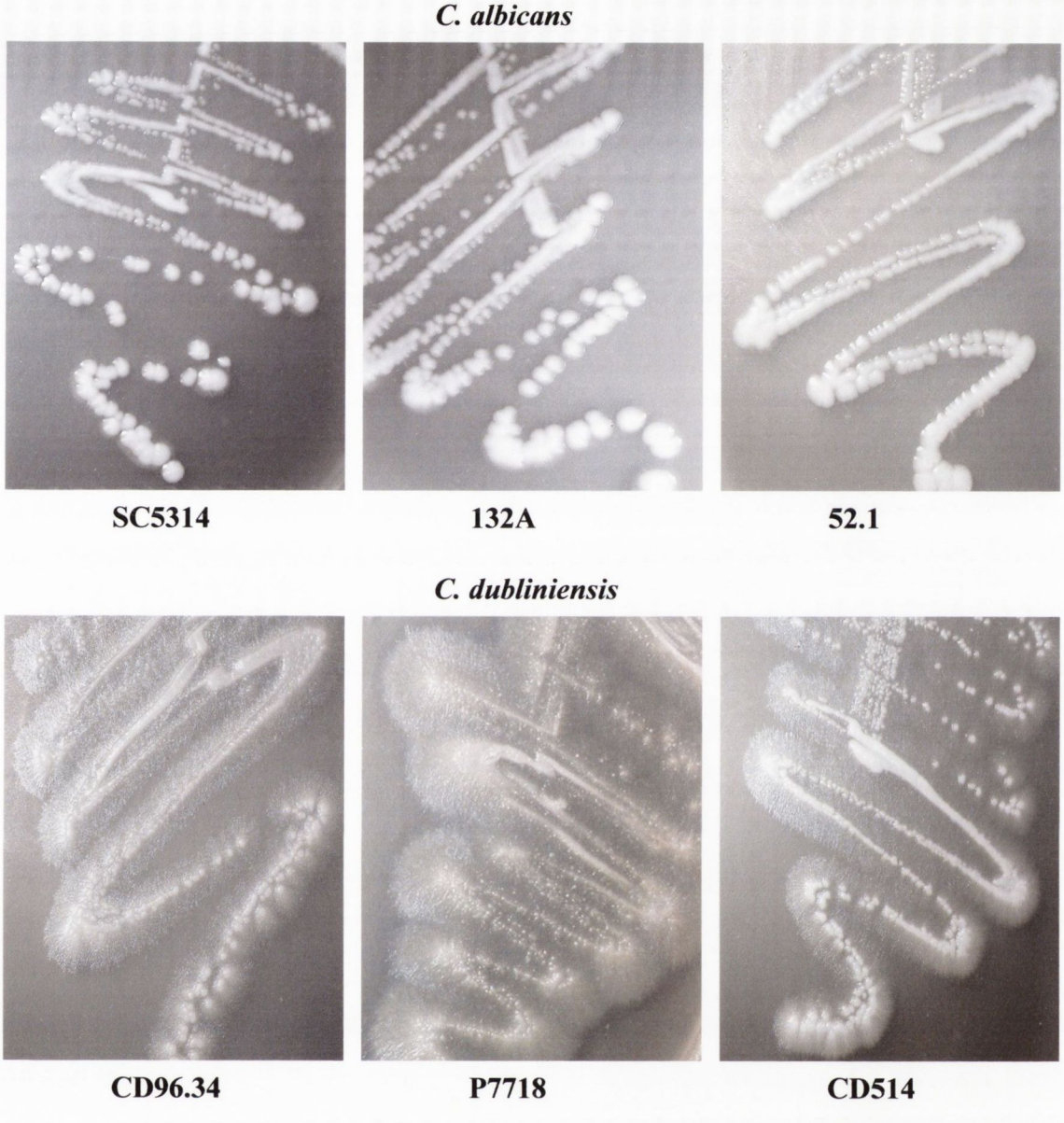


Figure 4.8. Appearance of culture growth of *C. albicans* strains (SC5314, 132A and 52.1) and *C. dubliniensis* strains (CD96.34, P7718 and CD514) following growth on Pal's agar after incubation at 30°C for 72 h.

4.3.2 Analysis of *HWP1*

4.3.2.2 Identification of *HWP1* in *C. dubliniensis*

The hyphal wall protein Hwp1 has been shown to be an important adhesin in *C. albicans* and is found solely on the surface of *C. albicans* hyphae and germ tubes. Due to the poor capacity of *C. dubliniensis* to produce hyphae it was decided to investigate the presence and expression of the hyphally regulated *HWP1* gene in *C. dubliniensis*. To investigate this. PCR primers were designed using the known nucleotide sequence of *CaHWP1* (Genbank accession no. U64206) (Table 4.2). It has previously been shown that it is relatively easy to amplify *C. dubliniensis* genes using primers designed on the corresponding *C. albicans* sequence (Donnelly *et al.*, 1999). However, numerous attempts to amplify the *CdHWP1* gene using primers based on internal regions of the *C. albicans* sequence failed. Eventually a single amplimer was obtained with *C. dubliniensis* template DNA using primers corresponding to the 5' and 3' ends of the *C. albicans* gene. The optimal conditions used are described in section 4.2.3 and the primer set used was HWP1F/HWP1R (Table 4.2). The forward primer HWP1F contained the start codon ATG and the reverse primer HWP1R contained the stop codon TAA in order to amplify the entire coding sequence of the gene. Amplification using the primer set HWP1F/HWP1R yielded amplimers of approximately 2 kb when DNA from *C. albicans* strains (132A, SC5314 and 103) was used as a template, as expected. However, when DNA from *C. dubliniensis* strains (CD36, CD33, P7718, CD519, CS1, CD539, CD506, CD514, P6265, P6785 and CD541) was used as a template the amplimers obtained with template DNA from individual strains ranged in size from 1.3-1.4 kb (examined in more detail in the next section, Fig. 4.9). Interestingly, *C. albicans* strains JP14CA, 52.1 and CA411 yielded two amplimers in comparison with the reference strain SC5314 which yielded a single amplimer. Further investigation was needed to elucidate why two products were amplified from these strains. In order to determine whether the amplimer obtained using the primer set HWP1F-HWP1R (Table 4.2) from *C. dubliniensis* CD36 template DNA was a homologue of the *C. albicans HWP1* gene it was necessary to clone and sequence the gene.

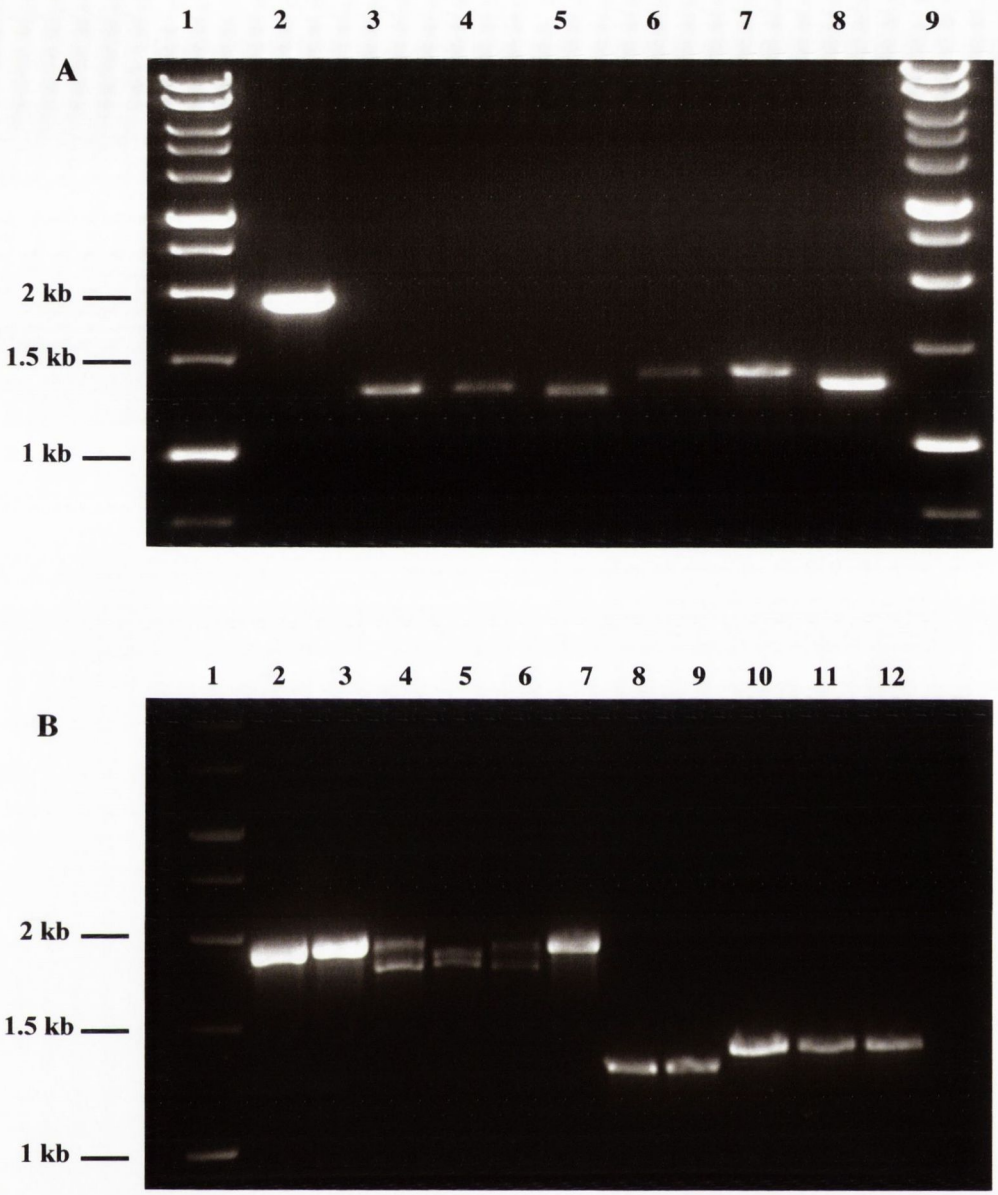
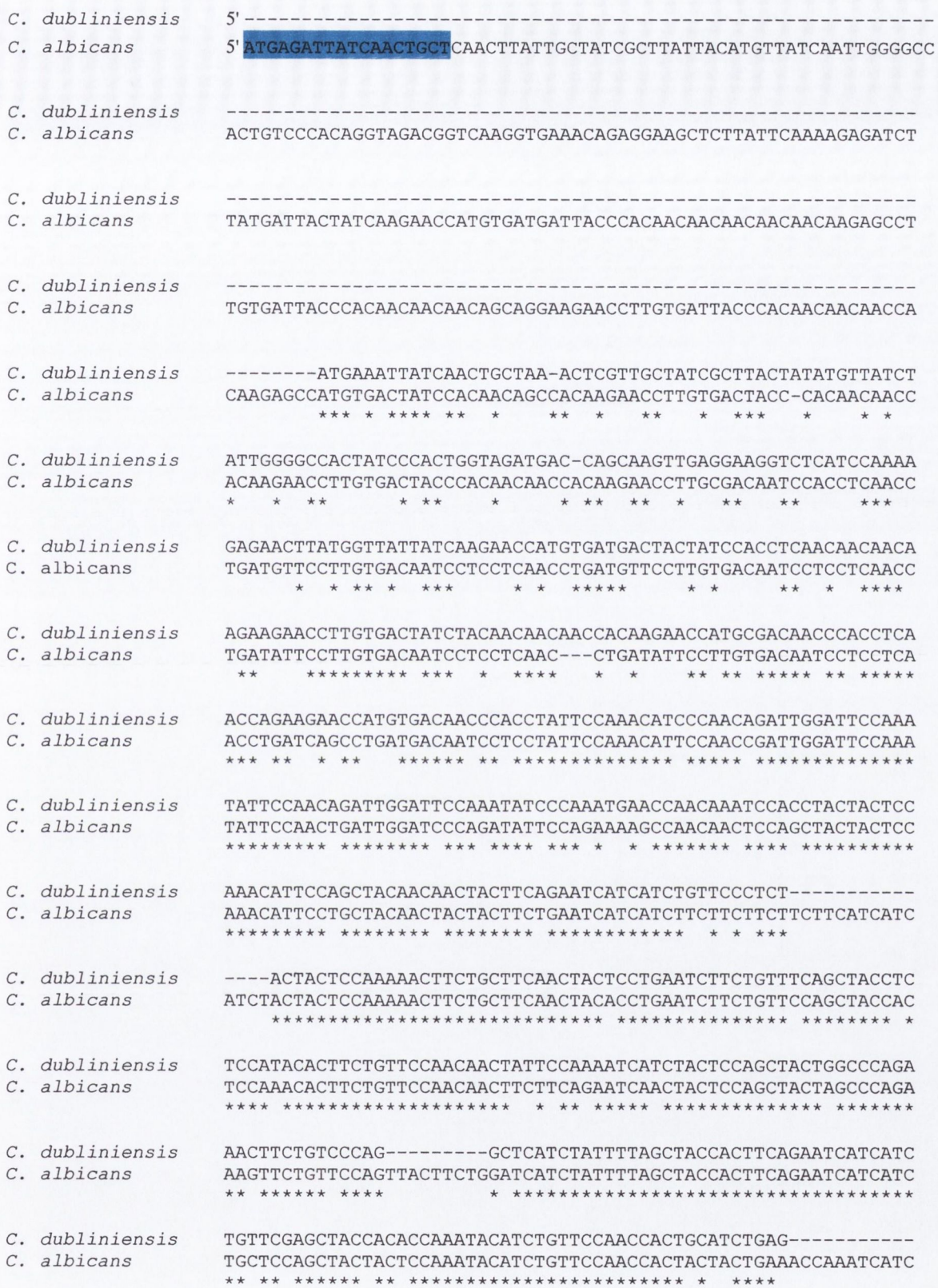


Figure 4.9. Agarose gels showing ethidium bromide-stained PCR-generated amplimers of both *C. albicans* and *C. dubliniensis* template DNA using the HWP1F/HWP1R primer pair (Table 4.2). The amplimers shown in the lanes were obtained from *Candida* strains as follows: (panel A) lane 2, *C. albicans* 132A; lanes 3-8 *C. dubliniensis* CD36, CD33, P7718, CD519, CS1, CD539; (panel B) lanes 2-7, *C. albicans* 132A, SC5314, JP14CA, 52.1, CA411, 103; lanes 8-12, *C. dubliniensis* CD506, CD514, P6265, P6785, CD541.

4.3.2.2 Sequence analysis of *HWP1* in *C. dubliniensis*

When sequenced the *C. dubliniensis HWP1* amplimer was found by BLAST analysis to exhibit extensive homology with the *C. albicans HWP1* gene (Fig. 4.10). However, although major regions of homology are evident between the two sequences, significant differences are also present, mainly due to the presence of extensive deletions in the *C. dubliniensis* sequence. The deduced amino acid sequence of the *C. dubliniensis* Hwp1 is only 421 amino acids long compared to 634 amino acids in *C. albicans* Hwp1p (Fig. 4.11). The N-terminus of Hwp1 in *C. albicans* contains numerous repeats of proline, aspartate and glutamine, which are thought to be crucial for the functioning of this protein, and to contain the recognition site of host transglutaminases. A large proportion of the region encoding the N-terminus is missing from the *CdHWP1* gene (Fig. 4.12). The initial 50 residues of each protein are highly homologous and both contain the KR signature of the Kex2 cleavage site. However, when the nucleotide sequences are compared using the DNA Strider^{TM} 1.3f11 software package, the results obtained show a low level of homology. Surprisingly, *CdHWP1* and *CaHWP1* were only 45.3% homologous at the nucleotide level and 49.3% homologous at the amino acid level.

Southern hybridisation analysis was also performed on strains of *C. albicans* and *C. dubliniensis* using the cloned *C. dubliniensis HWP1* amplimer (outlined in section 4.2.4) from *C. dubliniensis* CD36 as a probe. Genomic DNA from ten isolates, three *C. albicans* strains (132A, JP14CA and CA411) and seven *C. dubliniensis* strains (CD36, CD33, CD514, P7718, CM1, CD539 and CD541) were digested with the restriction enzyme *Eco*RI, the resulting fragments separated by electrophoresis, stained with ethidium bromide and transferred to nylon membranes as described in 2.3.2. The enzyme *Eco*RI was chosen as there is no cleavage site for this enzyme in either the *CaHWP1* or *CdHWP1* sequences. In all the strains tested only one fragment hybridised in all of the *C. dubliniensis* strains tested and in *C. albicans* 132A. However, there was a notable size difference in the hybridising band detected with the two species, with *C. albicans* yielding a band of approximately 4.5 kb and *C. dubliniensis* a band of approximately 9 kb (Fig. 4.13). For the two strains of *C. albicans,* JP14CA and CA411 two hybridising bands were detected with the *C. dubliniensis* probe, suggesting the presence of two alleles of the *HWP1* gene in these strains as was indicated by the PCR data described above (Fig 4.9). Southern analysis was also performed on genomic


C. dubliniensis 5'----------
C. albicans 5'ATGAGATTATCAACTGCTCAACTTATTGCTATCGCTTATTACATGTTATCAATTGGGGCC
C. dubliniensis ----------
C. albicans ACTGTCCCACAGGTAGACGGTCAAGGTGAAACAGAGGAAGCTCTTATTCAAAAGAGATCT
C. dubliniensis ----------
C. albicans TATGATTACTATCAAGAACCATGTGATGATTACCCACAACAACAACAACAACAAGAGCCT
C. dubliniensis ----------
C. albicans TGTGATTACCCACAACAACAACAGCAGGAAGAACCTTGTGATTACCCACAACAACAACCA
C. dubliniensis --------ATGAAATTATCAACTGCTAA-ACTCGTTGCTATCGCTTACTATATGTTATCT
C. albicans CAAGAGCCATGTGACTATCCACAACAGCCACAAGAACCTTGTGACTACC-CACAACAACC
C. dubliniensis ATTGGGGCCACTATCCCACTGGTAGATGAC-CAGCAAGTTGAGGAAGGTCTCATCCAAAA
C. albicans ACAAGAACCTTGTGACTACCCACAACAACCACAAGAACCTTGCGACAATCCACCTCAACC
C. dubliniensis GAGAACTTATGGTTATTATCAAGAACCATGTGATGACTACTATCCACCTCAACAACAACA
C. albicans TGATGTTCCTTGTGACAATCCTCCTCAACCTGATGTTCCTTGTGACAATCCTCCTCAACC
C. dubliniensis AGAAGAACCTTGTGACTATCTACAACAACAACCACAAGAACCATGCGACAACCCACCTCA
C. albicans TGATATTCCTTGTGACAATCCTCCTCAAC---CTGATATTCCTTGTGACAATCCTCCTCA
C. dubliniensis ACCAGAAGAACCATGTGACAACCCACCTATTCCAAACATCCCAACAGATTGGATTCCAAA
C. albicans ACCTGATCAGCCTGATGACAATCCTCCTATTCCAAACATTCCAACCGATTGGATTCCAAA
C. dubliniensis TATTCCAACAGATTGGATTCCAAATATCCCAAATGAACCAACAAATCCACCTACTACTCC
C. albicans TATTCCAACTGATTGGATCCCAGATATTCCAGAAAAGCCAACAACTCCAGCTACTACTCC
C. dubliniensis AAACATTCCAGCTACAACAACTACTTCAGAATCATCATCTGTTCCCTCT----------
C. albicans AAACATTCCTGCTACAACTACTACTTCTGAATCATCATCTTCTTCTTCTTCTTCATCATC
C. dubliniensis ----ACTACTCCAAAAACTTCTGCTTCAACTACTCCTGAATCTTCTGTTTCAGCTACCTC
C. albicans ATCTACTACTCCAAAAACTTCTGCTTCAACTACACCTGAATCTTCTGTTCCAGCTACCAC
C. dubliniensis TCCATACACTTCTGTTCCAACAACTATTCCAAAATCATCTACTCCAGCTACTGGCCCAGA
C. albicans TCCAAACACTTCTGTTCCAACAACTTCTTCAGAATCAACTACTCCAGCTACTAGCCCAGA
C. dubliniensis AACTTCTGTCCCAG---------GCTCATCTATTTTAGCTACCACTTCAGAATCATCATC
C. albicans AAGTTCTGTTCCAGTTACTTCTGGATCATCTATTTTAGCTACCACTTCAGAATCATCATC
C. dubliniensis TGTTCGAGCTACCACACCAAATACATCTGTTCCAACCACTGCATCTGAG----------
C. albicans TGCTCCAGCTACTACTCCAAATACATCTGTTCCAACCACTACTACTGAAACCAAATCATC

```
C. dubliniensis    ------------------------------------------------------------
C. albicans        AAGTACTCCATTAACTACTACTACTGAACATGATACAACTGTTGTCACTGTTACTTCATG

C. dubliniensis    ------------------------------------------------------------
C. albicans        TTCTAACAGTGTTTGTACCGAAAGTGAAGTTACTACTGGTGTTATTGTCATCACATCTAA

C. dubliniensis    ----------------------------------------------------TCTTCCACTTT
C. albicans        AGATACTATTTACACCACTTACTGTCCATTGACTGAAACTACTCCAGTTTCTACTGCTCC
                                                                      *** *  **

C. dubliniensis    GGAAACCAAAACATCAATTACTCCATTAACTACCTCTACTGAACATGATACAACTGTTAT
C. albicans        AGCCACTGAAACACCAACTGGTACAGTATCCACTTCTACTGAACAATCAACTACTGTTAT
                    *  **  ***** *** *  * ** ** * ** ***********    ** ********

C. dubliniensis    TACCGTTACTTCATGCTCCAACAGTGCATGTGCAGAAAGTGAAGTCACTACTGGTGTTGT
C. albicans        TACTGTTACTTCATGTTCTGAAAGCTCTTGTACCGAATCTGAAGTTACTACTGGTGTTGT
                   *** *********** **  * **  * *** * ***  ****** **************

C. dubliniensis    TGTCGTCACATCTGAAGATACTATTTACACTACTTACTGTCCTTTAACTGAAACTACTCC
C. albicans        TGTTGTTACTTCTGAGGAAACTGTCTACACTACATTCTGTCCATTGACTGAAAACACTCC
                   *** ** ** ***** ** *** * ******** * ****** ** *******  *****

C. dubliniensis    AGGTACTGAATCCACTCCAGAAGCTTCCACTCCAATCATGGAAACAATTCCAGCTGATTC
C. albicans        AGGTACTGATTCAACTCCAGAAGCTTCCATTCCACCTATGGAAACAATTCCTGCTGGTTC
                   ********* ** ***************** ****   ************** **** ***

C. dubliniensis    CGAACCATCAGTTCCTGCCAGTGAAACCTCAGCAGTTGTTCCAGAATTATCAGTCCCAAC
C. albicans        AGAATCATCCATGCCTGCCGGTGAAACCTCTCCAGCTGTTCCAAAATCAGATGTTCCAGC
                    *** ****  * ****** **********  *** ******* *** *   ** *** *

C. dubliniensis    TACAGAATCGGCTCCAGGTACAAAAATGACTCCAGGTGGTTTAGAACCATCC--------
C. albicans        TACTGAATCAGCTCCAGTTCCTGAAATGACTCCAGCTGGTTCACAACCATCTATTCCTGC
                   *** ***** ******* * *  ************ ***** * *******

C. dubliniensis    ----------------------------------------------------GCTCCAGT
C. albicans        CGGTGAAACCTCTCCAGCTGTTCCAAAATCAGATGTTCCAGCTACTGAATCTGCTCCTGC
                                                                     ***** *

C. dubliniensis    CTCTGAAATGACCCCAGCTGGTATTAAAACTAACCCAGCTGTTCCAGAATCTTCAATTCC
C. albicans        TCCTGAAATGACTCCAGCTGGTACTGAAACTAAACCAGCTGCTCCAAAATCATCAGCTCC
                     ********** ********** * ******* ******* **** **** ***  ***

C. dubliniensis    ------------------------------------------------------------
C. albicans        TGCCACTGAACCTTCCCCAGTTGCTCCAGGTACTGAATCCGCACCAGCTGGTCCAGGTGC

C. dubliniensis    ------------------------------------------------------------
C. albicans        TTCTTCTTCTCCAAAATCTTCTGTTTTGGCTAGTGAAACCTCACCAATTGCTCCAGGTGC

C. dubliniensis    ------------AGCTGGTTCAAGTGTTGCAATTACTACTCCGGTAAATAGTACTATCGT
C. albicans        TGAAACCGCTCCAGCTGGCTCAAGTGGTGCTATTACTATTCCGGAATCTAGTGCTGTCGT
                              ****** ******* *** ******* ***** *  **** ** ****

C. dubliniensis    CTCTACGACTGAAGGTGCTATTCCAACCACATTAGAATCTGTCCCAATCATGCAACCATC
C. albicans        CTCTACGACTGAAGGTGCTATTCCAACTACATTAGAATCAGTTCCACTCATGCAACCATC
                   *************************** ********** ** *** *************

C. dubliniensis    TGCCAATTATTCATCTAGTGTTGCCCCTGTTTCTACGTTTGAAGGTGTTGGTAACAACAT
C. albicans        TGCCAATTACTCA---AGTGTCGCTCCTATTTCTACATTTGAAGGTGCTGGTAACAACAT
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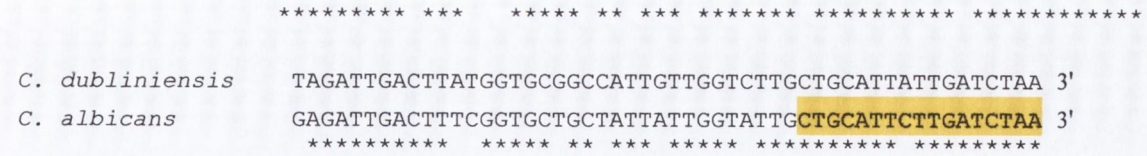

Figure 4.10. Nucleotide sequence alignment of *CdHWP1* from *C. dubliniensis* strain CD36 determined in the present study and *CaHWP1* from *C. albicans* strain SC5314 (Genbank accession no. U64206) generated with the CLUSTAL W sequence alignment program (Higgins & Sharp, 1988). Asterisks indicate identical residues, spaces indicate dissimilar residues and hyphens indicate gaps created to obtain alignment. The primers used to amplify the gene are highlighted, HWP1F in blue and HWP1R in yellow.



Figure 4.11. An alignment of the amino acid sequences of the *C. albicans* and *C. dubliniensis* Hwp1 proteins generated with the CLUSTAL W sequence alignment program (Higgins & Sharp, 1988). Asterisks indicate identical residues, spaces indicate dissimilar residues and hyphens indicate gaps created to obtain alignment. Large deletions are present throughout the *C. dubliniensis* protein, especially in the N-terminal and C-terminal regions which contain essential amino acids for the functioning of the protein. The presence of an isoleucine, valine or leucine 4-5 residues upstream from the ω site is characteristic of glycosylphosphatidylinositol-dependant cell wall proteins (GPI-CWP) proteins that become linked to β-1,6-glucan of the cell wall. The putative ω-site in the CaHwp1 and CdHwp1 proteins is highlighted in red and ω-5 is highlighted in blue.

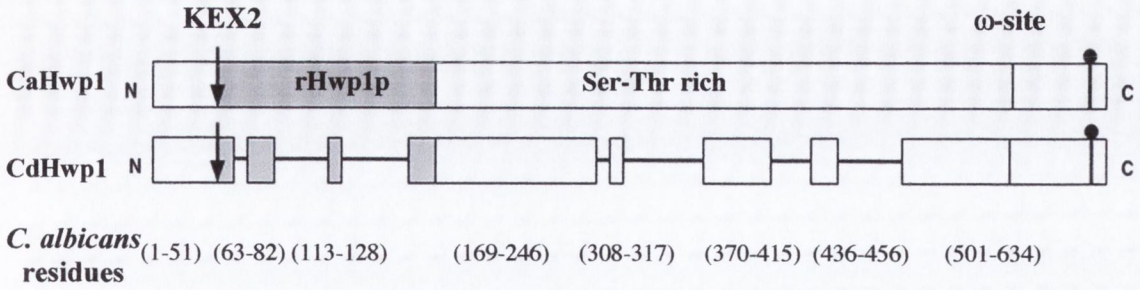

Figure 4.12. Diagram illustrating regions of homology to *C. albicans* CaHwp1 and the extent of deletions in the predicted CdHwp1 protein sequence. The upper rectangular box represents the CaHwp1 protein and shows the position of the KEX2 cleavage site (arrow), the recombinant rHwp1 domain (shaded area) shown to possess transglutaminase substrate activity (Sundstrum, 2002), the serine-threonine rich region (Ser-Thr rich) and the carboxy terminal-site. The lower boxes represent the homologous regions of the predicted *C. dubliniensis* CdHwp1 protein. The numbers below indicate the positions of the homologous *C. dubliniensis* protein domains relative to the corresponding *C. albicans* amino acid residues (presented with permission by Moran *et al.*, 2004).

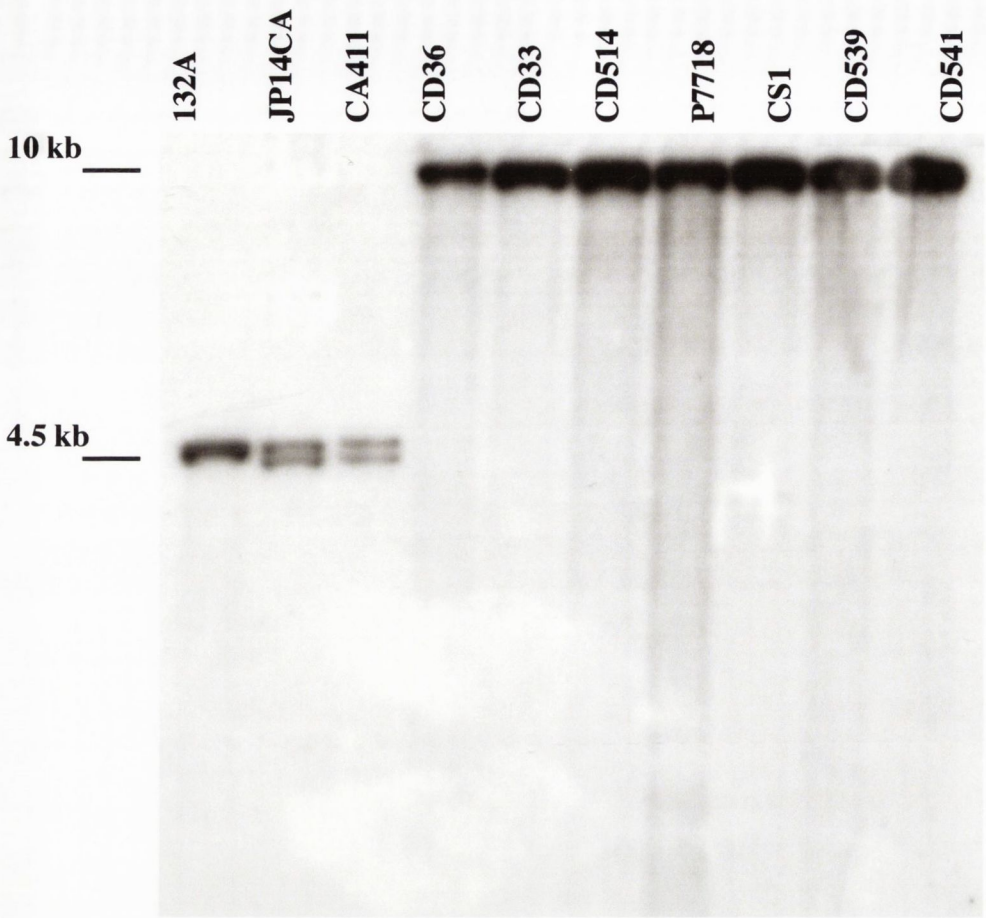

Figure 4.13. Southern blot of *Eco*RI digested genomic DNA from three *C. albicans* strains (132A, JP14CA and CD411) and seven *C. dubliniensis* strains (CD36, CD33, CD514, P7718, CS1, CD539 and CD541) following hybridisation analysis with the cloned PCR amplimer encoding *CdHWP1*.

DNA from five strains of *C. dubliniensis* (CD519, CD57, CS1, CD36 and P7718) digested with the enzyme *Hinc*II as there is also no cleavage site for this enzyme in the *CdHWP1* sequence. This was performed to confirm that only one gene is hybridising to the *CdHWP1* probe in *C. dubliniensis*. A single hybridising band was detected for each strain. However, strains as CD36, CD57 and P7718 yielded a hybridising band of approximately 4.5 kb, whereas CD519 and CS1 yielded a band of approximately 3.5 kb (data not shown).

4.3.2.3 Cloning and sequencing of the 5' upstream region of *CdHWP1*

In order to ensure that the amplification process amplified the entire *C. dubliniensis* homologue of *HWP1* it was necessary to clone and sequence the upstream region. In order to amplify this region primers were designed based on the sequence of the gene known to be immediately upstream from *CaHWP1,* i.e. *CaAPL6*. Due to the similarities between *C. albicans* and *C. dubliniensis* it was assumed that a similar gene order arrangement would be present in *C. dubliniensis*. In order to amplify the upstream region of the gene, primers were designed based on the sequence of *CaAPL6*. The primer sets APL6(1)F/HWPI(2)R, APL6(2)/HWP1(2)R, IV1F/HWP1(2)R and IV2F/HWP1(2)R (Table 4.2) which were designed based on the sequence of the *CaAPL6* gene upstream from *CaHWP1* and also on the flanking region between the two genes were used (Fig 4.14). A product of approximately 3 kb was obtained with the primer set APL6(1)F/HWPI(2)R, and 2.7 kb with the primer set APL6(2)/HWP1(2)R and approximately 1.5 kb with the primer set IV1F/HWP1(2)R following PCR (data not shown). No product was obtained with the primer set IV2F/HWP1(2)R. This could be because the primers were designed based on the sequence of the flanking region between *APL6* and *HWP1* in *C. albicans* and it should be noted that flanking regions are often not conserved regions. The products obtained were cloned and sequenced and compared with the corresponding *C. albicans* region.

The results obtained showed that the amplimer obtained from *C. dubliniensis* template DNA using the primer set HWP1F/HWP1R (Table 4.2) was the entire gene containing both the start and the stop codon. The *C. dubliniensis HWP1* sequence was then submitted to Genbank under the accession no. AJ63273. The upstream region which contains the promoter gives an insight into the regulation of the gene. It contains numerous E boxes, (consensus

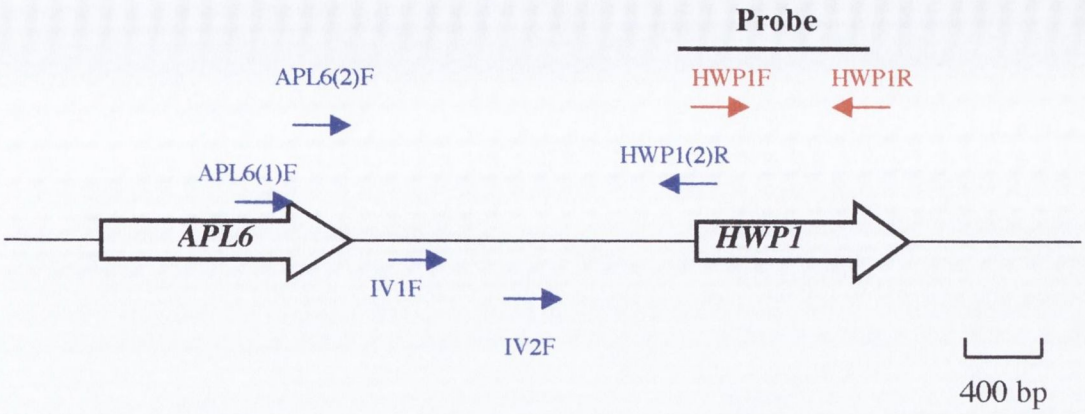

Figure 4.14. Illustration of the location and orientation of primers used to amplify the *C. dubliniensis HWP1* gene. Large black arrows represent the *APL6* and *HWP1* genes and indicate the direction of transcription. Small arrows indicate the positions of primer sequences used in this study. The primer set HWP1F/HWP1R highlighted in red was used to amplify the *CdHWP1* gene. The primer sets APL6(1)F/HWPI(2)R, APL6(2)/HWP1(2)R, IV1F/HWP1(2)R and IV2F/HWP1(2)R (Table 4.2), highlighted in blue, were used to amplify the upstream region of *CdHWP1*.

sequence, 5'-CANNTG-3'), which are also present in the *C. albicans* upstream region. Transcription factor Efg1 is thought to interact with the E box and regulate expression of promoters (Fig. 4.15). This may indicate that regulation of *C. dubliniensis HWP1* is due to Efg1, which is the case for *CaHWP1*. Hypha-specific genes carry E-boxes in their promoters and have been found in many genes involved in hyphal production in *C. albicans* such as *ALS8*, *HYR1*, *ALS3*, *HWP1*, *ECE1* and *RBT 4* (Leng *et al.*, 2001).

4.3.2.4 Comparison of sequence analysis of *HWP1* in *C. dubliniensis* strains

As mentioned earlier, when *C. dubliniensis* DNA was used as a template with the primer set HWP1F/HWP1R (Table 4.2) the amplimers obtained ranged in size from 1.3-1.4 kb, depending on the strain. In *C. dubliniensis* strain P7718 the amplimer obtained was smaller than the amplimer obtained with the reference strain CD36 (Fig 4.9). Strains CD519, CS1, P6265, P6785 and CD541 yielded larger amplimers than the reference strain (Fig. 4.9). In order to ascertain the reasons for the difference in size of amplimers obtained with different strains, the amplimers from three additional strains (CS1, CD519 and P7718) were examined by sequence analysis and all three were found to contain deletions relative to each other (Fig. 4.16). However, the differences were not as extensive as that found between the amplimers obtained from *C. dubliniensis* CD36 and *C. albicans*. The *HWP1* gene from the *C. dubliniensis* type strain CD36 encodes 9 more amino acids between residues 144 and 153 than the other three strains, whereas CD519 and CS1 contained 29 more amino acids between residues 352 and 381 than CD36 and P7718 (Fig. 4.17). These findings explain the small size difference observed in the amplimers yielded from the three strains when using the primer set HWP1F/HWP1R (Fig 4.9).

4.3.2.5 Comparison of sequence analysis of *HWP1* in *C. albicans* strains

As mentioned earlier, when *C. albicans* DNA was used as a template with the primer set HWP1F/HWP1R (Table 4.2) strains JP14CA, 52.1 and CA411 yielded two amplimers in comparison with the reference strain SC5314 which yielded a single amplimer. This result was reflected, in the Southern hybridisation experiments for two strains of *C. albicans,* JP14CA and CA411 where two hybridising bands were detected. In order to ascertain the reasons for the difference in size of amplimers obtained with different strains, the amplimers


C. albicans
C.dubliniensis
C. albicans
C. dubliniensis
C. albicans
C. dubliniensis
C. albicans
C. dubliniensis
C. albicans
C. dubliniensis
C. albicans
C. dubliniensis
C. albicans
C. dubliniensis
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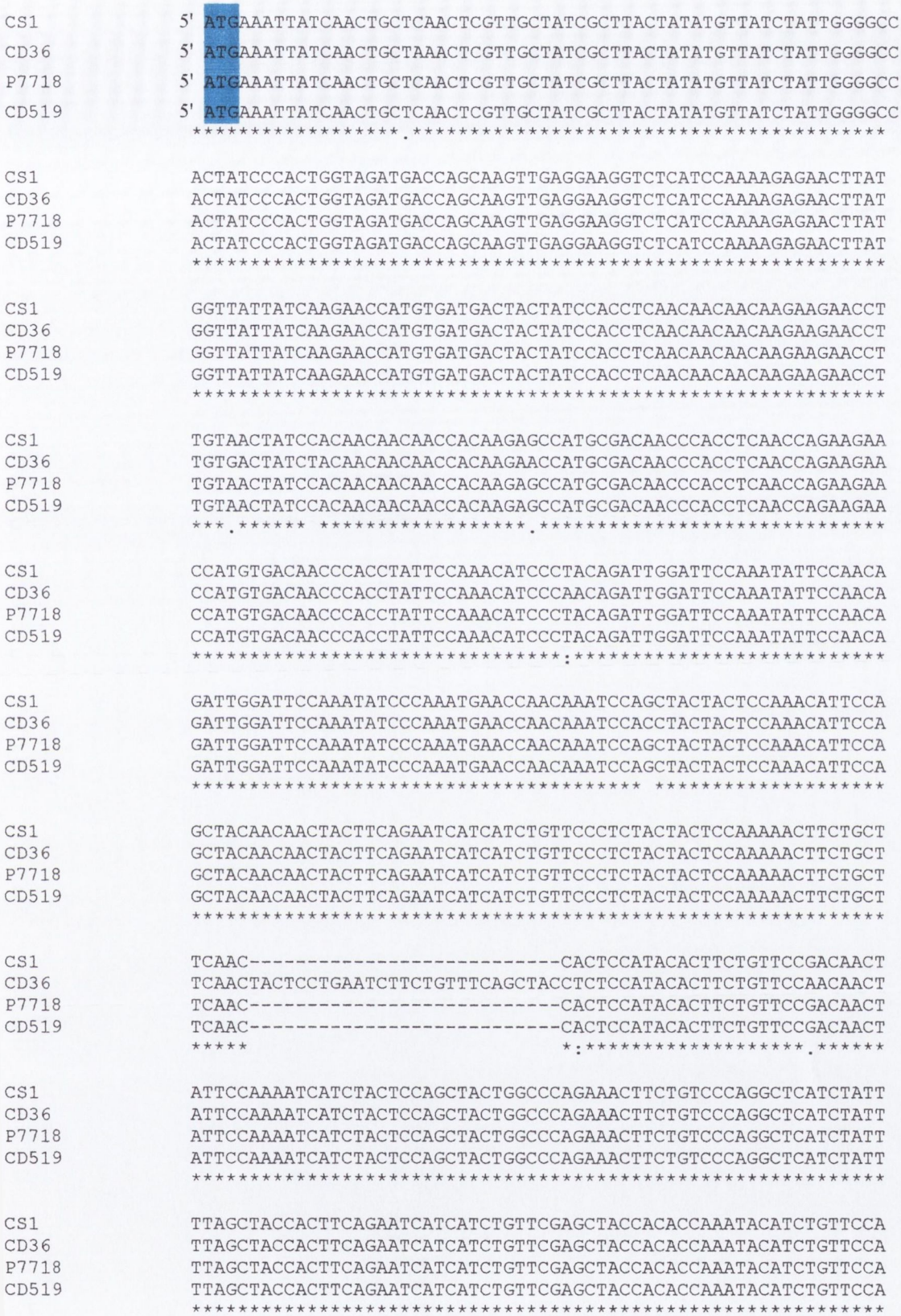
C. albicans
C. dubliniensis



Figure 4.15. Alignment of the nucleotide sequence of the upstream region of *HWP1* from *C. dubliniensis* strain CD36 and the corresponding region from *C. albicans* (Genbank accession no. U64206) generated with the CLUSTAL W sequence alignment program (Higgins & Sharp, 1988). Asterisks indicate identical residues, spaces indicate dissimilar residues and hyphens indicate gaps created to obtain alignment. The stop codon (TAA) of *APL6* in *C. dubliniensis* is highlighted in yellow. The start codon (ATG) of *HWP1* is highlighted in blue. Putative *EFG1* transcription motifs are highlighted in red.


CS1 5' ATGAAATTATCAACTGCTCAACTCGTTGCTATCGCTTACTATATGTTATCTATTGGGGCC
CD36 5' ATGAAATTATCAACTGCTAAACTCGTTGCTATCGCTTACTATATGTTATCTATTGGGGCC
P7718 5' ATGAAATTATCAACTGCTCAACTCGTTGCTATCGCTTACTATATGTTATCTATTGGGGCC
CD519 5' ATGAAATTATCAACTGCTCAACTCGTTGCTATCGCTTACTATATGTTATCTATTGGGGCC
ACTATCCCACTGGTAGATGACCAGCAAGTTGAGGAAGGTCTCATCCAAAAGAGAACTTAT
GGTTATTATCAAGAACCATGTGATGACTACTATCCACCTCAACAACAACAAGAAGAACCT
TGTAACTATCCACAACAACAACCACAAGAGCCATGCGACAACCCACCTCAACCAGAAGAA
TGTGACTATCTACAACAACAACCACAAGAACCATGCGACAACCCACCTCAACCAGAAGAA
CCATGTGACAACCCACCTATTCCAAACATCCCTACAGATTGGATTCCAAATATTCCAACA
GATTGGATTCCAAATATCCCAAATGAACCAACAAATCCAGCTACTACTCCAAACATTCCA
GCTACAACAACTACTTCAGAATCATCATCTGTTCCCTCTACTACTCCAAAAACTTCTGCT
TCAAC----------CACTCCATACACTTCTGTTCCGACAACT
TCAACTACTCCTGAATCTTCTGTTTCAGCTACCTCTCCATACACTTCTGTTCCAACAACT
ATTCCAAAATCATCTACTCCAGCTACTGGCCCAGAAACTTCTGTCCCAGGCTCATCTATT
TTAGCTACCACTTCAGAATCATCATCTGTTCGAGCTACCACACCAAATACATCTGTTCCA

```
CS1             ACCACTGCTTCTGAGTCTTCCACTTCGGAAACCAAAACATCAATTACTCCATTAACTACC
CD36            ACCACTGCATCTGAGTCTTCCACTTTGGAAACCAAAACATCAATTACTCCATTAACTACC
P7718           ACCACTGCTTCTGAGTCTTCCACTTCGGAAACCAAAACATCAATTACTCCATTAACTACC
CD519           ACCACTGCTTCTGAGTCTTCCACTTCGGAAACCAAAACATCAATTACTCCATTAACTACC
                ********:********** ************************************

CS1             TCTACTGAACATGATACAACTGTTATTACCGTTACTTCATGCTCCAACAGTGCATGTGCA
CD36            TCTACTGAACATGATACAACTGTTATTACCGTTACTTCATGCTCCAACAGTGCATGTGCA
P7718           TCTACTGAACATGATACAACTGTTATTACCGTTACTTCATGCTCCAACAGTGCATGTGCA
CD519           TCTACTGAACATGATACAACTGTTATTACCGTTACTTCATGCTCCAACAGTGCATGTGCA
                ************************************************************

CS1             GAAAGTGAAGTCACTACTGGTGTTGTTGTAGTCACATCTGAAGATACTATTTACACTACT
CD36            GAAAGTGAAGTCACTACTGGTGTTGTTGTCGTCACATCTGAAGATACTATTTACACTACT
P7718           GAAAGTGAAGTCACTACTGGTGTTGTTGTAGTCACATCTGAAGATACTATTTACACTACT
CD519           GAAAGTGAAGTCACTACTGGTGTTGTTGTAGTCACATCTGAAGATACTATTTACACTACT
                ****************************.*****************************

CS1             TACTGTCCTTTAACTGAAACTACTCCAGGTACTGAATCCACTCCAGAAGCTTCCACTCCA
CD36            TACTGTCCTTTAACTGAAACTACTCCAGGTACTGAATCCACTCCAGAAGCTTCCACTCCA
P7718           TACTGTCCTTTAACTGAAACTACTCCAGGTACTGAATCCACTCCAGAAGCTTCCACTCCA
CD519           TACTGTCCTTTAACTGAAACTACTCCAGGTACTGAATCCACTCCAGAAGCTTCCACTCCA
                ************************************************************

CS1             ATCATGGAAACAATTCCAGCTGATTCCGAACCATCAGTTCCTGCCAGTGAAACCTCACCA
CD36            ATCATGGAAACAATTCCAGCTGATTCCGAACCATCAGTTCCTGCCAGTGAAACCTCAGCA
P7718           ATCATGGAAACAATTCCAGCTGATTCCGAACCATCAGTTCCTGCCAGTGAAACCCCACCA
CD519           ATCATGGAAACAATTCCAGCTGATTCCGAACCATCAGTTCCTGCCAGTGAAACCTCACCA
                ********************************************************** ** **

CS1             GTTGTTCCAGAATTATCAGTCCCAACTACAGAATCGGCTCCAGGTACAAAAATGACTCCA
CD36            GTTGTTCCAGAATTATCAGTCCCAACTACAGAATCGGCTCCAGGTACAAAAATGACTCCA
P7718           GTTGTTCCAGAATTATCAGTCCCAACTACAGAATCGGCTCCAGGTACAAAAATGACTCCA
CD519           GTTGTTCCAGAATTATCAGTCCCAACTACAGAATCGGCTCCAGGTACAAAAATGACTCCA
                ************************************************************

CS1             GGTGGTTTAGAACCATCCGCTCCCGTCTCTGAAATGACCCCAGCTGGTATTAAAACTAAC
CD36            GGTGGTTTAGAACCATCCGCTCCAGTCTCTGAAATGACCCCAGCTGGTATTAAAACTAAC
P7718           GGCGGTTTAGAACCATCCGCTCCAGTCTCTGAAATGACCCCAGCTGGTATTAAAACTAAC
CD519           GGTGGTTTAGAACCATCCGCTCCCGTCTCTGAAATGACCCCAGCTGGTATTAAAACTAAC
                ** ********************.************************************

CS1             CCAGCTGTTCCAGAATCTTCAATTCCAGCTGGTACAGGTGCATCATCTACTTCAAAATCT
CD36            CCAGCTGTTCCAGAATCTTCAATTCCAGCTG----------------------------
P7718           CCAGCTGTTCCAGAATCTTCAATTCCAGCTG----------------------------
CD519           CCAGCTGTTCCAGAATCTTCAATTCCAGCTGGTACAGGTGCATCATCTACTTCAAAATCT
                ********************************

CS1             TCTGTTTTAGCTAGTGAAACTTCTTCAGTTGCTCCAGGTGCTGAAATCACTCTAGCTGGT
CD36            ------------------------------------------------------------GT
P7718           ------------------------------------------------------------GT
CD519           TCTGTTTTAGCTAGTGAAACTTCTTCAGTTGCTCCAGGTGCTGAAATCACTCTAGCTGGT
                                                                            **

CS1             TCAAGTGCTGCAATTACTACTCCGTTAAATAGTGCTATCGTCTCTACGACTGAAGGTGCT
CD36            TCAAGTGTTGCAATTACTACTCCGGTAAATAGTACTATCGTCTCTACGACTGAAGGTGCT
P7718           TCAAGTGTTGCAATTACTACTCCGGTAAATAGTGCTATCGTCTCTACGACTGAAGGTGCT
CD519           TCAAGTGCTGCAATTACTACTCCGTTAAATAGTGCTATCGTCTCTACGACTGAAGGTGCT
                ******* *************** ********.**************************
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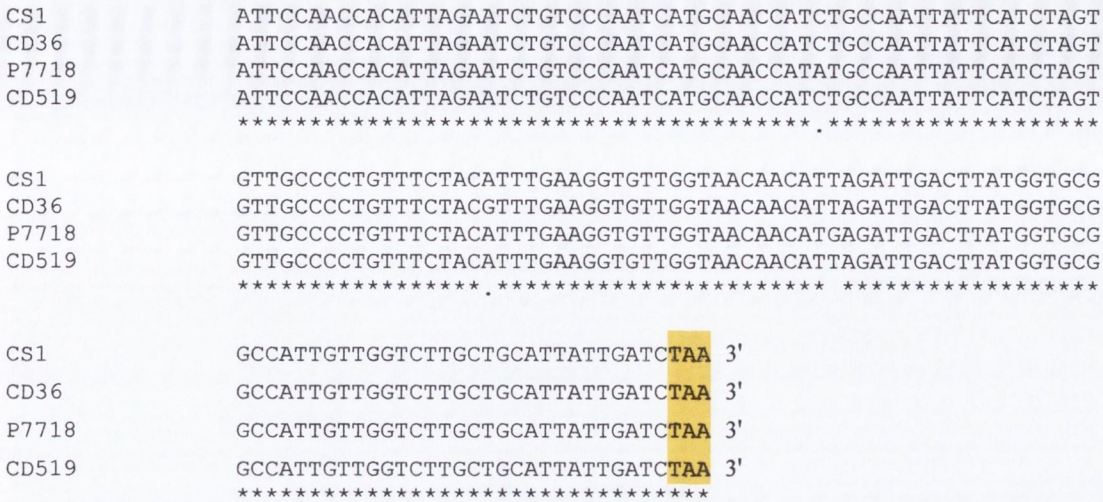

Figure 4.16. Alignment of the nucleotide sequence of *HWP1* from *C. dubliniensis* strains P7718, CD36, CD519 and CS1 generated with the CLUSTAL W sequence alignment program (Higgins & Sharp, 1988). Asterisks indicate identical residues, spaces indicate dissimilar residues and hyphens indicate gaps created to obtain alignment. The gaps indicate sequence differences between the strains and account for the difference in size of PCR amplimers obtained with HWP1F/HWP1R specific primers. The start codon (ATG) of *HWP1* is highlighted in blue and the stop codon (TAA) is highlighted in yellow.

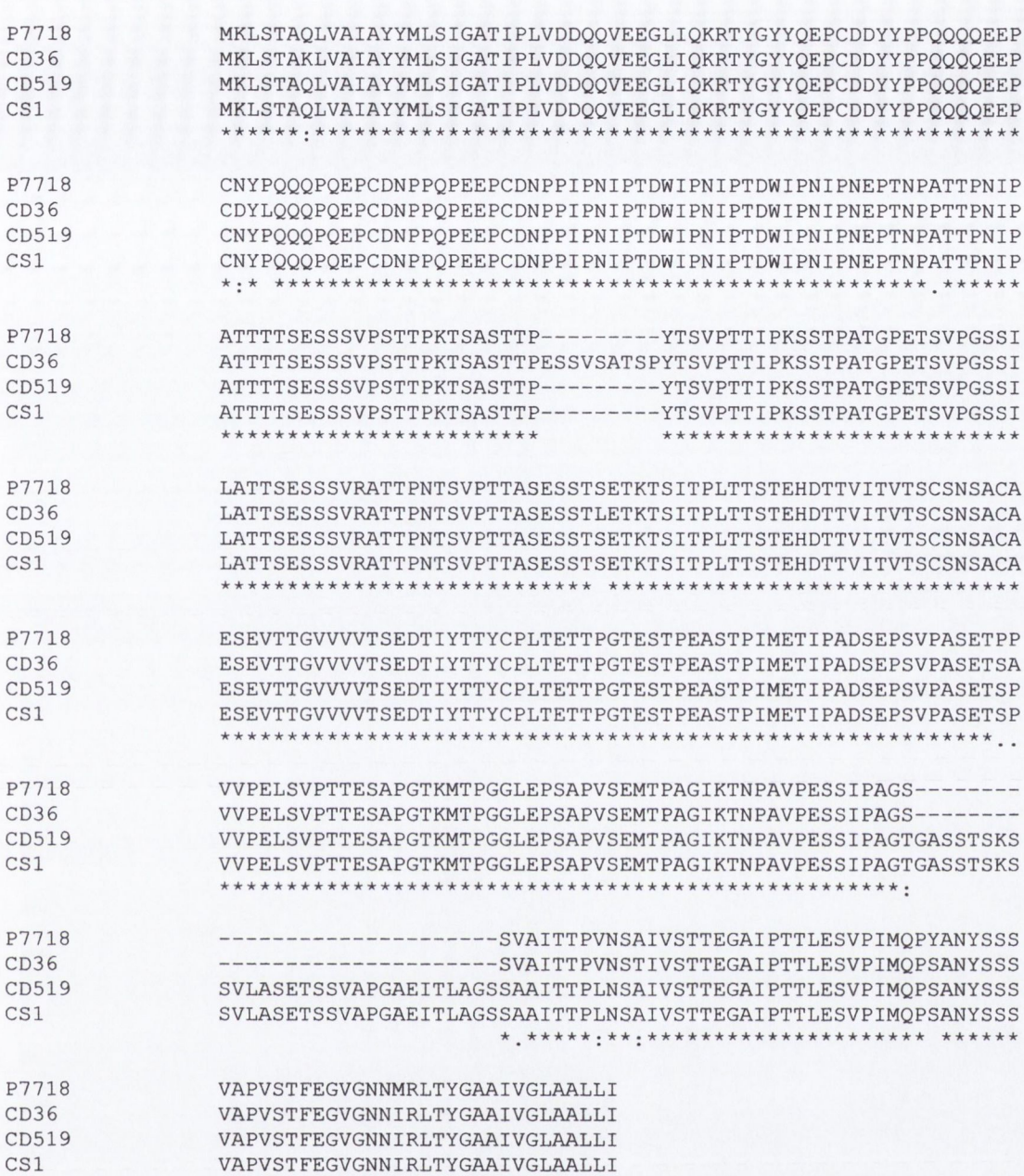

Figure 4.17. Alignment of Hwp1 from *C. dubliniensis* strains P7718, CD36, CD519 and CS1 generated with the CLUSTAL W sequence alignment program (Higgins & Sharp, 1988). Asterisks indicate identical residues, spaces indicate dissimilar residues and hyphens indicate gaps created to obtain alignment. The *C. dubliniensis* type strain CD36 contains 9 extra amino acids between residues 144 and 153 than the other strains. However strains CD519 and CS1 contain 29 extra amino acids between residues 352 and 381 than CD36 and P7718.

from strains CA411 and 52.1 were examined by sequence analysis. In each case two alleles of the gene were identified each differing from each other due to the presence of minor deletions (Fig. 4.18). The single *HWP1* allele in SC5314 corresponds to a protein of 634 amino acids, however in 52.1 the two alleles correspond to proteins of 614 (52.1 A) and 595 (52.1 B) amino acids, while the two alleles in CA411 correspond to proteins of 634 and 600 amino acids, respectively. The larger amplimer from strain CA411 was homologous to the amplimer obtained from strain SC5314. These findings explain the small size difference observed in the amplimers yielded from the three strains when using the primer set HWP1F-HWP1R and also the presence of two alleles for *HWP1* in some strains.

4.3.2.6 Analysis of *HWP1* expression in *C. dubliniensis* and *C. albicans* by Northern hybridisation

Expression of *CaHWP1* has previously been shown to occur during germ tube or hyphal growth. To investigate whether this also occurs in *C. dubliniensis,* three strains of *C. dubliniensis* (CD36, P7718 and CD519) and two strains of *C. albicans* (SC5314 and 132A) were grown in NYP, RPMI-1640 medium and water with 10% (v/v) serum, which are all media that have been shown to be conducive to hyphal production in *C. albicans*. These strains were chosen as they had previously been used in the kinetics of hyphal production experiments (see section 4.3.1.1 above). Total RNA was isolated at t=0,1,2,3,4,5,6 and t=24 h and probed consecutively with cloned amplimers of *CaHWP1* and *CdHWP1* (outlined in section 4.2.4). Cloned amplimers of *CdTEF3* and *CaTEF3* were also used as probes and their expression was used as an internal control for RNA loading. Northern hybridisation demonstrated that *HWP1* is expressed in both *C. albicans* strains and *C. dubliniensis* strains when cells are in the hyphal phase of growth. A *CaHWP1* transcript of approximately 2 kb was expressed by *C. albicans*, whereas a smaller transcript of approximately 1.3 kb was expressed by *C. dubliniensis.* When *C. albicans* strain SC5314 was examined in water with 10% (v/v) serum, a twelve-fold increase in expression was detected at t = 1 h, which rose to a twenty-fold increase in expression over the rest of the timepoints. As expected expression of *HWP1* was induced in *C. albicans* after 1 h in each medium and continued to be expressed at each of the timepoints sampled up to 24 h (Fig. 4.19). Time 0 h was used as a control as almost all cells were in the yeast phase and at which time no expression of HWP1 was

```
52.1 (B)        MRLSTAQLIAIAYYMLSIGATVPQVDGQGETEEALIQKRSYGYYQEPCDDYPQQQQQEEP
CA411           MRLSTAQLIAIAYYMLSIGATVPQVDGQGETEEALIQKRSYGYYQEPCDDYPQQQQQQEP
SC5314          MRLSTAQLIAIAYYMLSIGATVPQVDGQGETEEALIQKRSYDYYQEPCDDYPQQQQQQEP
52.1 (A)        MRLSTAQLIAIAYYMLSIGATVPQVDGQGETEEALIQKRSYGYYQEPCDEYPQQQQQ-EP
                ****************************************.*******:******* **

52.1 (B         CDYPQQQQQEEPCDYPQQQPQEPCDYPQQPQEPCDYPQQPQE------------------
CA411           CDYPQQQQQEEPCDYPQQQPQEPCDYPQQPQEPCDYPQQPQEPCDYPQQPQEPCDNPPQP
SC5314          CDYPQQQQQEEPCDYPQQQPQEPCDYPQQPQEPCDYPQQPQEPCDYPQQPQEPCDNPPQP
52.1 (A)        CDYPQQQQQEEPCDYPQQQPQEPCDYPQQPQEPCDYPQQPQEPCDYPQQPQEPCDNPPQP
                ****************************************

52.1 (A)        ----------------------PCDNPPQPDIPCDNPPQPDQPDDNPPIPNIPTDWIPNI
CA411           DVPCDNPPQPDVPCDNPPQPDIPCDNPPQPDIPCDNPPQPDQPDDNPPIPNIPTDWIPNI
SC5314          DVPCDNPPQPDVPCDNPPQPDIPCDNPPQPDIPCDNPPQPDQPDDNPPIPNIPTDWIPNI
52.1 (B)        DVPCDNPPQP--------------------DIPCDNPPQPDQPDDNPPIPNIPTDWIPNI
                                              ******************************

52.1 (A)        PTDWIPDIPEKPTTPATTPNIPATTTTSESSSSSSSSSSSSTTPKTSASTTPESSVPATT
CA411           PTDWIPDIPEKPTTPATTPNIPATTTTSESSSSSSSSSSS-TTPKTSASTTPESSVPATT
SC5314          PTDWIPDIPEKPTTPATTPNIPATTTTSESSSSSSSSSSS-TTPKTSASTTPESSVPATT
52.1 (B)        PTDWIPDIPEKPTTPATTPNIPATTTTSESSSSSSSSSSSSTTPKTSASTTPESSVPATT
                **************************************** ******************

52.1 (A)        PNTSVPTTSSESTTPATSPESSVPVTSGSSILATTSESSSAPATTPNTSVPTTTTETKSS
CA411           PNTSVPTTSSESTTPATSPESSVPVTSGSSILATTSESSSAPATTPNTSVPTTTTEAKSS
SC5314          PNTSVPTTSSESTTPATSPESSVPVTSGSSILATTSESSSAPATTPNTSVPTTTTETKSS
52.1 (B)        PNTSVPTTSSESTTPATSPESSVPVTSGSSILATTSESSSAPATTPNTSVPTTTTETKSS
                ************************************************************:***

52.1 (A)        STSLTTTTEHDTTVVTVTSCSNSVCTESEVTTGVIVITSKDTIYTTYCPLTETTPVSTAP
CA411           STPLTTTTEHDTTVVTVTSCSNSVCTESEVTTGVIVITSKDTIYTTYCPLTETTPVSTAP
SC5314          STPLTTTTEHDTTVVTVTSCSNSVCTESEVTTGVIVITSKDTIYTTYCPLTETTPVSTAP
52.1 (B)        STPLTTTTEHDTTVVTVTSCSNSVCTEREVTTGVIVITSKDTIYTTYCPLTETTPVSTAP
                **.********************** ********************************

52.1 (A)        ATETPTGTVSTSTEQSTTVITVTSCSESSCTESEVTTGVVGVTSEETVYTTFCPLTENTP
CA411           ATETPTGTVSTSTEQSTTVITVTSCSESSCTESEVTTGVVVVTSEETVYTTFCPLTENTP
SC5314          ATETPTGTVSTSTEQSTTVITVTSCSESSCTESEVTTGVVVVTSEETVYTTFCPLTENTP
52.1 (B)        ATETPTGTVSTSTEQSTTVITVTSCSESSCTESEVTTGVVVVTSEETVYTTFCPLTENTP
                **************************************** ******************

52.1 (A)        GTDSTPEASIPPMETIPAGSEPSMPAGETSPAVPKSDVPATESAPVPEMTPAGSQPSIPA
CA411           GTDSTPEASIPPMETIPAGSEPSMPAGETSPAVPKPEVPATESAPVPEMTPAG-------
SC5314          GTDSTPEASIPPMETIPAGSESSMPAGETSPAVPKSDVPATESAPVPEMTPAGSQPSIPA
52.1 (B)        GTDSTPEASIPPMETIPAGSEPSMPAGETSPAVPKSDVPATESAPVPEMTPAGSQPSIPA
                ********************.*************.:****************

52.1 (A)        GETSPAVPKSDVSATESAPAPEMTPAGTETKPAAPKSSAPATEPSPVAPGTESVPAGPGA
CA411           --------------------------TETKPAAPKSSAPATEPSPVAPGTESAPAGPGA
SC5314          GETSPAVPKSDVPATESAPAPEMTPAGTETKPAAPKSSAPATEPSPVAPGTESAPAGPGA
52.1 (B)        GETSPAVPKSDVPATESAPAPEMTPAGTETKPAAPKSSAPATEPSPVAPGTESAPAGPGA
                                        **************************.******

52.1 (A)        SSSPKSSVLASETSPIAPGAETAPAGSSGAITIPESSAVVSTTEGAIPTTLESVPLMQPS
CA411           SSSPKSSVLASETSPIAPGAETAPAGSSGAITIPESSAVVSTTEGAIPTTLESVPLMQPS
SC5314          SSSPKSSVLASETSPIAPGAETAPAGSSGAITIPESSAVVSTTEGAIPTTLESVPLMQPS
52.1 (B)        SSSPKSSVLASETSPIAPGTETAPAGSSGAITIPESSAVVSTTEGAIPTTLESVPLMQPS
                ******************:*****************************************
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```
52.1 (A)        ANYSSVAPISTFEGAGNNMRLTFGAAIIGIAAFLI
CA411           ANYSSVAPISTFEGAGNNMRLTFGAAIIGIAAFLI
SC5314          ANYSSVAPISTFEGAGNNMRLTFGAAIIGIAAFLI
52.1 (B)        ANYSSVAPISTFEGAGNNMRLTFGAAIIGIAAFLI
                *********************************
```

Figure 4.18. Alignment of Hwp1 from *C. albicans* strains (52.1, CA411 and SC5314) generated with the CLUSTAL W sequence alignment program (Higgins & Sharp, 1988). Asterisks indicate identical residues, spaces indicate dissimilar residues and hyphens indicate gaps created to obtain alignment.

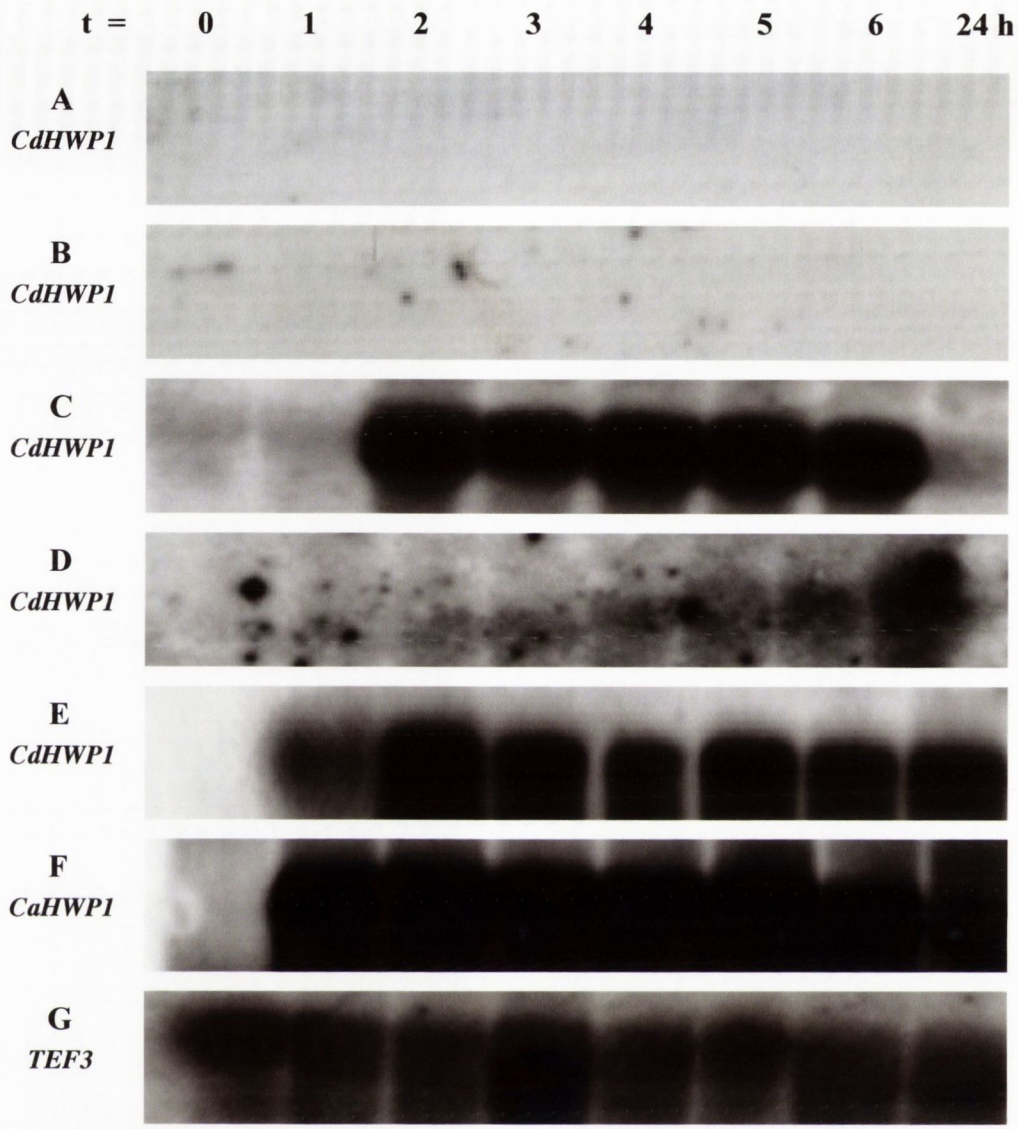

Figure 4.19. Expression of *CdHWP1* and *CaHWP1* in *C. dubliniensis* (CD36, P7718 and CD519) and *C. albicans* (SC5314) strains detected by Northern blot analysis of total RNA. Total RNA was extracted from cultures grown for t= 0,1,2,3,4,5,6 and t=24 h, respectively, in (A) RPMI-1640 medium (CD519), (B) NYP medium (CD519), (C) Water with 10% (v/v) serum (CD36), (D) RPMI-1640 (CD36), (E) water with 10% (v/v) serum (P7718) and (F) water with 10% (v/v) serum (SC5314). A total of 15 µg of each was electrophoresed on a denaturing agarose gel. Following transfer to a nylon membrane the blots were sequentially probed with radiolabeled DNA probes homologous to the cloned amplimers of *CdHWP1* and *CaHWP1* and the constitutively expressed *CdTEF3* gene. Expression of (G) *TEF3* was used as an internal control for RNA loading.

detected. In contrast, the levels of *HWP1* expression in each of the *C. dubliniensis* strains tested varied considerably. *Candida dubliniensis* strain CD519 showed no expression of *HWP1* when examined in NYP and RPMI-1640 media. Strain CD36 also showed no expression of *HWP1* when grown in NYP medium and very weak expression during later timepoints sampled during growth in RPMI-1640 medium. Expression of *HWP1* by *C. dubliniensis* strain CD36 following growth in water with 10% (v/v) serum was detected at t = 2,3,4,5 and 6 h. There was a fifteen-fold increase in expression after 2 h which was maintained until t = 6 h where it dropped to ten-fold and then dropped again to a two-fold increase in expression at t = 24 h in comparison with t =0 h. Expression of *HWP1* by *C. dubliniensis* strain CD36 following growth in RPMI-1640 medium was detected at t = 2,3,4,5,6 and 24 h. However, expression was very low in comparison with t = 0 h, with a five-fold increase in expression at t = 6 and a ten-fold increase at t = 24 h. *Candida dubliniensis* strain P7718 showed expression of *HWP1* in both NYP and water with 10% (v/v) serum after 1 h incubation and showed a twenty-four fold increase in expression in comparison with t = 0 h. This high level of expression continued over the rest of the timpoints and decreased to a eighteen-fold at t = 24 h. When the levels of *HWP1* expression were compared with the kinetics of hyphal production a strong correlation between the level of hyphae and *HWP1* transcript was found.

It is clear from these results that in *C. albicans HWP1* expression occurs faster and also at higher levels than in *C. dubliniensis*. No expression of *HWP1* was found for any of the *C. dubliniensis* or *C. albicans* strains tested at t=0.

4.3.3 Annotation of the *C. dubliniensis* genome sequence

Due to the recent *C. dubliniensis* genome sequencing project it is now possible to use the computer programme Artemis, to annotate individual *C. dubliniensis* genes in the raw sequence data. In order to begin to investigate the basis of the reduced capacity of *C. dubliniensis* to produce hyphae *in vitro* a survey of the recently acquired data from the *C. dubliniensis* genome sequencing project was examined using this programme. Artemis allows visualisation of sequence features and the results of analyses within the context of the sequence, and its six-frame translation (Rutherford *et al.*, 2000). The presence of sequence homology to each *C. albicans* gene known to be involved in hyphal production was first

determined (Fig. 4.20). In nearly every case a homologue was identified in *C. dubliniensis* (Table 4.6). The identity of each *C. dubliniensis* gene was then confirmed on the basis of its position in relation to neighbouring genes; i.e. its synteny.

An unusual observation was found for the Sra1 protein (cAMP-dependent protein kinase) in *C. dubliniensis*. When it was compared with the *C. albicans* protein, it was found that the *C. dubliniensis* protein was 132 amino acids larger than the *C. albicans* protein and was only 68.2 % homologous at the amino acid level. The sequence for the *C. dubliniensis* protein was then shortened to the next methionine due to the large size difference and compared again with the *C. albicans* protein, however, the resulting *C. dubliniensis* protein was 180 amino acids smaller in comparison with the *C. albicans* protein and only 5.1 % homologous at the amino acid level. The results regarding Sra1 are ambiguous and further analysis would be necessary to confirm which of these proteins if either are involved in the regulation of hyphae. These results show the presence of many homologues of the genes involved in hyphal regulation in *C. albicans*, in *C. dubliniensis*.

The presence of homologues of *C. albicans* genes expressed in the hyphal phase namely, *HWP1, ECE1, RBT4* and *ALS3* were also examined in the *C. dubliniensis* genome sequence. Use of the *C. dubliniensis* genome sequence confirmed the low homology of HWP1 (49%) between the *C. albicans* and *C. dubliniensis* genes. Many of these proteins had a homologue in the unannotated *C. dubliniensis* sequence, Ece1 69% and Rbt4 67%. However, difficulty was incurred in investigation of the presence of a homologue in *C. dubliniensis* of Als3 which may be due to the results published by Hoyer *et al.,* which indicate that *CdALS3* is closely related to *CaALS4* which would account for the difficulty incurred in trying to find a homologue in *C. dubliniensis* to *CaALS3* (Hoyer *et al.*, 2001).

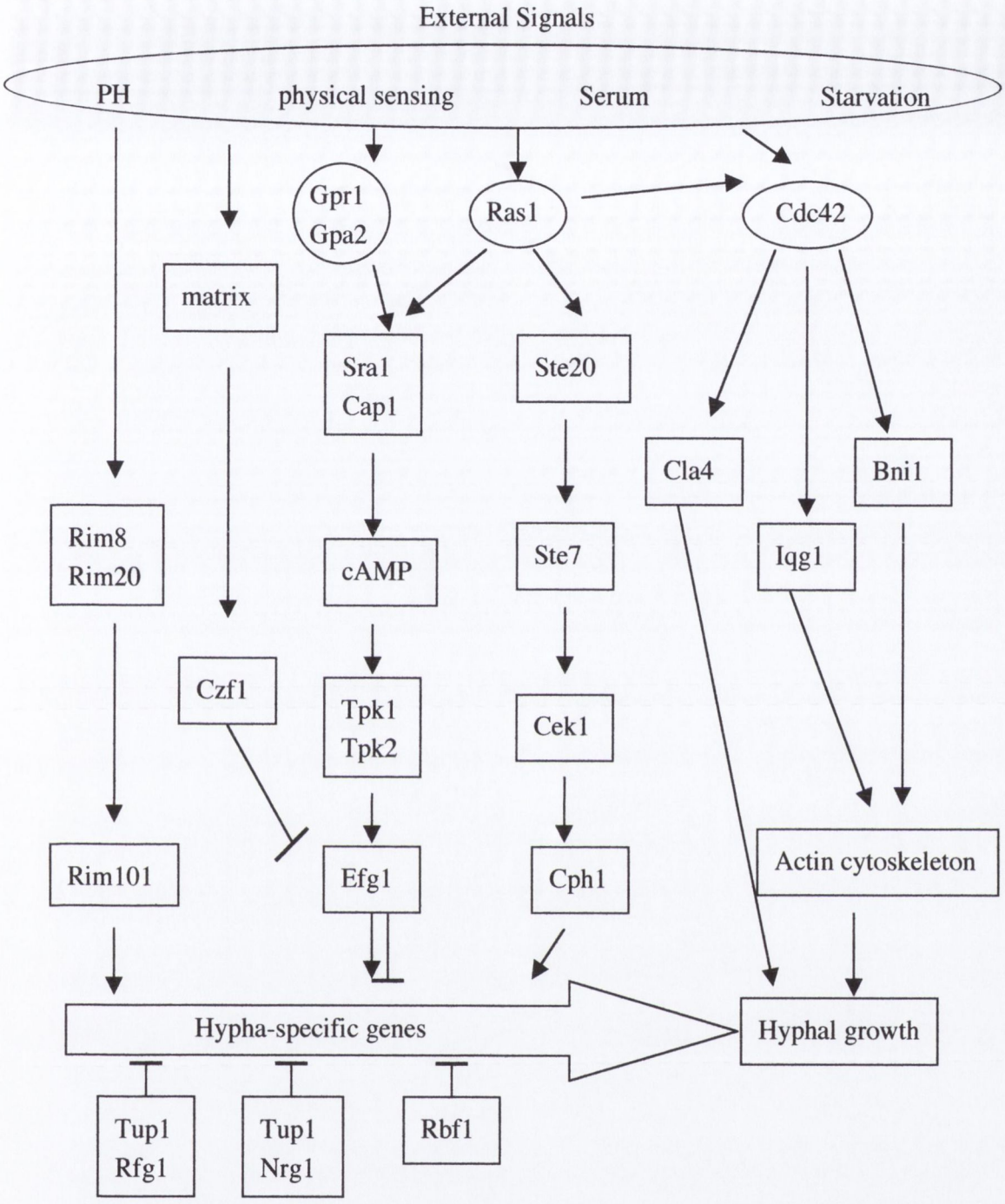

Figure 4.20. A schematic representation of the regulation of the yeast to hyphae transition *in C. albicans*, based on figures presented in (Bassilana *et al.*, 2003; Lane *et al.*, 2001; Liu, 2001; Rottmann *et al.*, 2003; Sohn *et al.*, 2003). Blunt ended arrows represent negative regulation.

Table 4.6. Genes involved in the regulation of the yeast to hyphae transition in *C. albicans* and their similarity to genes in *C. dubliniensis*.

		Gene	Protein
Name	Function	% similarity between *C. albicans* and *C. dubliniensis*	
BNI1	Regulator of budding	89	93
CAP1	Transcriptional activator	87	91
CEK1	Mitogen-activated protein kinase	83	90
CDC42	Cell division control	97	100
CLA4	Protein kinase homologue	87	91
CPH1	Transcription factor	88	90
CZF1	Canal zinc finger protein	79	82
EFG1	Enhanced filamentous growth factor	65	71
GPA2	Nucleotide-binding regulatory protein	87	92
GPR1	Similar to *S. cerevisiae* Gpr1p G-protein coupled receptor	80	88
IQG1	RAS GTPase-activating-like protein	89	94
NRG1	Transcriptional repressor	67	79
RAS1	GTP-binding protein	86	88
RBF1	Transcription factor	79	90
RFG1	Transcriptional regulator of filamentous growth and hyphal genes	74	76
RIM8	Regulator of pH response	79	90
RIM20	Rim101 activating protein	85	86
RIM101	Zinc finger transcription factor homologue; regulator of pH response	78	84
SRA1	cAMP-dependent protein kinase	49 or 65[a]	5 or 68[a]
STE7	MAP kinase kinase	87	93
STE20	Serine/threonine-specific protein kinase	79	85
TPK1	cAMP-dependent protein kinase 2	91	98
TPK2	cAMP-dependent protein kinase 2	89	94
TUP1	General transcriptional repressor	91	75

[a] The results for *SRA1* are ambiguous due to the unconfirmed position of the ATG start codon.

4.4 Discussion

4.4.1 Induction of hyphae under selected environmental conditions

4.4.1.1 Hyphal induction in liquid medium

One of the most important virulence factors of *C. albicans* is its ability to form true unconstricted germ tubes (Lo *et al.*, 1997). This is a trait shared by the closely related species, *C. dubliniensis*. However, although this feature has been examined extensively in *C. albicans*, a comparison of hyphal induction in each species has only been performed under a limited number of conditions (Gilfillan *et al.*, 1998). The aim of this part of the present study was to perform a thorough comparison of the kinetics of hyphal production under a wide range of conditions previously shown to induce hyphae in *C. albicans*.

In this study hyphal production following incubation in hyphal-inducing media was found to be rapid for all *C. albicans* strains tested and within 3 h 50% or more of cells were in a hyphal phase in 9/10 media examined. In the remaining medium (YNB) >50% of cells were found to be in the hyphal phase after 5 h for all *C. albicans* strains. In contrast in *C. dubliniensis* where very few strains reached a level whereby 50% or more of cells were in a hyphal phase e.g in water with 10% serum which showed the greatest levels of hyphal production, 8/11 strains of *C. dubliniensis* produced > 50 % of cells in a hyphal phase after 5 h (Figs. 4.3 and 4.4.). *Candida dubliniensis* strain P7718 (genotype IV) was an unusual isolate as it produced hyphae at levels equivalent to *C. albicans* in many of the hyphal inducing media examined.

In addition, in all of the hyphal inducing media examined all strains of *C. albicans* produced significant amounts (> 50%) of hyphae (Table 4.3), whereas in many cases only some strains of *C. dubliniensis* produced hyphae in each of the media examined and in most cases where hyphae were present they were usually in low amounts (<30%). The difference between both species was found to be significant (P <0.05) in all of the media examined at both 2 and 6 h except in RPMI-1640 medium (Table 4.4), which showed that after 2 h the difference between the species was not significant. This discrepancy is due to the low levels of hyphal production shown by *C. albicans* strains 52.1 and JP14CA in RPMI-1640 medium. The data obtained from this study clearly indicate that induction of hyphae is less efficient

and less rapid for *C. dubliniensis* than *C. albicans* when examined using all of the media listed in Table 4.1.

These results confirm the findings of Gilfillan *et al.*, who showed that hyphal production in *C. dubliniensis* was slower than *C. albicans* when hyphae were induced in water with 10% (v/v) serum and Lee's medium under a pH and temperature shift (Gilfillan *et al.*, 1998). However, in the Gilfillan *et al.* study only two strains of *C. albicans* and two strains of *C. dubliniensis* were examined with three hyphal inducing media (Gilfillan *et al.*, 1998). In the present study for both species the addition of serum to induction media resulted in an increased production of hyphae, however, the amount of hyphal production by *C. dubliniensis* does not approach that of *C. albicans*. Serum induces the yeast to hyphal form in *C. albicans* via several pathways (Madhani & Fink, 1998). Due to the complex nature of serum no single component has been identified that induces the morphological change in *Candida*. Quinghua *et al.*, showed that serum albumin was not the likely inducing component of hyphae but rather other molecules that are co-purified with albumin as small molecules which had been filtered out of the serum did induce hyphae in *Candida* (Qinghua *et al.*, 1999). Recently Hudson *et al.* suggested that the hyphal-inducing ability of serum is due to its nutrient properties, mainly nitrogen and carbon (Hudson *et al.*, 2004). It has also been shown though that farnesol a quorum sensing molecule may be responsible for inhibiting hyphal formation in stationary phase cells (Hornby *et al.*, 2001).

In conclusion, these findings confirm that *C. dubliniensis* strains are capable of producing hyphae under specific conditions, but that hyphal production is significantly less efficient and more restricted in comparison with *C. albicans*. Induction of hyphae was both strain and medium dependent for *C. dubliniensis* as not all strains tested produced hyphae in the media examined. The results also indicate that the presence of serum in the medium had a significant positive effect on the amount of hyphae that were produced in *C. dubliniensis* and also on the speed of hyphal production in *C. albicans*.

4.4.1.2 Hyphal induction on solid media

Solid media were also used to compare hyphal production between *C. albicans* and *C. dubliniensis* (Table 4.5). Spider medium was examined as it has previously been shown to induce hyphal formation via different pathways compared to liquid medium for *C. albicans*

(Liu *et al.*, 1994). The results show that hyphal induction was strain dependent on Spider medium and no direct correlation could be made between the ability of strains of *C. dubliniensis* to produce hyphae compared to strains of *C. albicans* on this medium as strains of both species produced hyphae. Embedded conditions were also examined using a sandwich of yeast extract-peptone-sucrose (YPS) medium whereby cells were plated between two solidified layers of YPS medium and incubated at 25°C for five days (Fig. 4.6). Eight out of eleven strains of *C. dubliniensis* and 4/4 strains of *C. albicans* tested produced hyphae when embedded in this medium. Previously, these conditions have been used for *C. albicans* and filamentous growth was found after 3-4 days only when cells were sandwiched (Douglas *et al.*, 1999). No hyphae were observed in this study after 5 days for *C. albicans* and *C. dubliniensis* growing on the surface of YPS medium. The data from this part of the study show that hyphal production was less efficient for strains of *C. dubliniensis* as not all strains produced hyphae, in comparison all strains of *C. albicans* produced hyphae when grown in the embedded situation between two slabs of YPS agar. The results suggest that it was not the chemical composition of YPS medium that induces filament formation but rather the conditions of growth. Indeed, it has been hypothesised that embedded condition *in vitro* resemble those encountered by *C. albicans in vivo* (Brown & Gow, 1999; Doedt *et al.*, 2004; Ernst, 2000), where the organism grows under reduced oxygen tension and an increasing CO_2 concentration in contact with surrounding extracellular matrix.

While *C. dubliniensis* seems to be less inclined to produce hyphae in liquid and solid media routinely used for hyphal induction in *C. albicans*, it is interesting to note that *C. dubliniensis* can produce hyphae under specific conditions where *C. albicans* strains are unable to produce hyphae at all. Recently Pal's and Staib media have been described as being useful tests for discrimination between *C. albicans* and *C. dubliniensis* isolates based on colony morphology. When incubated on Staib and Pal's agar *C. albicans* grows as smooth, glistening colonies that are comprised solely of yeast cells. In contrast, *C. dubliniensis* grows as rough colonies with a hyphal fringe which are comprised of pseudohyphae, hyphae and chlamydospores (Al Mosaid *et al.*, 2003; Al Mosaid *et al.*, 2001). Staib *et al.* have shown that when grown on Staib medium, *NRG1* (general transcriptional repressor) acts as a repressor of chlamydospore formation in *C. albicans* and expression of *CaNRG1* in *C. dubliniensis* repressed pseudohyphae and chlamydospore formation, whereas expression of

CaPDE2 (high affinity cAMP phosphodiesterase) only reduced the extent of filamentous growth but did not affect chlamydospore formation (Staib & Morschhauser, 2005).

In conclusion, strains of *C. dubliniensis* are capable of producing hyphae. However, both the physical and chemical environment used in inducing hyphae are crucial for filament formation. These results may suggest that the pathways controlling filament formation in response to different inducing conditions in *C. dubliniensis* are different to *C. albicans.* The results from this part of the present study reflect clinical observations whereby *C. dubliniensis* is clearly less important than *C. albicans* and may also indicate that the inability of *C. dubliniensis* to produce significant amounts of hyphae under certain conditions may be a contributing factor as to why *C. dubliniensis* is not as readily seen in systemic infection as it may not be as efficient as *C. albicans* at penetrating epithelial layers.

4.4.2 Analysis and investigation of *HWP1* in *C. dubliniensis*

4.4.2.1 Investigating the presence of a homologue of *HWP1* in *C dubliniensis*

Adherence is one of the most important determinants of microbial pathogenesis, yet very few adhesins have been identified from fungal pathogens. Four structurally related adhesins, Hwp1, Ala1/Als5, Als1, from *C. albicans* and Epa1 from *C. glabrata* are members of a class of proteins termed glycosylphosphatidylinositol-dependant cell wall proteins (GPI-CWP). These proteins possess distinguishing features including N-terminal signal peptides and C-terminal features that mediate glycosylphosphatidylinositol (GPI) membrane anchor addition that leads to attachment to cell wall glucan. The hyphal wall protein Hwp1 has been described in *C. albicans* as an important adhesin and is thought to be a significant virulence factor (Sundstrom, 2002). The importance of Hwp1 in systemic candidiasis has been clearly demonstrated with *C. albicans* derivatives where the *HWP1* gene was deleted. In these experiments mice infected with null mutant strains did not succumb to candidiasis. However, when inoculated with isogenic strains harbouring a single copy of the *HWP1* gene the mice survived only 2-3 weeks (Sundstrom, 2002). Due to the importance of Hwp1 in virulence and the fact that it is also produced by hyphal cells in *C. albicans,* the purpose of this part of the present study was to determine whether a homologue of *HWP1* is present in *C. dubliniensis* and if so, to compare its sequence with that of *C. albicans*.

4.4.2.2 Sequence analysis of *HWP1*

A homologue of *HWP1* was identified in *C. dubliniensis* in this study, but, was found to be significantly different in size (1.3 kb) compared to *CaHWP1* (2 kb), (Fig. 4.9). Nucleotide sequence analysis of the *CdHWP1* gave an insight into the differences between the *C. albicans* and *C. dubliniensis* genes. *Candida albicans HWP1* encodes a series of tandem repeats 10 amino acids in length with a 27% proline, 16% glutamine and 12% aspartate content (Sundstrom, 1999). The NH_2-terminal domain of Hwp1 is rich in proline and glutamine making the region very similar to mammalian transglutaminase (Tgase) substrates. Transglutaminases are capable of cross-linking small proline rich proteins, such as that found in the N-terminus of *C. albicans* Hwp1 to human proteins in keratinised epithelia. All genes described to date from *C. dubliniensis* are approximately 90% identical to those in *C. albicans*. Despite this numerous attempts were made to amplify *HWP1* from *C. dubliniensis* using primers designed on the internal sequence of *C. albicans*. Finally, the primer set HWP1F/HWP1R was used whereby the forward primer HWP1F contained the start codon ATG and the reverse primer HWP1R contained the stop codon TAA in order to amplify the entire coding sequence of the gene. The sequence of the amplimer described above using the type strain CD36 was compared with the published sequence of *C. albicans HWP1* (Genbank accession no. U64206), and *CdHWP1* was found to contain numerous deletions (Fig. 4.10). The predicted Hwp1 protein from *C. dubliniensis* is only 49.3% homologous to the *C. albicans* Hwp1. Interestingly, many of the deletions in the *C. dubliniensis* Hwp1 are in the proline and glutamine rich region in the N-terminal domain of the protein and these deletions are in the region that have been reported in the literature to mediate the adhesive properties of the protein suggesting that the *C. dubliniensis* Hwp1 could have reduced functionality in comparison to *C. albicans.*

Candida albicans Hwp1 is a member of the glycosylphosphatidylinositol-dependent cell wall proteins (GPI-CWP) which contain N-terminal signal peptide and C-terminal features that mediate glycosylphosphatidylinositol (GPI) membrane anchor addition (Sundstrom, 2002). The GPI-signal region is composed of three domains a GPI attachment region comprising ω, ω+1, and ω +2 sites: a spacer of 5-10 amino acids; and a hydrophobic stretch of 10-15 amino acids. A protein containing the GPI-signal is cleaved at the ω site, and the resulting carboxyl terminus of the protein becomes covalently bound to a GPI moiety

(Hamada *et al.*, 1998). Proteins that become cross-linked to cell wall β-1,6-glucan are a subset of GPI-proteins, which contain isoleucine, valine or leucine 4-5 residues upstream from the ω site. On examination of the sequence of the *C. albicans* Hwp1, an isoleucine residue is present at ω-5 whereas *C. dubliniensis* has a valine residue, which indicates that it falls into the subset of these proteins that are incorporated into the cell wall (Fig. 4.15). Therefore it is possible that the *C. dubliniensis* Hwp1p may like *C. albicans* Hwp1 become cross linked to β-(1,6)-glucan of the cell wall. Other proteins, which share this property, are Rbt1, Als3, Ala1/Als5, Als6, Als8, Als9 and Hyr1 (Sundstrom, 2002).

In order to confirm that the entire *CdHWP1* gene had been amplified and sequenced it was necessary to sequence the region upstream of the ORF. While confirming that the entire ORF sequence had been obtained this also allowed the promoter of *CdHWP1* to be compared with the *C. albicans* promoter. A number of hypha-specific genes harbour E-boxes (5'-CANNTG-3') in their promoters. Several signaling pathways appear to activate hyphal development, and one of these pathways is defined by the factor Efg1. Mutations that inactivate Efg1 block hyphal development under most experimental conditions *in vitro* and *in vivo* (Lo *et al.*, 1997; Stoldt *et al.*, 1997). Promoters which contain E-boxes include *ALS8*, *HYR1*, *ALS3*, *HWP1*, *ECE1*, *RBT4* (Leng *et al.*, 2001). On analysis of the 5' upstream sequence of *CdHWP1*, 5 putative E-boxes are present upstream of the start codon, however, only 2 of these are 1000 bp upstream of the start codon, which would be the putative promoter region (Fig. 4.9). *Candida albicans HWP1* contains 6 E-boxes based on the published sequence of *CaHWP1* (Genbank accession no. U64206) however, only 3 of these are 1000 bp upstream. It has been confirmed that Efg1 is a sequence-specific DNA binding protein that is capable of binding the E-box, and that most hypha-specific genes contain E-boxes in their promoters. However, *RBT1* is the exception as it doesn t contain an E-box in the 5' region even though it is regulated by Efg1, although there may be an indirect regulation (Leng *et al.*, 2001). It has also been suggested that although the E-box sequence is present at multiple sites upstream of hyphae-specific genes, it also appears at approximately the same frequency upstream of randomly chosen genes (Liu, 2001). These results suggest that Hwp1

in *C. dubliniensis* may be regulated by Efg1 like *C. albicans*, however further work is necessary to confirm this.

In this study there was also a size difference (1.3 kb-1.4 kb) observed for *HWP1* between different strains of *C. dubliniensis* when amplified with the primer set HWP1F/HWP1R designed on the entire *C. albicans* coding (Fig. 4.16). The *HWP1* gene from the *C. dubliniensis* type strain CD36 was found to encode 9 more amino acids than *C. dubliniensis* strains CS1, CD519 and P7718, whereas CD519 and CS1 contained 29 more amino acids between residues 352 and 381 than CD36 and P7718 (Fig. 4.17). This level of variation is quite high and may be due to inaccurate replication due to the presence of repeats or deletions.

Also there were two amplimers obtained from *C. albicans* strains JP14CA, CA411 and 52.1 when amplified with the primer set HWP1F/HWP1R, which indicates the presence of two alleles and therefore heterozygosity. These amplimers were cloned and sequenced and it was revealed that they contained deletions in comparison with the reference strain SC5314. Southern hybridisation analysis showed that for two strains of *C. albicans* JP14CA and CA411 two hybridising bands were detected with the *C. dubliniensis* probe. This confirms the presence of two alleles, which was outlined earlier. A similar difference between two alleles has also been found for *ALS7* in *C. albicans*. Zhang *et al.*, found 60 alleles of the *ALS7* open reading frame upon examination of 66 *C. albicans* strains and these deletions were mainly due to rearrangements of repeat elements in the tandem repeat domain (Zhang *et al.*, 2003)

In conclusion, a homologue of *HWP1* exists in *C. dubliniensis,* however, it contains numerous deletions in comparison with *CaHWP1*. These deletions account for the difference in size of amplimers obtained for *CaHWP1* (2 kb) and *CdHWP1* (1.3 kb) using the primer set HWP1F-HWP1R. These deletions of proline and glutamine residues were also found to be in the N-terminal region, which is crucial for the function of the protein in *C. albicans*. A small size difference was also observed between amplimers obtained when genomic DNA from strains of *C. dubliniensis* were amplified with the primer set HWP1F-HWP1R and also the presence of two alleles when strains of *C. albicans* were examined. The presence of these deletions may explain why *C. dubliniensis* is not detected as readily as *C. albicans* as it may not be able to adhere to host tissue and penetrate epithelium as efficiently as *C. albicans*.

4.4.2.3 Expression of *HWP1*

Expression of *HWP1* in *C. dubliniensis* was found to occur in cells in the hyphal phase and not in cells which were in a yeast phase. Similar findings have previously been reported for *C. albicans* (Sharkey *et al.*, 1999). The level of expression of *HWP1* was also significantly greater for *C. albicans* than for *C. dubliniensis.*

In conclusion, these results show that like *CdHWP1*, like *CaHWP1* is expressed only on the surface of germ tubes and hyphae for *C. dubliniensis,* as strains that were in a yeast phase did not show expression of *HWP1* whereas cells which were in a hyphal phase showed expression of *HWP1* when probed with the cloned amplimer of *CdHWP1.* These results may suggest that *C. albicans HWP1* is regulated differently and also to different levels than in *C. dubliniensis HWP1.* In addition, the results also suggest that *HWP1* is regulated conditionally as there is no expression of *HWP1* in *C. albicans* or in *C. dubliniensis* when cells are in the yeast phase and so it is upregulated in response to the hyphal inducing media.

4.4.3 Annotation of the *C. dubliniensis* genome sequence

Candida albicans has many genes that are known to be associated with the transition and regulation of the change from yeast to hyphal cells. In this study homologues of genes encoding many of these proteins in *C. dubliniensis*, with many of the proteins showing a high homology to those found in *C. albicans* (Table 4.6). This may indicate a similar regulation of genes involved in the hyphal pathway in *C. dubliniensis,* however, more work is necessary to elucidate the regulation of these genes in *C. dubliniensis.* Examination of proteins expressed in the hyphal phase namely, Hwp1, Ece1, Rbt4 and Als3 were also examined in *C. dubliniensis.* All of this proteins showed high levels of homology to the corresponding proteins in *C. albicans* except Hwp1 which was only 49% homologous and no homologue was observed for Als3 however this is not surprising as previous studies have incurred difficulty in identifying the *ALS* gene family in *C. dubliniensis* (Hoyer *et al.*, 2001; Moran *et al.*, 2004), however, it has been suggested that that *CdALS3* is closely related to *CaALS4* and hence this is why no homologue to Als3 was found in *C. dubliniensis.*

The results from this part of the present study indicate the usefulness of the DNA viewer programme Artemis, and also signifies the advent of the fully annotated *C.*

dubliniensis genome to compare with the already annotated *C. albicans* genome, which will allow further comparisons between the two species.

Chapter 5

Comparison of growth of *C. albicans* and *C. dubliniensis* in an *ex vivo* model

5.1 Introduction

5.1.1 Reconstituted Human Epithelium (RHE)

The majority of studies designed to investigate the virulence of *Candida* strains and specific mutants have relied exclusively upon the use of animal infection models, in particular the systemic mouse infection model. However, this model is difficult to standardise and there are increasing difficulties in performing animal based studies. For these reasons, researchers have recently begun to investigate the use of *ex vivo* infection models to study virulence. One of the most commonly used *ex vivo* models is based on the use of Reconstituted Human Epithelium (RHE) which is commercially available.

The RHE models available resemble a wide variety of epithelial surfaces including oral, vaginal, skin and other cell types. Oral RHE was obtained by culturing transformed human keratinocytes of the TR146 cell line which was derived from squamous cell carcinoma cells from the buccal mucousa on an inert supporting membrane (Rupniak *et al.*, 1985). The benefits of using the RHE model is that the model can be standardised, is relatively inexpensive, can be used with a large number of strains, is readily available, reproducible, and allows direct analysis of the interaction between *C. albicans* and epithelial cells at the mucousal surface. An important advantage of the RHE model is that its multilayer structure, which can consist of up to 12 layers, closely resembles the oral epithelium, is in stark contrast to traditional single layer tissue culture systems. Furthermore, the RHE model closely resembles the internal milieu of the oral cavity within a pH range of 7.0-7.4. Korting *et al.* have also shown that histologically RHE resembles the normal human oral epithelium and that pathological changes associated with human disease are similar to what occurs when RHE is infected with *C. albicans* (Korting *et al.*, 1998).

The RHE model has previously been used to examine the function of the various Als proteins and the role each plays in the biology and pathogenesis of *C. albicans* (Zhao *et al.*, 2005; Zhao *et al.*, 2004) and also the role of the secreted aspartyl proteinases (Saps) in tissue damage (Korting *et al.*, 1998; Korting *et al.*, 1999; Korting *et al.*, 2003; Schaller *et al.*, 2004). The role of the immune system has also been examined using the RHE model by supplementing the model with polymorphonuclear leukocytes (Schaller *et al.*, 2004), and

more recently, an investigation of ultrastructural and histochemical features of invasive candidiasis in humans has been performed using the RHE model (Jayatilake *et al.*, 2005).

One of the great advantages of the RHE model is its potential use to quantify tissue damage. Lactate dehydrogenase (LDH) is a cytosolic enzyme that is present in all mammalian cells. The normal plasma membrane is impermeable to LDH, but damage to the cell membrane results in a change in the membrane permeability and subsequent leakage of LDH into the extracellular fluid. This release of LDH provides an accurate measure of cell membrane integrity and viability and has previously been examined in *C. albicans* (Korting *et al.*, 2003).

Previous studies have examined the ability of *C. albicans* cells to attach and penetrate through the epithelial layers and cause membrane damage. This part of the present study is the first study to my knowledge to examine the ability of *C. dubliniensis* to attach, penetrate and cause cell damage using the RHE model and LDH assay and allows a direct and accurate comparison to be made with the effects of *C. albicans* on this tissue.

5.2 Materials and Methods

5.2.1 *Candida* isolates

The *Candida* strains used in this part of the study are listed in Table 3.1. Derivatives of two strains of *C. albicans* SC5314 and 132A which had been tagged with a molecular marker cassette containing MPA^R and GFP were used and a derivative of *C. dubliniensis* strain CD36 which was also tagged with the molecular marker cassette were used. The methods used to develop this cassette and insert it into these strains is outlined in Chapter 3. Two other *C. dubliniensis* strains CD519 and P7718 were also examined.

5.2.2 Culture media and growth conditions

Inocula were prepared by culturing yeast cells for 24 h at 37°C on PDA. Cultures were washed three times in PBS and counted using a haemocytometer (Neubauer). A sample of approximately 2 x 10^5 cells was suspended in 10 ml of YPD and cultured for 16 h at 25°C in a shaking Clifton waterbath (Nickel-Electro Ltd. Weston-Super-Mare, North Somerset, UK). Following growth a suspension of 4 x 10^6 cells was transferred to fresh YPD medium and incubated with shaking for 24 h at 37°C, washed three times in PBS and resuspended in 1 ml of PBS and the final inoculum was adjusted to the desired density with PBS solution.

5.2.3 Reconstituted Human Epithelium (RHE)

The oral human epithelium for the *in vitro* model of oral candidiasis was purchased from Skinethic Laboratory (Nice, France). It was originally obtained by culturing transformed human keratinocytes of the TR146 cell line which was derived from squamous cell carcinoma cells from the buccal mucousa on an inert supporting membrane (Rupniak *et al.*, 1985). Cultures were incubated in serum-free conditions in a defined maintenance medium based on the MCDB-153 medium (Clonetics, San Diego, California, USA), containing 5 µg/ml of insulin, on a 0.5 cm^2 microporous polycarbonate filter for 7 days. Each RHE sample was placed in 6 well tissue culture plates (Corning Inc., Corning, NY, USA) with 2 ml of maintenance medium. This fully defined medium feeds the basal cells

through the filter substratum (Fig. 5.1). The *in vitro* model and all culture media were prepared without antibiotics and antimycotics.

Triplicate infection experiments were performed for each strain and each timepoint. Reconstituted epithelium samples were infected with 2 x 10^6 yeast cells in 50 µl of PBS and controls were inoculated with 50 µl of PBS alone. Inoculated cultures were incubated at 37°C with 5% CO_2 at 100% humidity for 12, 24, 36, 48, 60 and 72 h. Each experiment was performed in triplicate and at each time point 50 µl of PBS was added to the supernatant of two of these infections for each strain, the third was used for histological analysis. Phosphate buffered saline was used to wash the non-adherent cells from the surface of the tisssue. The supernatant was plated out onto YNB media and incubated for 48 h at 37°C to determine the level of *Candida* cells present, which were located in the supernatant and had not attached to the epithelial surface.

5.2.4 Lactate dehydrogenase assay

The release of lactate dehydrogenase (LDH) from epithelial cells into the surrounding medium was monitored as a measure of epithelial cell damage. Lactate dehydrogenase release into the maintenance media of cultures from infected and uninfected epithelial cells was measured at 12, 24, 36, 48, 60 and 72 h. To determine the amount of LDH, which is a stable cytosolic enzyme that is released upon cell lysis, the CytoTox 96® non-radioactive cytotoxicity asssay (Promega) was used. This assay is based upon a coupled enzymatic assay involving the conversion of a tetrazolium salt, 2-ρ-(iodophenyl)-3-(ρ-nitrophenyl)-5-phenyltetrazolium chloride (INT), into a red formazan product. The amount of colour formed is proportional to the number of lysed cells. At each timepoint 500 µl of culture medium was removed and stored in the dark at -20°C. This was then thawed and 50 µl was placed in a 96 well round bottomed plate (Greiner bio-one Ltd., Stonehouse, Gloucestershire, UK) and used in the assay. The reaction was stopped by the addition of 50 µl of stop solution and the absorbance at 480_{nm} was determined in a Genios plate reader (Tecan UK Ltd., Reading, UK). One unit of LDH activity is equivalent to 1 mmol of formazan formed

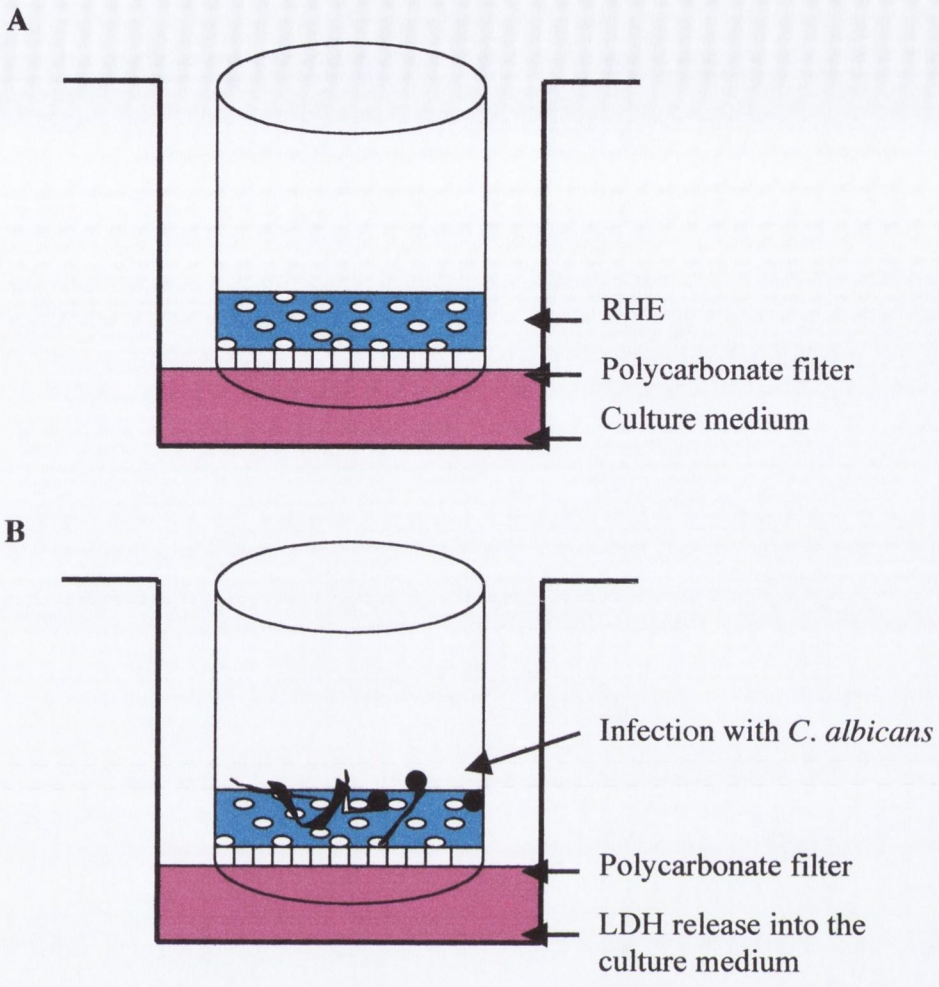

Figure 5.1. Schematic diagram of the experimental design in this study. (A) RHE on a microporous polycarbonate filter, fed by a fully defined nutrient medium through the filter substratum (B) Infection of RHE by *C. albicans*.

min/l. Mean values were calculated from triplicate values of tripicate experiments, therefore the mean values are indicative of nine replicates ± standard deviation.

5.2.5 Histological analysis of RHE tissue

At each time point tissues were cut away from the plastic casing and placed in 4% paraformaldehyde (v/v in PBS; 0.1 M, pH 7.4) and stored at 4°C. Tissues were then washed with buffer, dehydrated in ethanol and embedded in paraffin wax. Sections were stained with Periodic Acid Schiff (PAS) reagent for demonstration of fungal elements (Luna, 1968). Smears of the tissue were also created by using a forceps and rubbing the tissue onto a microscope slide. Tissues were examined using a Nikon Eclipse 600 microscope (Nikon Europe). Fluorescence was detected using a Nikon Eclipse 600 microscope (Nikon) fitted with a super high power mercury lamp (Nikon) and the GFP specific filter set (Endow GFP Bandpass Emission (FGP®-BP) filter combination), (Nikon).

5.2.6 Statistical analysis

Statistical analysis was carried out using the two sample *t*-test assuming unequal variances. *P* values of <0.05 were considered significant.

5.3 Results

5.3.1 Morphology of uninfected and infected RHE

Reconstituted human epithelium which is derived from the buccal mucousa of the squamous cell carcinoma cell line TR146 that had been cultured on an inert supporting membrane was used as a model of the oral epithelium as it has previously been shown that pathological changes that occur using this model shows major similarity with what occurs during human oral infection (Korting *et al.*, 1998). Reconstituted human epithelium cells were inoculated with *C. albicans* derivatives SC5314 (M/G) and 132A (M/G) and one *C. dubliniensis* derivative CD36 (M/G) and two *C. dubliniensis* strains CD519 and P7718. Strains SC5314 (M/G), 132A (M/G) and CD36 (M/G) had been tagged with the molecular marker cassette to aid visualisation of the cells. *Candida dubliniensis* strains P7718 and CD519 were chosen as they are clinical isolates which have been used previously in the kinetics of hyphal induction. In section 4.3.1 it was shown that strain CD519 does not produce hyphae readily under a wide range of hyphal inducing conditions whereas strain P7718 showed high levels of hyphal production, and using some media, produced levels similar to *C. albicans.* In order to investigate the comparative effect of *C. albicans* and *C. dubliniensis* when incubated with oral RHE, 2 x 10^6 cfu/ml of each species was added to each RHE well and incubated for 72 h. At specific time points every 12 h the level of damage done to the RHE by *Candida* cells was examined microscopically and by measuring LDH levels in the surrounding culture medium.

In general over the time course the structural features of RHE resembled the normal oral epithelium (Fig 5.2 A). Both *C. albicans* derivatives SC5314 (M/G) and 132A (M/G) produced yeast and hyphal cells after 12 h on RHE (Fig. 5.2 B). Hyphal elements were visible penetrating the epithelial cells and changing their direction of penetration. After 12 and 24 h post-infection with *C. albicans*, the RHE showed signs of tissue damage, characterised by oedema, vacuolisation and detachment of the keratinocytes in all cell layers. Invasion of deeper parts of the epithelium by *C. albicans* was observed after 24 h, whereby hyphal cells had penetrated through the epithelium and were found lying on top of the inert supporting membrane (Fig. 5.2 B). These results are in agreement with the findings reported by Schaller *et al.* (Schaller *et al.*, 1998a). After 60 h for *C. albicans* derivative SC5314

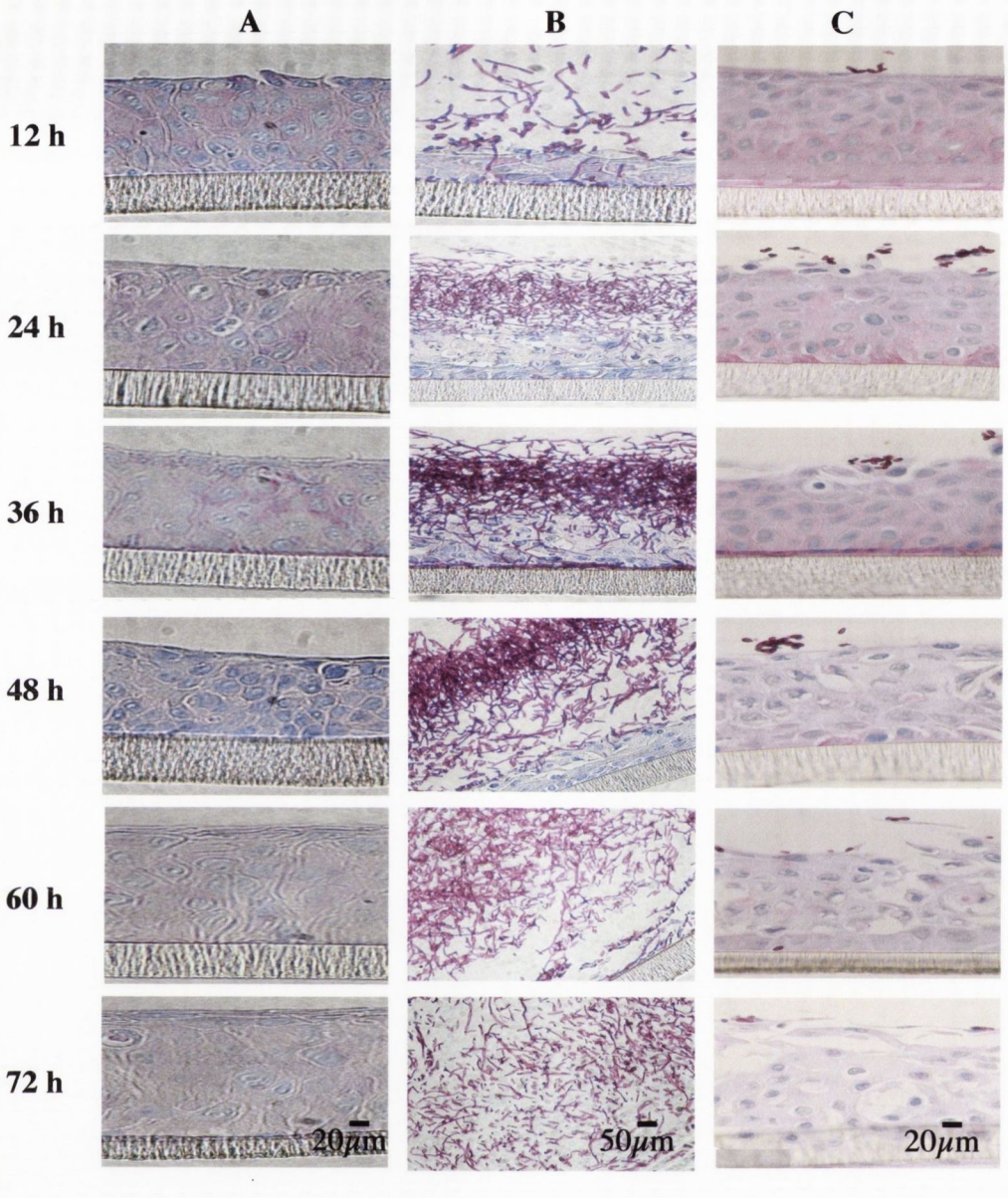

Figure 5.2. Light micrographs of RHE. (A) RHE, uninfected control, (B) RHE infected with *C. albicans* strain SC5314 and (C) RHE infected with *C. dubliniensis* strain CD36. The RHE specimens were incubated at 37°C with 5% CO_2 and saturated humidity. The polycarbonate filter serves as a support for growth of the epithelial cells. Histological sections were stained with Periodic-Acid Schiff reagent. The scale bars are representative of all the light micrographs in each column.

(M/G) there are very few epithelial cells left attached to the membrane. Similar results were also found for *C. albicans* derivative 132A (M/G), although more yeast cells were observed than with SC5314 (M/G).

Another striking difference between infection of RHE with *C. dubliniensis* in comparison with *C. albicans* is that only very few yeast cells were attached to the epithelium on examination of the entire surface in *C. dubliniensis,* whereas hundreds of attached yeast cells were visible for *C. albicans*. The pattern of damage caused by *C. dubliniensis* was strikingly different to that caused by *C. albicans*. After 12 h RHE cells which had been inoculated with *C. dubliniensis* showed no oedema and also showed very few yeast cells attaching to the epithelium in contrast with *C. albicans* (Fig. 5.2 C). After 24 h there was slight oedema with some of the top layer of epithelial cells becoming detached and after 36 h there were large holes visible within the epithelial layers. After 72 h a layer of epithelial cells was still visible over the membrane, in contrast to *C. albicans* at 72 h where no epithelial cells were still visible attached to the membrane. All of the *C. dubliniensis* cells present were also in the yeast phase with no detectable hyphae visible (Fig. 5.2). These results reflect those found in the kinetic of hyphal induction whereby *C. dubliniensis* strains were found to be less efficient at producing hyphae than *C. albicans*. A similar result was found for the two other strains of *C. dubliniensis,* CD519 and P7718. Despite this during preliminary work 54 h after infection an unusual result was found for *C. dubliniensis* derivative CD36 (M/G) whereby small microcolonies were observed along the epithelial surface (Fig. 5.3). The microcolonies consisted solely of yeast cells and two or three were present along the length of the tissue. On repeating the experiment these microcolonies were only observed on one other occasion, however, 72 h after infection.

The results from this study indicate that *C. dubliniensis* is less adherent to oral epithelial cells using the RHE model in comparison with *C. albicans* and cause less destruction of the tissue over 72 h. Destruction of the tissue also occurred more slowly with *C. dubliniensis*. These results also confirm that the RHE model is a suitable model to examine the effects of infection of humans with *Candida*.

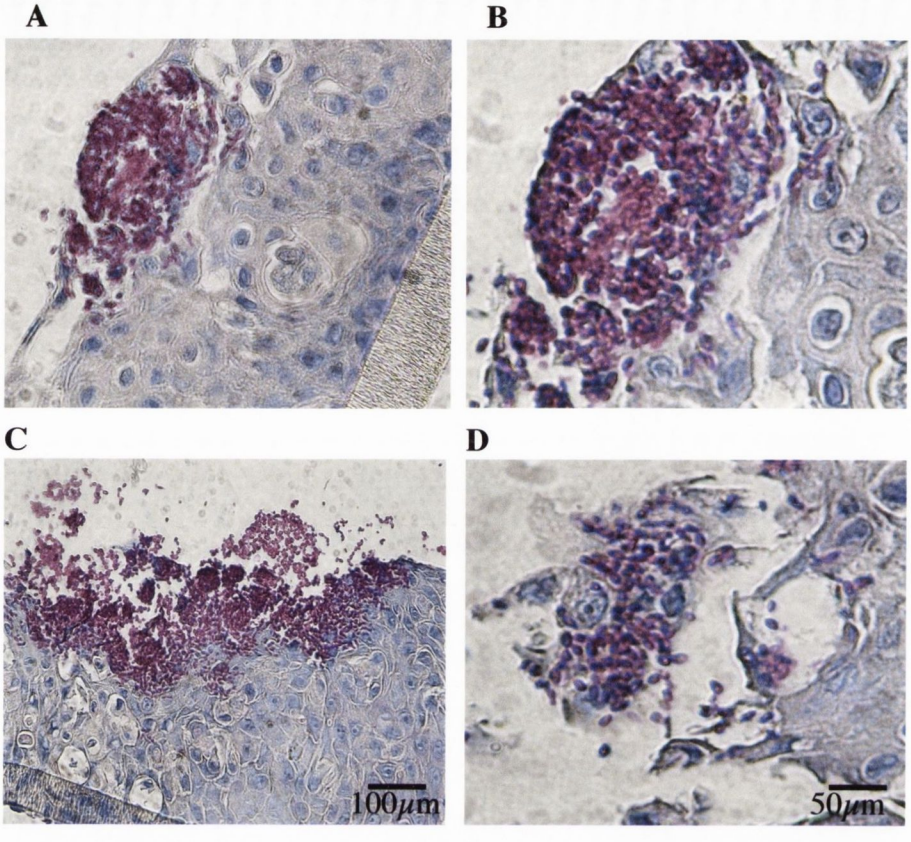

Figure 5.3. Light micrographs of RHE infected with *C. dubliniensis* derivative CD36 (M/G), 54 h after infection. Histological sections were stained with Periodic-Acid Schiff reagent. The scale bar of 50μm is representative of light micrographs A, B and D and the scale bar of 100μm is representative of light micrograph C.

5.3.2 Examination of the unattached yeast cells of each species

To examine whether there was a difference in adherence between the two species, the supernatant which lies on top of the RHE epithelium was examined for the presence of *Candida* at each timepoint. After addition of 50 µl of PBS the supernatant was removed and plated onto YNB agar and incubated at 48 h at 37°C to determine the level of non-adherent *Candida* cells present which were present in the supernatant. It is clear from Table 5.1 that even after 12 h there was an approximately 10-fold difference between *C. albicans* derivative SC5314 (M/G) and all other strains tested. *Candida albicans* derivative SC5314 (M/G) showed the lowest levels of non-adherent *Candida* cells as it was between 10- and 100-fold lower across the timepoints. All of the strains showed high cell densities in the supernatant; e.g. 132A (M/G) between 5.1 x 10^7 and 21.6 x 10^7, and CD36 (M/G) between 5.2 x 10^7 and 26.3 x 10^7over 72 h. After 48 h incubation *C. albicans* strains were present in lower densities in comparison with *C. dubliniensis* and this continued up to 72 h. To determine whether cells in the supernatant were in a yeast or a hyphal phase smears of the tissue at each of the timepoints were examined (Fig. 5.4 and 5.5). Examination of the smears revealed after 12 h almost all of *C. albicans* SC5314 (M/G) cells were in the hyphal phase. A similar result was also found for derivative 132A (M/G), however, slightly more yeast cells were observed (Fig. 5.6 A). On examination of the supernatant removed from *C. dubliniensis* derivative CD36 (M/G), it was found that almost all cells are in the yeast phase of growth. A similar result was found for strain CD519; however, strain P7718 showed some evidence of hyphal cells after 48 h (Fig. 5.6 B and C).

The results from this part of the present study confirmed that *C. dubliniensis* cells were present predominantly in the supernatant and simply did not attach to the top layers of the RHE epithelium, like *C. albicans*. Furthermore, *C. dubliniensis* cells were present in the yeast phase of growth, in contrast to strains of *C. albicans,* which were generally in a hyphal phase of growth.

5.3.3 Lactate dehydrogenase assay

Lactate dehydrogenase is a marker of cell injury and release into the maintenance media of cultures from infected and uninfected RHE cells was measured at 12, 24, 36, 48, 60

Table 5.1. Levels of *C. albicans* derivatives SC5314 (M/G) and 132A (M/G), *C. dubliniensis* derivative CD36 (M/G) and *C. dubliniensis* strains CD519 and P7718 in the supernatant above the RHE model at each timepoint.

	Time (h)					
	12	24	36	48	60	72
Strain	x 10^7 cfu/ml					
SC5314 (M/G)	0.7 ± 0.8[a]	0.3 ± 2.3	0.4 ± 1.3	0.4 ± 3.0	0.2 ± 1.4	0.7 ± 2.3
132A (M/G)	5.1 ± 1.8	5.6 ± 0.5	21.6 ± 1.9	9.9 ± 4.1	17.3 ± 2.1	14.4 ± 0.9
CD36 (M/G)	5.2 ± 1.0	8.8 ± 0.7	13.9 ± 0.5	14.6 ± 1.2	26.3 ± 10.0	20.7 ± 3.5
CD519	5.6 ± 0.7	9.6 ± 1.3	13.6 ± 1.6	15.5 ± 2.1	22.5 ± 4.1	18.3 ± 4.3
P7718	9.0 ± 0.9	19.4 ± 1.6	18.3 ± 2.0	20.3 ± 2.5	27.2 ± 3.7	18.0 ± 2.8

[a] Mean values were calculated from triplicate plates of duplicate experiments, therefore the mean values are indicative of six replicates ± standard deviation

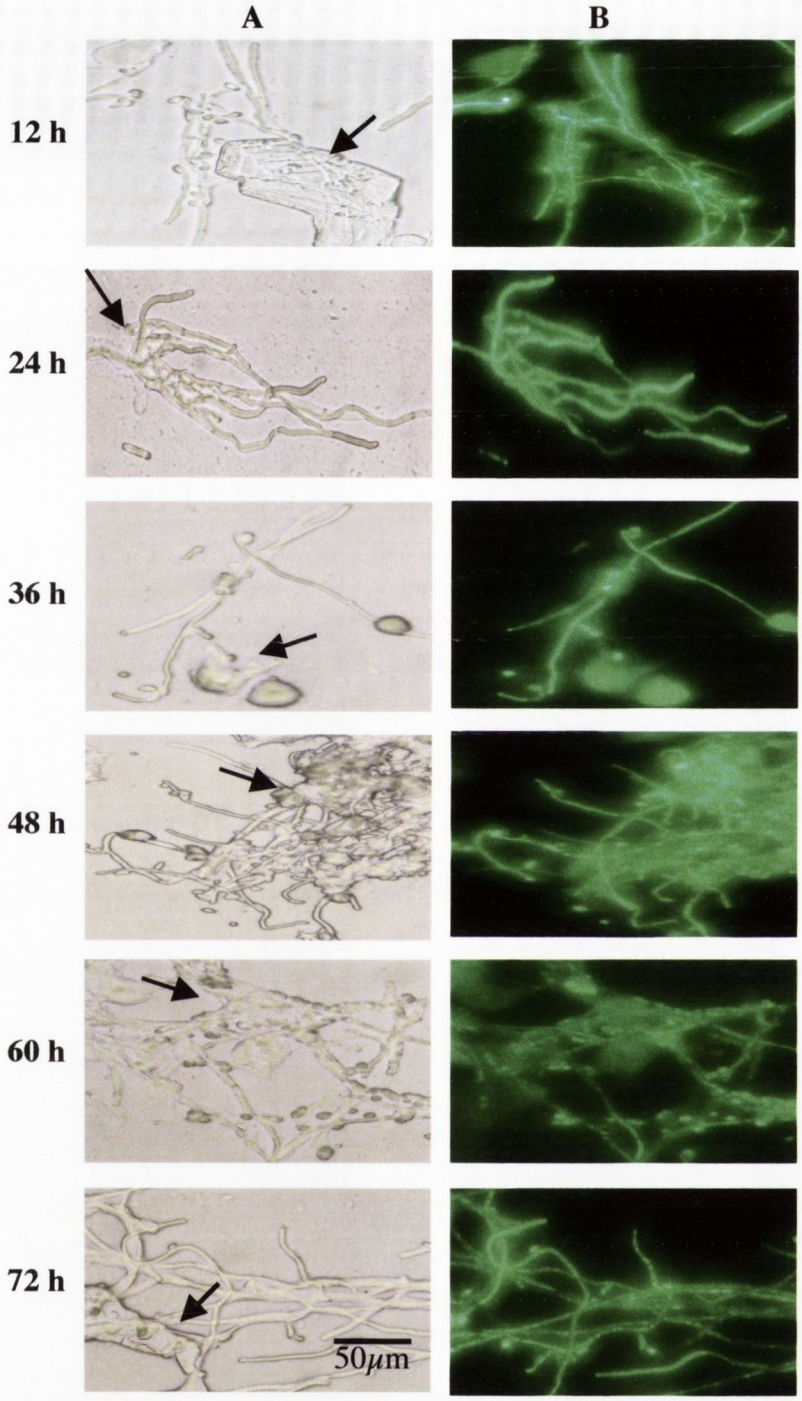

Figure 5.4. Light (A) and fluorescent (B) micrographs of *C. albicans* derivative SC5314 (M/G) present in the supernatant at each timepoint above the epithelium of RHE cells (A) without and (B) with fluorescent filters to aid visualisation of the cells. A large proportion of the cells are in the hyphal phase and are visible attached (represented by arrows) to loose epithelial cells. The scale bar is representative of each of the light micrographs.

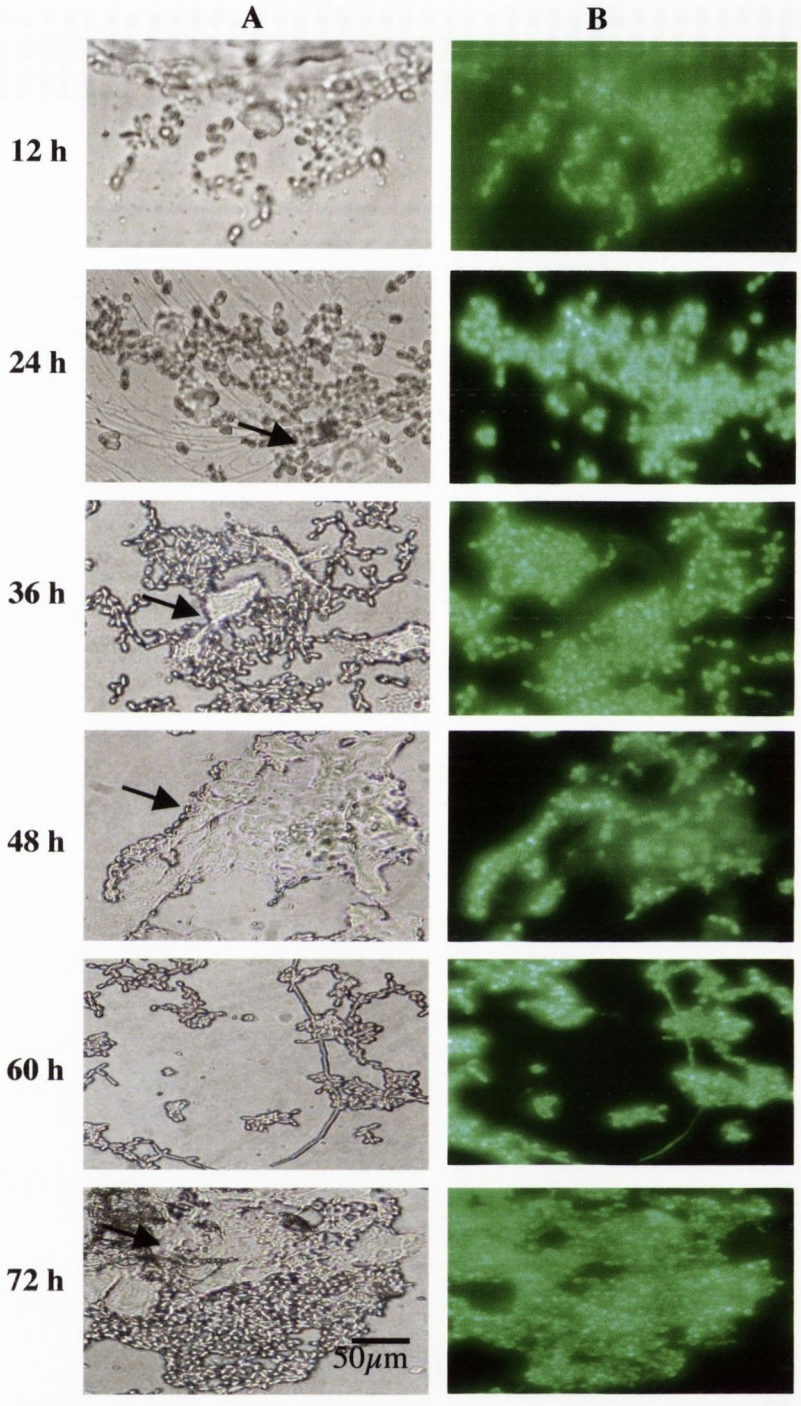

Figure 5.5. Light (A) and (B) fluorescent micrographs of *C. dubliniensis* derivative CD36 (M/G) present in the supernatant at each timepoint above the epithelium of RHE cells (A) without and (B) with fluorescent filters to aid visualisation of the cells. A large proportion of the cells are in the yeast phase and are visible attached (represented by arrows) to loose epithelial cells. The scale bar is representative of each of the light micrographs.

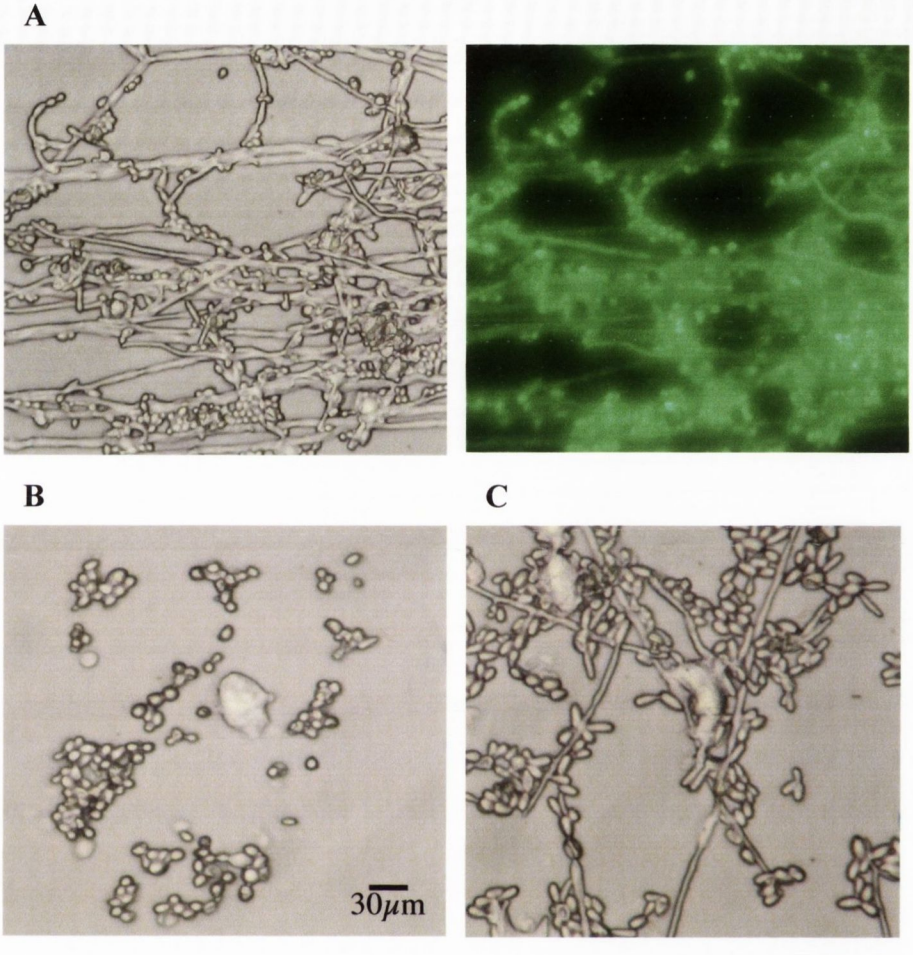

Figure 5.6. Light micrographs of *C. albicans* derivative 132A (M/G) (A) without and with fluorescent filters and *C. dubliniensis* strains (B) CD519 and (C) P7718 present in the supernatant 48 h after inoculation of RHE cells. The scale bar is representative of each of the light micrographs.

and 72 h. *Candida albicans* derivatives 132A (M/G) and SC5314 (M/G) exhibited twice the level of LDH activity than *C. dubliniensis* strains after 12 h, and three times the level after 24 h (Fig. 5.7). After 36 h, LDH activity in response to *C. albicans* strains was only two fold higher than *C. dubliniensis* as the activity level in response to *C. dubliniensis* strains started to increase. These data correlate with the results from the histological analysis described above as after 36 h *C. dubliniensis* strains started to show oedema and vacuolisation of the epithelia in response to *C. dubliniensis*. However, after 60 h, RHE infected by *C. dubliniensis* showed similar LDH activity compared to *C. albicans.* This is in contrast to the results shown in Fig. 5.2 as there is significantly more membrane damage observed due to *C. albicans* in comparison with *C. dubliniensis* at this timepoint. After 72 h *C. albicans* strains elicited higher levels of LDH activity from infected RHE in comparison with *C. dubliniensis*, however, the difference between the two species might have been expected to be higher since no RHE membrane was visible for strains of *C. albicans* in contrast to *C. dubliniensis* where intact RHE membrane was still visible.

The results from this part of the present study confirmed the results found in the histological analysis over the earlier timepoints whereby *C. albicans* strains show higher levels of cell damage and leakage of LDH into the culture medium than *C. dubliniensis* strains.

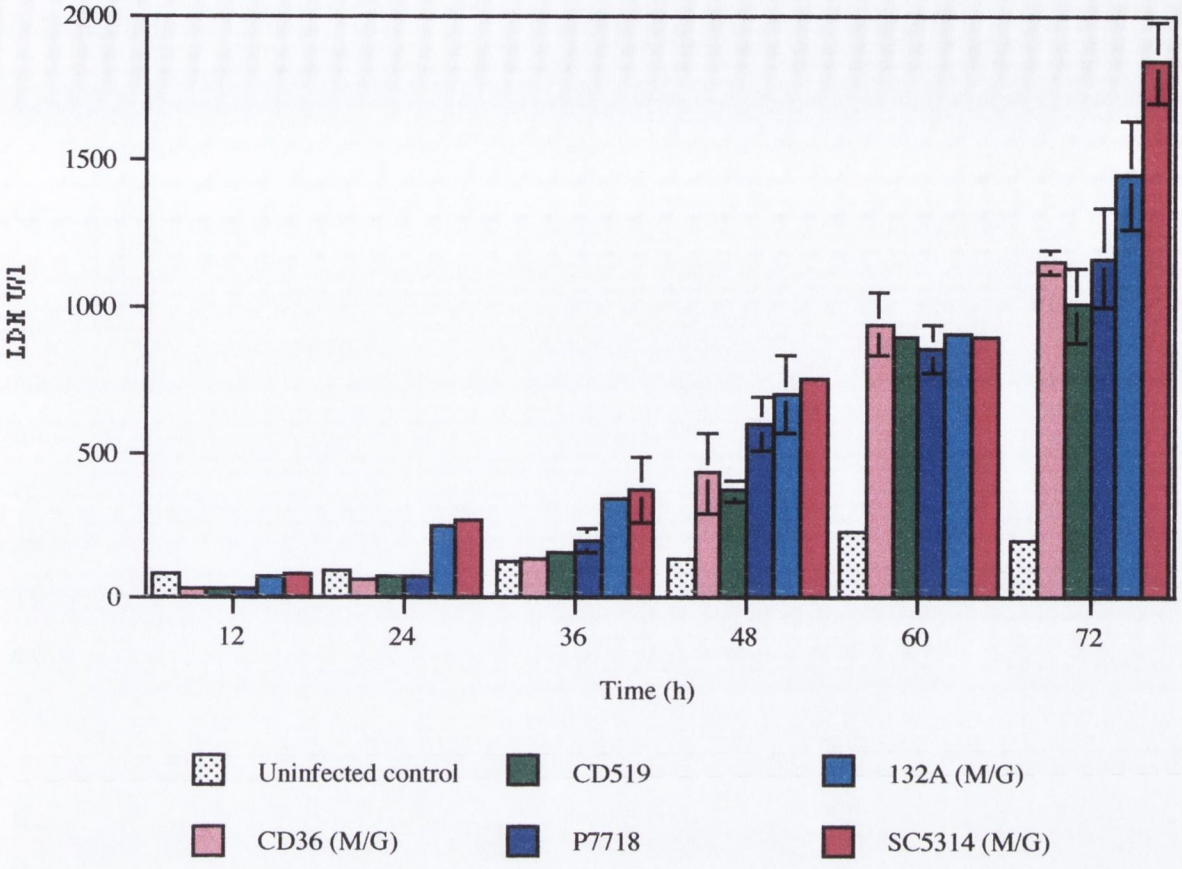

Figure 5.7. Graph showing LDH U/l of *C. albicans* derivatives 132A (M/G) and SC5314 (M/G) and *C. dubliniensis* strains CD519 and P7718 and *C. dubliniensis* derivative CD36 (M/G) over 72 h. Mean values were calculated from triplicate values of tripicate experiments, therefore the mean values are indicative of nine replicates ± standard deviation.

5.4 Discussion

5.4.1 Morphology of uninfected and infected RHE

Reconstituted human epithelial tissues closely resemble the *in vivo* human oral epithelium. Infection models based on these tissues represent an excellent model for human oral candidiasis. The RHE model used in the present study is not complicated by non-epithelial-cell factors, which allows the direct analysis of the interaction between *Candida* and epithelial cells at the mucousal surface. The epidermal differentiation, skin permeability, and the protein and lipid metabolism of this tissue culture system have been shown to resemble the *in vivo* situation (Rosdy *et al.*, 1993). Previous studies have examined the ability of *C. albicans* to adhere and to penetrate through the epithelial layers (Korting *et al.*, 1999; Schaller *et al.*, 1998a; Schaller *et al.*, 1999a) however, *C. dubliniensis* has never been examined using the RHE model. In the present study the ability of *C. albicans* and *C. dubliniensis* to adhere to and penetrate through the epithelial layers was compared.

From the histological and biochemical data obtained using the RHE model it is clear that *C. albicans* has a far greater capacity to damage human tissue. The primary causes for this appear to be two-fold. Firstly, *C. albicans* appears to adhere more successfully to RHE cells than *C. dubliniensis*. It appears that adhesion may be strain dependent in *C. dubliniensis* (personal communication Rachael Jordan). Secondly, the higher capacity for *C. albicans* to produce hyphae also appears to be an important factor in tissue damage. Previously Gilfillan *et al.* showed that, when grown in the presence of glucose, *C. dubliniensis* isolates adhered to buccal epithelial cells to a greater extent than *C. albicans* reference isolates (Gilfillan *et al.*, 1998). However, other studies have shown that *C. albicans* strains adhere better then *C. dubliniensis* strains, (Borg-Von Zepelin *et al.*, 2002; Vidotto *et al.*, 2003). These discrepancies are potentially due to the use of different strains of both species and also to differences in the techniques used to measure adhesion.

Over a period of 72 h, *C. albicans* strains invaded the RHE and histological analysis revealed the characteristic hallmarks of pathological tissue invasion (Fig. 5.2). After 12 h, RHE which had been infected with *C. albicans* and started to show signs of vacuolisation and oedema. In contrast, a similar situation was only observed in *C. dubliniensis* after 36 h. After 72 h there were no epithelial cells left attached to the membrane with *C. albicans*,

whereas for *C. dubliniensis* the presence of attached epithelial cells was extensive. The most striking difference between the two species was the significantly lower densities of *C. dubliniensis* cells adhering to the epithelium in contrast to *C. albicans* (Fig. 5.2). However, the presence of microcolonies was observed for *C. dubliniensis* derivative CD36 (M/G) (Fig. 5.3). The microcolonies were made up of yeast cells and some of these seem to have penetrated through the epithelial cells. Adherence and microcolony formation are thought to be the earliest stages of biofilm formation (Kumamoto & Vinces, 2005). Biofilms are the preferred mode of living for microbes in the natural world (Baillie & Douglas, 1998; Gilbert *et al.*, 1997). The presence of *C. albicans* cells resting above the RHE surface suggests that cells may be embedded in a matrix, which is also a feature consistent with a biofilm (Fig. 5.2 C). This structure has been reported in the literature as a pseudomembrane (Schaller *et al.*, 1998a). Both *C. albicans* and *C. dubliniensis* have been shown to produce biofilms *in vitro* (Kirkpatrick *et al.*, 2000). These results suggest that production of biofilms may be necessary for both *C. albicans* and *C. dubliniensis* to establish infection on oral epithelium.

It has been reported that *C. albicans* adhesion to mucousal cells is enhanced by several factors such as phospholipase and protease production, carbohydrates, pH and temperature and germ tube production (Ghannoum & Abu Elteen, 1986; Hube, 1996). It is therefore possible that these factors may contribute to the ability of *C. dubliniensis* cells to adhere to human tissue. Investigation into the presence of agglutinin-like sequence (Als) in *C. dubliniensis* has also been performed. These proteins have been implicated in *C. albicans* in mediating adherence to host cells. Hoyer *et al.* have identified three distinct *ALS*-like sequences in *C. dubliniensis*, *ALDS*1-3 (Hoyer *et al.*, 2001). These genes were also found to be divergent from *C. albicans* particularly at the 3' ends using comparative genomic microarray hybridisations. According to the literature, genetic, environmental and phenotypic factors such as pH, temperature, anaerobic conditions and nutritional factors can contribute to tissue digestion thus enhancing penetration through mucousal cells (Calderone *et al.*, 2000; Staib *et al.*, 2000).

Another striking difference between the two species was the presence of yeast cells solely on the surface of the epithelium on RHE infected with *C. dubliniensis* in comparison with RHE infected with *C. albicans* which showed the presence of both yeast and hyphal cells. These data reflect what was found previously in this study whereby, *C. dubliniensis*

strains exhibited significantly less hyphal production when examined in eleven different hyphal inducing conditions in comparison with *C. albicans* (section 4.3.1.1). Hyphal invasion of *C. albicans* in this study was seen at the cell surface and also at intercellular cell junctions. Hyphae may simply penetrate the cell membrane or alternatively traverse the intercellular spaces of the artificial epithelium where the surface integrity is week. This thigmotropism has previously been shown for *C. albicans,* whereby hyphal cells on Nucleopore membrane filters entered the filter pores thereby sensing the surface topography (Gow *et al.*, 1994). Ultrastructural observations have revealed areas of clearing around penetrating hyphae, supporting a role for lytic enzymes during invasion (Montes *et al.*, 1968; Scherwitz, 1982; Sweet, 1997). Phospholipase activity has been found at the tips of hyphae and initial sites of bud formation in a RHE model using *C. albicans* (Jayatilake *et al.*, 2005).

Examination of the cells which had not attached to the RHE epithelium showed high cell densities of both species present in the supernatant. (Table 5.1). *C. albicans* derivative SC5314 (M/G) showed the lowest density of *Candida* cells in the supernatant, which were between 10-and 100-fold lower in comparison with the rest of the strains examined across the timepoints. Examination of smears of the tissues showed that *C. dubliniensis* derivative, CD36 (M/G) (Fig 5.5) and CD519 (Fig. 5.6 B) were in the yeast phase, whereas *C. dubliniensis* strain P7718 showed the presence of hyphal cells (Fig. 5.7 C) which were not observed in the histological sections of RHE infected with this organism. The results for strain P7718 are surprising as this strain showed high levels of hyphal production under many of the hyphal inducing media examined in section 4.3.1, however, under these conditions no hyphae were visible at all. In contrast, *C. albicans* derivatives SC5314 (M/G) (Fig. 5.4) and 132A (M/G) (Fig. 5.6 A) were found to be mainly in the hyphal phase.

The findings of this part of the present study confirm that the RHE model can be used as a flexible, simple model for the study of experimental candidiasis. However, this is the first study to examine the effects of *C. dubliniensis* with this model. Although RHE mimicks the *in vivo* situation parameters such as pH changes, competition with oral flora, nutrient limitation, humoral and cell mediated immunity are absent and allow *Candida* to grow unchallenged on the mucousal surface.

5.4.2 Lactate dehydrogenase assay

The release of lactate dehydogenase (LDH) from epithelial cells into the surrounding medium was monitored as a measure of epithelial cell damage. Lactate dehydrogenase release in the maintenance media of the cultures from uninfected and infected epithelial cells was measured at 12, 24, 36, 48, 60 and 72 h. The marked histological differences in tissue damage corresponded with significant differences of LDH release. The release of LDH (U/ l, mean ± SD) for *C. albicans* derivatives SC5314 (M/G) (82.8 ± 9) and 132A (M/G) (74.1 ± 20) after 12 h is double that found for *C. dubliniensis* derivative CD36 (M/G) (33 ±1), CD519 (37.9 ± 2) and P7718 (32.8 ± 0.4) (Fig. 5.7). After 24 h the difference is almost fourfold however, after 36 h *C. dubliniensis* as observed in the histological analysis began to create oedema and vacuolisation of the membrane and so the levels of LDH increased to less than threefold less than *C. albicans*, e.g. *C. albicans* derivative SC5314 (M/G) (374 ± 12) and *C. dubliniensis* derivative CD36 (M/G) (141 ± 13). The result found for *C. albicans* derivative SC5314 (M/G) after 36 h correlates with what was found previously by Korting *et al.* who reported a LDH value of (391 ± 15) despite using a different assay method (Korting *et al.*, 2003). After 48 h the difference between the two species drops to less than two fold and after 72 h a similar result was found whereby *C. albicans* derivative SC5314 (M/G) (1837 ± 14) is less than twofold greater than *C. dubliniensis* derivative CD36 (M/G) (1151 ± 39). When statistical analysis was performed comparing *C. albicans* strains with *C. dubliniensis* strains over each timepoint the difference was significant ($P < 0.05$) between 12 and 48 h while no significant difference ($P > 0.05$) was observed between 60 and 72 h.

The results from the LDH assay at 60 and 72 h are unusual as there is no statistically significant difference between the two species, however, histological analysis shows a striking difference between the two species at these two timepoints. No membrane is visible attached to the polycarbonate filter in *C. albicans* at 60 and 72 h in contrast to *C. dubliniensis* where layers of membrane are still visible attached to the polycarbonate filter although disruption of the membrane is visible. This discrepancy may be due to an initial large production of hydrolytic enzymes by *C. albicans*, which may level off at 60 and 72 h due to a cell density phenomenom in comparison with *C. dubliniensis* whereby there is a gradual

increase in production of hydrolytic enzymes. In *C. dubliniensis*, although the damage to the membrane is not as extensive as *C.albicans* there is still visible (Fig. 5.2) disruption of the membrane which would allow leakage of LDH into the surrounding medium.

The results from this part of the present study show that more LDH is released into the culture medium for *C. albicans* over early timepoints in comparison with *C. dubliniensis* which is indicative of greater membrane damage as a result of release of phospholipases, Saps and hydrolytic enzymes. Despite the inability of *C. dubliniensis* to produce hyphae and penetrate the epithelium it is still able to cause vacuolisation of the membrane and membrane damage resulting in release of LDH. It would appear that the reduced damage caused by *C. dubliniensis* is due to a reduced capacity to adhere to human cells and to produce hyphae.

Chapter 6

Comparison of growth of *C. albicans* and *C. dubliniensis* in an *in vivo* model

6.1 Introduction

6.1.1 The role of the gastrointestinal tract in candidiasis

The leading causative agent of candidiasis, *C. albicans*, can reside as a harmless commensal of the human mucousa and gastrointestinal tract in healthy people. Gastrointestinal carriage of *Candida* spp. is common and fungi have been found in 80% of faecal samples from healthy adults (Gorbach *et al.*, 1967). In these individuals, the gastrointestinal tract (GIT) wall and the immune system prevent the overgrowth of *Candida* species. However, especially in immunocompromised patients, *C. albicans* can develop from a harmless commensal to an opportunistic pathogen that can overgrow and penetrate the gut wall and gain entry to the vascular system leading to systemic infection. Populations at risk of serious disseminated disease include immunosuppressed individuals who may be predisposed by granulocytopenia, or following treatment with broad spectrum antibiotics, cytotoxic drugs or corticosteroids and include cancer patients, transplant recipients, and especially leukemia patients (Klein *et al.*, 1984).

Passage across the bowel wall into the bloodstream is an important portal of entry for this opportunistic pathogen especially in the neutropenic host, leading to systemic and disseminated candidiasis (Walsh & Merz, 1986). The molecular mechanisms enabling transition from the gastrointestinal tract to the bloodstream are poorly understood, but experimental evidence has supported the roles of secretion of aspartyl proteinases that facilitate invasion through host tissues (Hube, 1996), hyphal differentiation (Soll, 2002) and specific adhesion receptors that recognise host ligands (Calderone *et al.*, 2000).

6.1.2 Animal models available for use in studying infections caused by *Candida*

An increasingly diverse array of clinically relevant animal models of candidiasis have been established that mimic the immune perturbations of the host and tissue-specific features of candidiasis in humans (de Repentigny, 2004). The principal advantage of studying animals instead of humans is that we have more control over an animal and its environment (Allen, 1994). Although a human model has been used to evaluate the natural history of local immmune responsiveness to *Candida* at the vaginal mucousa (Barousse, 2005). In choosing an animal model to study a particular disease it is necessary that the model chosen

should reproduce as closely as possible the process of colonisation and invasion at a specific portal of entry, but also closely match the specific immune defects or hormonal conditions associated with infection at that particular site. Retention of natural host barriers is important for studies of colonisation or virulence.

Murine models of candidiasis are widely used due to the availabilty of methods for immunological analysis, as well as the abiltiy to generate knockout and transgenic mice that reproduce specific immune perturbations of the host (de Repentigny, 2004). Mice are not normally colonised by *C. albicans* as colonisation is inhibited by the host s innate defenses and by the mucousal barrier. Furthermore, competition between the yeast and the existing murine microbial flora through antagonism of *C. albicans* growth and blockage of host receptor molecules that could otherwise bind *C. albicans* adhesins preventing *Candida* colonisation (Erkenna & Sheretz, 1987; Kennedy, 1988). Therefore mice need to be experimentally infected. The infant mouse model however, closely mimics what is found in humans whereby humans are colonised shortly after birth. The infant gastric mouse model allows establishment of *C. albicans* in the mouse prior to immunocompromising procedures reflecting the situation in human patients where the immune system is impaired (de Repentigny *et al.*, 1992; Guentzel & Herrera, 1982; Pope *et al.*, 1979). Innate defenses are minimal in the infant mouse and the microbial flora consists mainly of lactobacilli, which *C. albicans* is able to successfully compete with for sites of adherence and colonisation. The advantages of the infant mouse model are due to the technique used for inoculation, precise control of challenge dose, retention of natural host barriers including gastric and intestinal secretions, peristalsis, the mucous layer and indigenous flora. It also provides a model to study events leading to sytemic spread by persorption and dissemination, and for studying compromising agents and procedures that may precipitate candidiasis in humans.

6.1.3 Histology of the murine stomach

The murine stomach is separated into a keratinised, stratified cornified squamous epithelium similar to the lining of the esophagous and a glandular portion, which contains the digestive glands. At the boundary of the glandular and non-glandular regions the mucous membrane forms a ridge which is termed the cardial-atrium fold. The keratinised epithelium is continuous through the esophagous to the cardial-atrium fold. The cardial-atrium fold is

where hyphal proliferation and invasion generally occur in experimental infection (Cole *et al.*, 1996). Mice are usually rendered immunocompromised by administration of cyclophosphamide and cortisone acetate. Cyclophosphamide is known to be toxic to dividing cells in the GI tract and can alter the normal integrity of the mucojsal epithelium of the gut (Cole *et al.*, 1990b). The toxic effects of the use of cyclophosphamide and cortisone acetate which induce leukopenia acccounts for the proliferation of *C. albicans* and their ability to invade the GI mucosa (Bistoni *et al.*, 1984).

The aims of this part of the present study were to compare the ability of *C. dublininiensis* and *C. albicans* to colonise the gastrointestinal tract and disseminate to other organs in the infant mouse model when inoculated separately and together in co-infection studies.

6.2 Materials and Methods

6.2.1 *Candida* isolates

The *Candida* strains used in this part of the present study are listed in Table 3.1. Two strains of *C. albicans* (SC5314 and 132A) and one strain of *C. dubliniensis* (CD36) were examined separately and in co-infection studies with one another. A derivative of *C. dubliniensis* strain CD36, which had been tagged with the molecular marker cassette containing MPA^R and GFP was also examined (CD36 M/G).

6.2.2 Animal models

Crl:CFW(SW)BR mice obtained from Charles River Laboratories (Wilmington, MA, USA), were used to establish a breeding colony and the offspring of these animals were used in all experiments. Experiments were carried out with the help of Dr. K. R. Seshan in the Medical College of Ohio, USA under the license of Prof. Garry Cole. Infant mice (6-9 days old) derived from these animals were isolated from their mothers 3-4 h before inoculation and held at room temperature to enable clearance of the mothers milk from the infant stomach.

6.2.3 Inoculum and inoculation

A subculture of the *Candida* strains grown on Sabouraud dextrose agar (SDA) for 48 h at 30°C was harvested with nonpyrogenic saline (Travenol; Travenol laboratories Inc., Deerfield, ILL. USA), washed twice and counted with a haemocytometer to an inoculum size of 2.0×10^8 yeast cells suspended in 0.05 ml of sterile saline (Travenol). The inoculum was delivered by the oral-intragastric route with a 24-gauge feeding needle attached to a 1.0 ml syringe (Popper and sons, Rhode Island, USA).

6.2.4 Faecal pellet assays

Mice infected by oral intra-gastric inoculation and subjected to immunocompromising treatment were examined for the presence of *Candida* in their faecal pellets. Animals were marked for identification with picric acid at the time of the assay. Fresh faecal pellets

collected from mice were immmediately homogenised in 1.0 ml of chilled, sterile saline and 0.1 ml of the homogenate was plated on SDA containing 50 μg/ml chloramphenicol (Sigma). The plates were incubated at 30°C for 48 h.

6.2.5 Immunosuppression

Stock solutions of the two drugs were prepared at concentrations of 20 mg/ml cyclophosphamide in sterile saline and 25 mg/ml cortisone acetate in sterile saline. A compromising treatment was initiated on day 11 after oral-intragastric challenge of infant mice with *C. albicans*. Immunocompromising drugs were administered by intraperitoneal (IP) injection. Animals selected on the basis of their positive faecal pellets received cyclophosphamide (Mead Johnson, BMS Co.; New Jersey, USA) 0.2 mg/ml plus 1.25 mg/ml cortisone acetate (Westward Pharmaceutical Corporation, New Jersey USA), followed by a second administration of cyclophosphamide (0.1 mg/ml), and cortisone acetate (1.25 mg/ml) on day 14 post-challenge. Mice used in the colonisation experiment were not immunocompromised in any way and were simply inoculated and then sacrificed every two days over a period of 10 days.

6.2.6 Sacrifice

Animals which had been subjected to immunocompromising treatment were sacrificed at 20-24 days post challenge by asphyxiation with carbon dioxide. Mice were sacrificed earlier if they were unresponsive. Mice were immediately dissected to remove the entire gastrointestinal tract and selected organs were prepared for homogenisation and plating, or for histological studies as described below.

6.2.7 Enumeration of *C. albicans* and *C. dubliniensis* in body organs

Animals were dissected under aseptic conditions. The stomach, liver and kidney were examined for the presence of abscesses and homegenised separately in chilled sterile saline (4°C) using a tissue grinder equipped with a Teflon pestle. The enumeration of colony forming units (cfu) of *Candida* in homogenised stomach and liver were performed by dilution plating on YNB agar (Sigma) containing chloramphenicol (50 μg/ml) and incubated

at 30°C for 2 days. When differentiation was necessary between tagged and untagged strains the level of each species was determined by dilution plating on YNB agar containing chloramphenicol (50 μg/ml) and YNB agar containing chloramphenicol (50 μg/ml) and MPA (0.01 mg/ml).

The indigenous yeast, *Candida pintolopesii*, was rarely detected in the young animals used in these experiments. Typically this yeast requires prolonged incubation (more than 5 days) to appear in plate culture. When *C. pintolopesii* does appear in plate culture it can be readily distinguished from *C. albicans* by its much smaller colony diameter.

6.2.8 Photography

Whole mounts and dissected preparations of the stomach, liver and kidneys were examined using a Polaroid MP-3 Land Camera equipped with a 127 mm lens.

6.2.9 Histological analysis of organs

Animals were sacrificed at 20 days post-challenge and prepared for histological examination. Following removal organs were placed in 4% paraformaldehyde (v/v in PBS; 0.1 M, pH 7.4) for 12 h at 4°C. Tissues were then washed with buffer, dehydrated in ethanol, embedded in paraffin wax. Sections were stained with Periodic acid Schiff (PAS) reagent for demonstration of fungal elements (Luna, 1968) and examined with a Zeiss Photomicroscope II. Fluorescence was detected using a FITC filter.

A selection of organs were chemically fixed for microscopy. Organs were chemically fixed for 12 h at 4°C in glutaraledyhyde (3%, v/v) and paraformaldeyhyde (2% v/v), each prepared separately in cacodylate buffer (0.1 M, pH 7.4) and mixed just before use. The tissues were rinsed in buffer, post fixed in 2% osmium tetroxide (2 h) prepared in the same buffer, dehydrated and embedded in Spurr's low viscosity resin as described previously (Seshan & Cole, 1994). Thick sections (approximately 1 μm) were stained with azure 11-methylene blue for light microscopy as reported (Cole *et al.*, 1989). Smears of organs were also examined without any fixative.

6.2.10 Statistical analysis

The data were compared using the nonparametric Mann-Whitney U test. *P* values of <0.05 were considered significant. Statistical comparisons were performed using SPSS version 9.0 software package for Windows.

6.3 Results

6.3.1 Examination of the ability of *C. albicans* and *C. dubliniensis* to colonise the intestine and stomach in the infant mouse model

6.3.1.1 Comparison of the ability of *C. albicans* and *C. dubliniensis* to colonise when inoculated separately into the infant mouse model

The aim of this part of the present study was to compare the ability of *C. albicans* and *C. dubliniensis* to colonise and infect the infant mouse model when inoculated separately and together in co-infection. Infant mice (6-9 days old) were isolated from their mothers 3-4 h before inoculation and held at room temperature. Clearance of milk from the stomachs and intestines of the neonates during this preinoculation period enhances the ability of *Candida* to interact with the mucousal surface of the host. Infant mice were inoculated with 2.0 X 10^8 yeast cells suspended in 0.05 ml of sterile saline by the oral-intragastric route with a 24-gauge feeding needle attached to a 1.0 ml syringe. Mice were then sacrificed by asphyxiation with carbon dioxide every two days over a period of 10 days and enumeration of colony forming units (cfu) of *Candida* in homogenised tissues was determined by dilution plating on YNB agar (Sigma) containing chloramphenicol (50 μg/ml) and incubated at 30°C for 2 days. Six mice were used per timepoint per group except for the competition experiment whereby 5 mice were used for days 8 and 10.

Candida albicans strain SC5314 and *C. dubliniensis* derivative CD36 (M/G), which had as previously outlined been tagged with the molecular marker cassette containing GFP and MPA^R to enable differentiation from *C. albicans* when inoculated together were used. When mice were infected with either *C. albicans* or *C. dubliniensis* alone there was ten-fold less *C. dubliniensis* present in the stomach after 2 and 4 days and similar levels at day 6. However, at days 8 and 10 no *C. dubliniensis* were detected and 2.0 x 10^3 and 1.5 x 10^3 cfu/ml of *C. albicans* were detected on days 8 and 10, respectively (Table 6.1). In the intestine a similar result was found as there was ten-fold less *C. dubliniensis* than *C. albicans* detected at days 2-6 and a hundred-fold less at days 8 and 10 (Fig. 6.1).

Table 6.1. *Candida* colony forming units (cfu) recovered from the stomach and intestine of yeast infected infant mice inoculated with *C. albicans* and *C. dubliniensis* separately and sacrificed every two days.

	C. albicans SC5314		*C. dubliniensis* CD36 (M/G)[a]	
Day				
	Stomach	Intestine	Stomach	Intestine
	(cfu/ml)			
2	1.4×10^{4} [b] 6/6 [c] (2×10^{2}-9.5×10^{4}) [d]	1.5×10^{5} [b] 6/6 [c] (1.4×10^{3}-9.2×10^{5}) [d]	3.4×10^{3} [b] 6/6 [c] (8×10 -1.7×10^{4}) [d]	1.7×10^{4} [b] 6/6 [c] (3.4×10^{2}-9.2×10^{4}) [d]
4	1.3×10^{3} [b] 6/6 [c] (7×10^{2}-2.7×10^{3}) [d]	1.7×10^{4} [b] 6/6 [c] (1.1×10^{3}-4.3×10^{4}) [d]	2.0×10^{2} [b] 5/6 [c] (0 -7.5×10^{2})	1.1×10^{3} [b] 6/6 [c] (1.4×10^{2}-4.8×10^{3})
6	1.1×10^{3} [b] 6/6 [c] (4.1×10^{2}-2.1×10^{3})	1.1×10^{3} [b] 6/6 [c] (3×10^{2}-2.3×10^{3})	1.4×10^{3} [b] 5/6 [c] (0 -7.8×10^{3}) [d]	4.2×10^{2} [b] 6/6 [c] (5×10 -1.2×10^{3}) [d]
8	2.0×10^{3} [b] 6/6 [c] (3.1×10^{2}-5×10^{3}) [d]	2.2×10^{3} [b] 6/6 [c] (1×10^{2}-1.8×10^{4}) [d]	0 [b] 0/6 [c] 0 [d]	7.2×10 [b] 6/6 [c] (0 -1.8×10^{3}) [d]
10	1.5×10^{3} [b] 6/6 [c] (1.3×10^{3}-1.6×10^{3}) [d]	2.2×10^{2} [b] 6/6 [c] (7×10 -2.2×10^{2}) [d]	0 [b] 0/6 [c] 0 [d]	1 [b] 1/6 [c] (0 -2×10) [d]

[a] The *C. dubliniensis* strain used was CD36, which had been tagged with a molecular marker cassette containing MPA^{R} and GFP.

[b] Mean values for all animals in each treatment group.

[c] Number of culture positive for *Candida* over total tested.

[d] Range of cfu of *Candida* for positive samples.

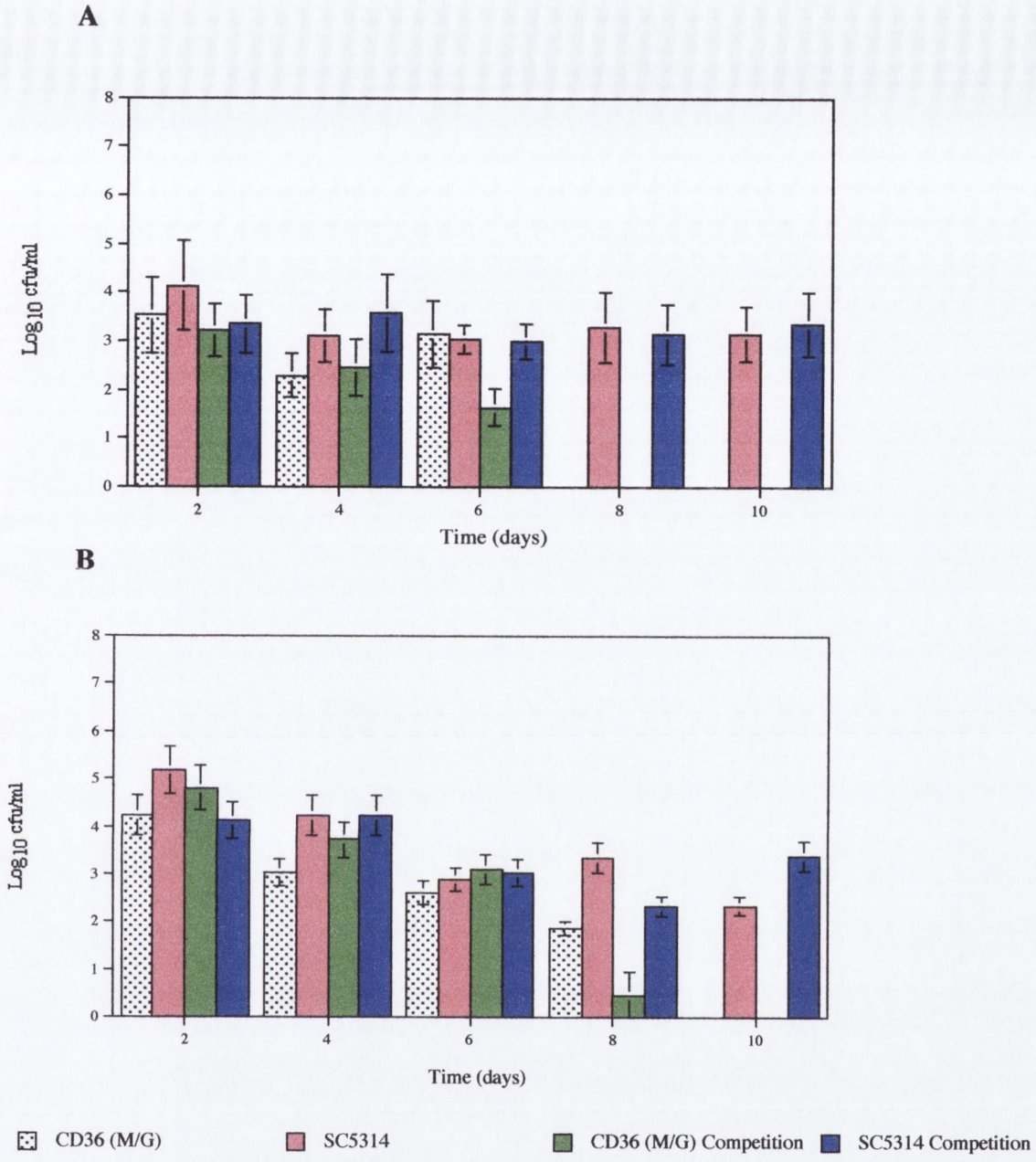

Figure 6.1. Graphs showing the levels of colonisation (cfu/ml) of *C. albicans* strain SC5314 and *C. dubliniensis* derivative CD36 (M/G) when inoculated separately and in co-infection together in both the (A) stomach and (B) intestine when mice were sacrificed every two days over 10 days.

6.3.1.2 Comparison of the ability of *C. albicans* and *C. dubliniensis* to colonise when co-inoculated in competition

The ability of *C. albicans* and *C. dubliniensis* to colonise, when inoculated separately into the infant mouse model was examined above. The aim of this part of the study was to examine what happened when *C. albicans* and *C. dubliniensis* were inoculated in competition with one another in an *in vivo* animal model. *Candida dubliniensis* derivative CD36 (M/G) and *C. albicans* strain SC5314 were used in this part of the study as they are reference strains which are used worldwide. It has already been shown *in vitro* in section 3.3.2 that when inoculated in competition at 37°C, that after 48 h *C. albicans* is always present at higher levels than *C. dubliniensis*. The inoculum used in this part of the study was as above 2.0 X 10^8 yeast cells suspended in 0.05 ml of sterile saline, and contained 1.0 X 10^8 of each species. Differentiation was necessary between tagged and untagged strains due to the close similarity of the two species. The level of each species was determined by dilution plating on YNB agar containing chloramphenicol (50 μg/ml) and YNB agar containing chloramphenicol (50 μg/ml) and MPA (0.01 mg/ml).

The results in Table 6.2 show that when inoculated in competition with one another *C. albicans* is present in higher numbers than *C. dubliniensis*. After day 2, *C. albicans* was present in ten-fold higher levels in the stomach and intestine, after day 6 it was a hundred-fold higher in the stomach, although similar levels of each species were recovered from the intestine. After day 8 *C. albicans* was a thousand-fold higher in the stomach and a hundred-fold higher in the intestine and after 10 days it was a thousand-fold higher in the stomach and intestine, in comparison with *C. dubliniensis* which showed no colonisation (Fig. 6.1). These results reflect what was found above, the lack of *C. dubliniensis* present after day 6 is not due to it being completely out-competed by *C. albicans* but simply due to clearance of *C. dubliniensis* from the stomach and intestine faster than *C. albicans*.

Table 6.2. *Candida* colony forming units (cfu) recovered from the stomach and intestine of yeast infected infant mice inoculated with *C. albicans* and *C. dubliniensis* together and sacrificed every two days.

	SC5314 + CD36 (M/G) [a]			
Day				
	SC5314		CD36 (M/G) [a]	
	Stomach	Intestine	Stomach	Intestine
	(cfu/ml)			
2	2.3×10^{3} [b] 6/6 [c] $(2.6 \times 10^{3} \times 1.0 \times 10^{4})$ [d]	1.3×10^{4} [b] 6/6 [c] $(1.4 \times 10^{3}\text{-}1.0 \times 10^{4})$ [d]	1.6×10^{3} [b] 6/6 [c] $(7 \times 10^{2}\text{-}3 \times 10^{3})$ [d]	6.4×10^{4} [b] 6/6 [c] $(5 \times 10\text{ -}1.3 \times 10^{4})$ [d]
4	3.8×10^{3} [b] 6/6 [c] $(5.5 \times 10^{2}\text{-}2.0 \times 10^{4})$ [d]	1.7×10^{4} [b] 6/6 [c] $(8.6 \times 10^{2}\text{-}5.1 \times 10^{3})$ [d]	2.9×10^{2} [b] 4/6 [c] $(0\text{-}2.4 \times 10^{3})$ [d]	5.4×10^{3} [b] 4/6 [c] $(0\text{-}3.7 \times 10^{4})$ [d]
6	1.0×10^{3} [b] 6/6 [c] $(5.5 \times 10^{2}\text{-}2.56 \times 10^{3})$ [d]	1.1×10^{3} [b] 6/6 [c] $(2 \times 10^{2} \times 1.3 \times 10^{4})$ [d]	4.4×10 [b] 2/6 [c] $(0\text{ -}4.1 \times 10^{2})$ [d]	1.3×10^{3} [b] 2/6 [c] $(0\text{ -}1.2 \times 10^{4})$ [d]
8	1.4×10^{3} [b] 5/6 [c] $(1.1 \times 10^{2}\text{-}1.4 \times 10^{3})$ [d]	2.2×10^{2} [b] 5/6 [c] $(2 \times 10\text{ -}4.8 \times 10^{2})$ [d]	0 [b] 0/5 [c] $(0\text{ -}2 \times 10)$ [d]	3 [b] 2/5 [c] $(0\text{ -}1 \times 10)$ [d]
10	2.3×10^{3} [b] 5/6 [c] $(1.1 \times 10^{3}\text{-}3.4 \times 10^{3})$ [d]	2.2×10^{3} [b] 5/6 [c] $(3 \times 10\text{ -}2.4 \times 10^{3})$ [d]	0 [b] 0/5 [c] 0 [d]	0 [b] 0/5 [c] 0 [d]

[a] The *C. dubliniensis* strain used was CD36, which had been tagged with a molecular marker cassette containing MPA^{R} and GFP.

[b] Mean values for all animals in each treatment group.

[c] Number of culture positive for *Candida* over total tested.

[d] Range of cfu of *Candida* for positive samples.

6.3.2 Examination of the ability of *C. albicans* and *C. dubliniensis* to colonise the stomach and disseminate in the infant mouse model

6.3.2.1 Comparison of the ability of *C. albicans* and *C. dubliniensis* to disseminate from the stomach when inoculated separately from one another

The ability of *C. albicans* to disseminate from the stomach to other organs such as the liver, kidney and brain has been examined in *C. albicans* in previous studies (Cole *et al.*, 1990a; Cole *et al.*, 1989). The aim of this part of the present study was to examine the ability of *C. dubliniensis* alone and in co-culture with *C. albicans* to disseminate from the stomach to other organs. Mice were inoculated as outlined above and immunocompromising drugs were administered by intraperitoneal (IP) injection. Animals selected on the basis of their positive faecal pellets received cyclophosphamide (Mead Johnson) 0.2 mg/ml plus 1.25 mg/ml cortisone acetate (Westward Pharmaceutical Corporation), followed by a second administration of cyclophosphamide (0.1 mg/ml), and cortisone acetate (1.25 mg/ml) on day 14 post-challenge. Faecal pellets were also examined for the presence of fluorescent cells, which confirmed that passage through the GI tract had no deleterious effect on GFP in the molecular cassette. Animals were sacrificed at 20-24 days post challenge by asphyxiation with carbon dioxide. A schematic representation of this protocol is demonstrated in Fig. 6.2.

In this part of the present study *C. dubliniensis* derivative CD36 (M/G) and *C. albicans* strain SC5314 were examined in the infant mouse model. Strain CD36 and derivative SC5314 (M/G) were also examined to confirm that the tag had no affect on the strains in the infant mouse model. The results are summarised in Table 6.3. *Candida dubliniensis* strains CD36 and derivative CD36 (M/G) showed mean values of 4.8×10^4 and 6.4×10^2 cfu/ml, respectively, in the stomach and 8.3×10^5 and 2.2×10^3 cfu/ml in the liver. The difference between the tagged and untagged strains was deemed not to be significant with a P value of >0.05, this was the same for all comparisons between tagged and untagged strains except for the comparisons between the tagged and untagged SC5314 strain in the liver where a P value of 0.021 was obtained. This is likely to be due to the difference in the levels of colonisation i.e. 5/5 mice were colonised with strain SC5314 and 4/5 mice were colonised with SC5314 (M/G) (Fig. 6.3). This indicated that tagging of these strains with the molecular marker cassette conferred no advantage or disadvantage in comparison to the parent for any of the strains.

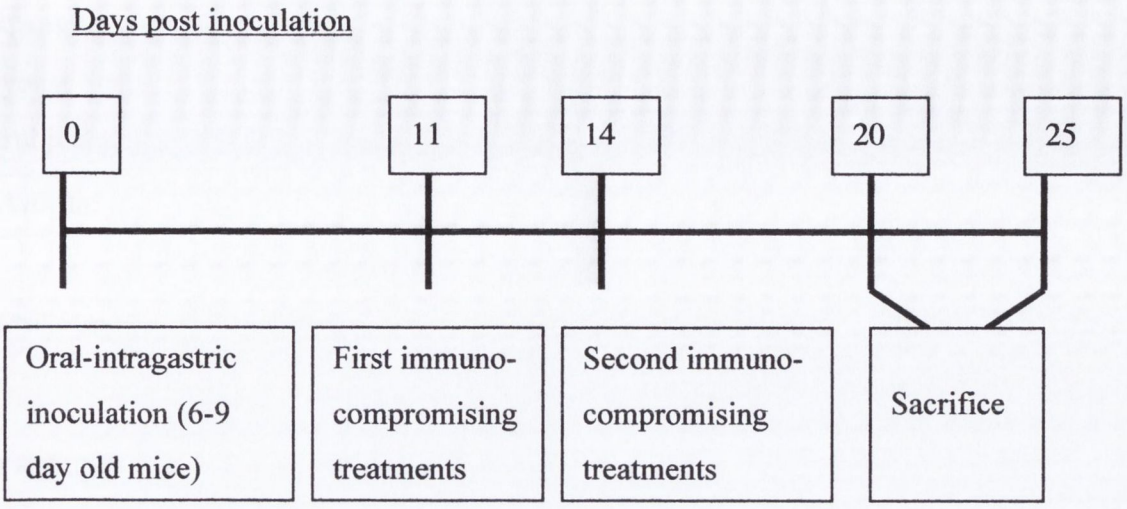

Figure 6.2. Protocol for inoculation of mice. Treatment of animals with immunocompromising drugs (cyclophosphamide plus cortisone acetate) occurred at days 11 and 14. Animals were sacrificed after 20 days post inoculation.

Table 6.3 *Candida* colony forming units (cfu) recovered from the stomach and liver of yeast infected mice inoculated with *C. albicans* and *C. dubliniensis* separately and subjected to immunocompromising procedures.

Animal treatment	**Stomach**	**Liver**
	(cfu/ml)	
Challenged with *C. dubliniensis* strain CD36	4.8×10^4 [a] 2/5 [b] $(0 - 1.8 \times 10^5)$ [c]	8.3×10^5 [a] 2/5 [b] $(0 - 4.2 \times 10^6)$ [c]
Challenged with *C. dubliniensis* strain CD36 (M/G)	6.4×10^2 [a] 3/5 [b] $(0 - 3 \times 10^3)$ [c]	2.2×10^3 [a] 3/5 [b] $(0 - 1.1 \times 10^4)$ [c]
Challenged with *C.albicans* strain SC5314	2.8×10^6 [a] 5/5 [b] $(4.5 \times 10^5 - 3.8 \times 10^6)$ [c]	1.1×10^5 [a] 5/5 [b] $(1.3 \times 10^4 - 4.2 \times 10^5)$ [c]
Challenged with *C. albicans* strain SC5314 (M/G)	2×10^6 [a] 5/5 [b] $(3.3 \times 10^5 - 4.1 \times 10^6)$ [c]	3.1×10^3 [a] 4/5 [b] $(0 - 1.2 \times 10^4)$ [c]

[a] Mean values for all animals in each treatment group.

[b] Number of culture positive for *Candida* over total tested.

[c] Range of cfu of *Candida* for positive samples.

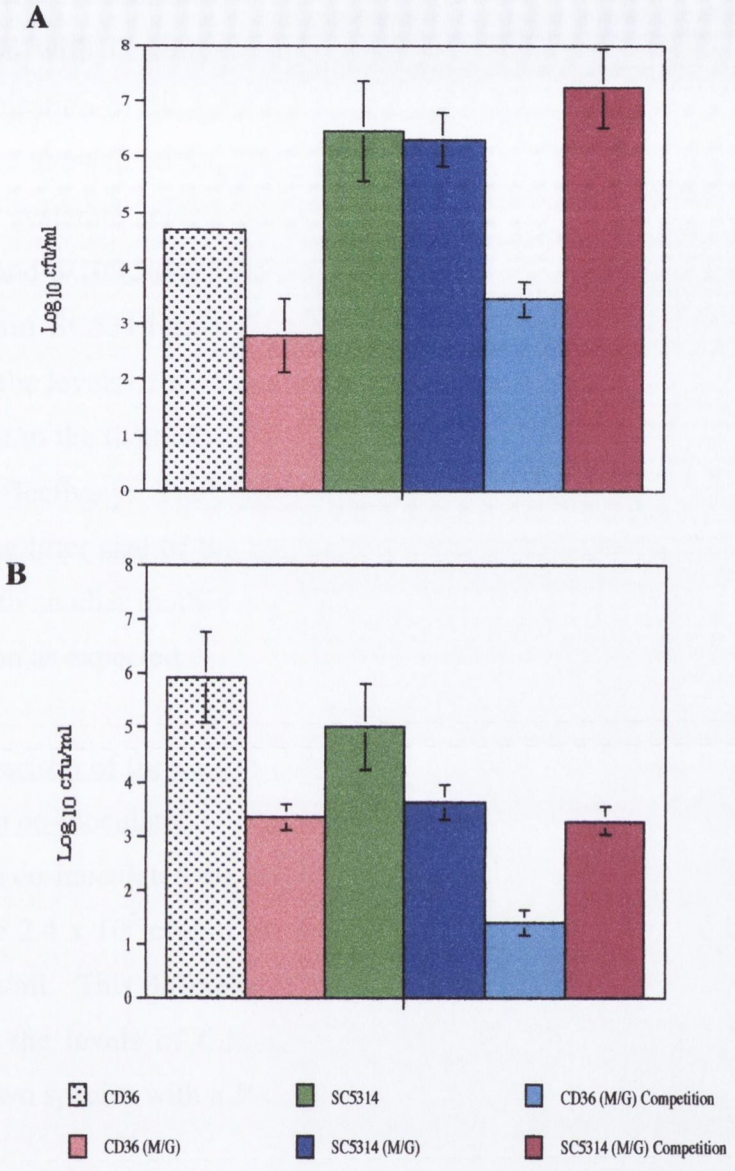

Figure 6.3. Graphs showing the levels of colonisation (cfu/ml) of *C. albicans* strain SC5314 and derivative SC5314 (M/G) and *C. dubliniensis* strain CD36 and derivative CD36 (M/G) when inoculated separately and together in both the (A) stomach and (B) liver after immunocompromising treatment. Strains SC5314 and CD36 (M/G) were used in the co-infection study.

Comparison of the results of *C. albicans* and *C. dubliniensis* indicates that as outlined in section 6.3.1.1, *C. albicans* is much better at colonising organs in the infant mouse model in comparison with *C. dubliniensis*. All mice infected with *C. albicans* 10/10 (100%), showed colonisation of the stomach in comparison with *C. dubliniensis* whereby only 5/10 (50%) of mice showed colonisation in the stomach. The presence of *Candida* in the liver indicates that systemic spread has occurred and 9/10 (90%) mice showed colonisation with *C. albicans* and 5/10 (50%) with *C. dubliniensis*. Comparison of the results between *C. albicans* strain SC5314 and *C. dubliniensis* derivative CD36 (M/G) showed that the difference in the levels of the two species was significant in the stomach (*P* value 0.007) and not significant in the liver (*P* value 0.113). These findings show that two species are able to disseminate effectively. The reason for the different numbers of mice treated in each group was due to the litter size of the mother as it was deemed that removal from the mother and placement with another mother may lead to death of the animal. Uninfected controls showed no colonisation as expected.

6.3.2.2 Comparison of the ability of *C. albicans* and *C. dubliniensis* to disseminate from the stomach when co-inoculated

When co-inoculated the levels of *C. dubliniensis* derivative CD36 (M/G) found in the stomach were 2.4 x 10^2 cfu/ml in comparison with *C. albicans* strain SC5314, which were 1.7 X 10^7 cfu/ml. This difference was significant with a *P* value < 0.001 (Table 6.4). The difference in the levels of *Candida* found in the liver was also found to be significant between the two species with a *P* value of 0.042.

6.3.3 Histological analysis of organs

Histological sections of the murine stomach of infected, immunocompromised mice which were sacrificed 20 days post challenge shows the presence of the cardial atrium fold (CAF) which is clearly evident in 3 week-old mice and separates the keratinised epithelium and the glandular epithelium. The CAF in Fig. 6.4 is visibly swollen due to infection with *C. albicans* from Cole *et al.* with permission (Cole *et al.*, 1990a).. An outline of the different layers of the murine stomach is represented in Fig. 6.5 (Cole *et al.*, 1996). Penetration of the stomach by *Candida* cells was evident as *C. albicans* strain SC5314 is clearly visible in both

Table 6.4. *Candida* colony forming units (cfu) recovered from the stomach and liver of yeast infected mice inoculated with *C. albicans* and *C. dubliniensis* together and subjected to immunocompromising procedures.

Animal treatment	**Stomach**	**Liver**
	(cfu/ml)	
Challenged with an inoculum containing *C. albicans* strain SC5314 and *C. dubliniensis* strain CD36 (M/G)	2.4×10^2 [a]	1.5 x 10
	2/10 [b]	1/10
C.F.U. levels of CD36 (M/G)	$(0 - 2 \times 10^3)$ [c]	$(0 - 1.5 \times 10^2)$
Challenged with an inoculum containing *C. albicans* strain SC5314 and *C. dubliniensis* strain CD36 (M/G)	1.7×10^7	1.9×10^3
	10/10	7/10
C.F.U. levels of SC5314	$(1.1 \times 10^3 - 4.3 \times 10^6)$	$(0 - 1.3 \times 10^4)$

[a] Mean values for all animals in each treatment group.

[b] Number of culture positives for *Candida* over total tested.

[c] Range of cfu of *Candida* for positive samples.

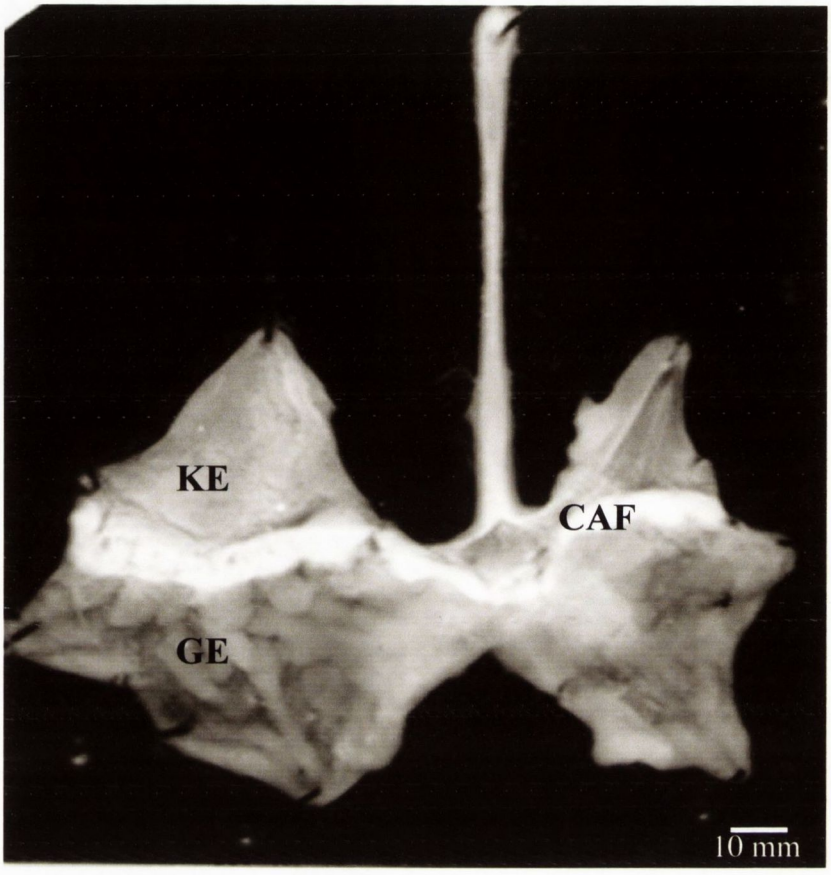

Figure 6.4. Photograph of macroscopic features of a longitudinally bisected stomach. The cardial-atrium fold (CAF) is slightly thickened, and is separated by the glandular epithelium (GE) and squamous epithelium (SE), presented with permission (Cole *et al.*, 1990).

the yeast and hyphal phase in the stomach submucosa (Fig 6.6 B & C) and in the gastric lumen (Fig. 6.6 D). Unfortunately due to the low colonisation levels of *C. dubliniensis,* 50%, stomachs removed from mice infected with *C. dubliniensis* and prepared for light microscopy showed no apparent colonisation, however, these mice were also negative in enumeration of cfu/ml in the stomach. Smears of stomachs of mice that had been infected with *C. dubliniensis* strain CD36 tagged with the molecular marker cassette and *C. albicans* strain SC5314 tagged with the molecular marker cassette and examined using FITC filters shows the presence of *C. dubliniensis* cells in the yeast phase, with no hyphae present and *C. albicans* cells in the hyphal phase (Fig. 6.7).

Examination of body organs during dissection of infected, immunocompromised mice sacrificed 20 days post-challenge revealed the presence of macro-abscesses on the liver and also on the kidney of some mice infected with *C. albicans* and *C. dubliniensis* (Fig. 6.8 A & B). Thin sections of liver abscesses demonstrated the presence of both yeast and hyphae cells in both species (Fig. 6.8 C & D). Thin sections of the kidney demonstrate the presence of an abscess consisting mainly of yeast cells for *C. dubliniensis* strain CD36 (Fig. 6.8 E) and yeast and hyphal cells for a mouse which had been infected with *C. albicans* strain SC5314 (Fig. 6.4 F).

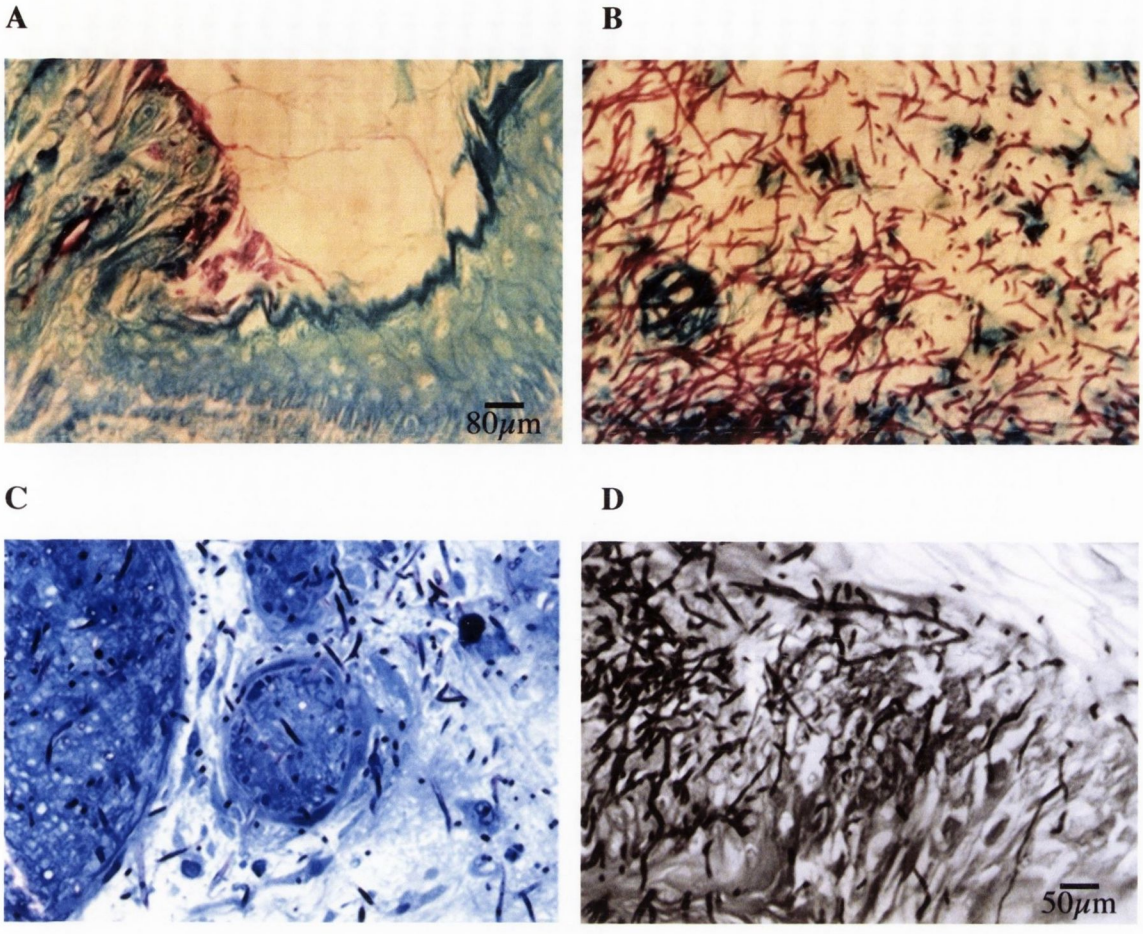

Figure 6.6. Light micrographs of histological preparations of the stomachs of *Candida* infected, immunocompromised mice 20 days post challenge. (A) Uninfected control stomach. Penetration of the stomach is evident by the presence of both yeast and hyphae of *C. albicans* strain SC5314 in the (B & C) submucosa and (D) lumen. (A & B) were PAS stained and (C & D) were azure 11-methylene blue stained. The scale bar of 80μm is representative of the light micrograph A and the scale bar of 50μm is representative of the light micrographs B-D.

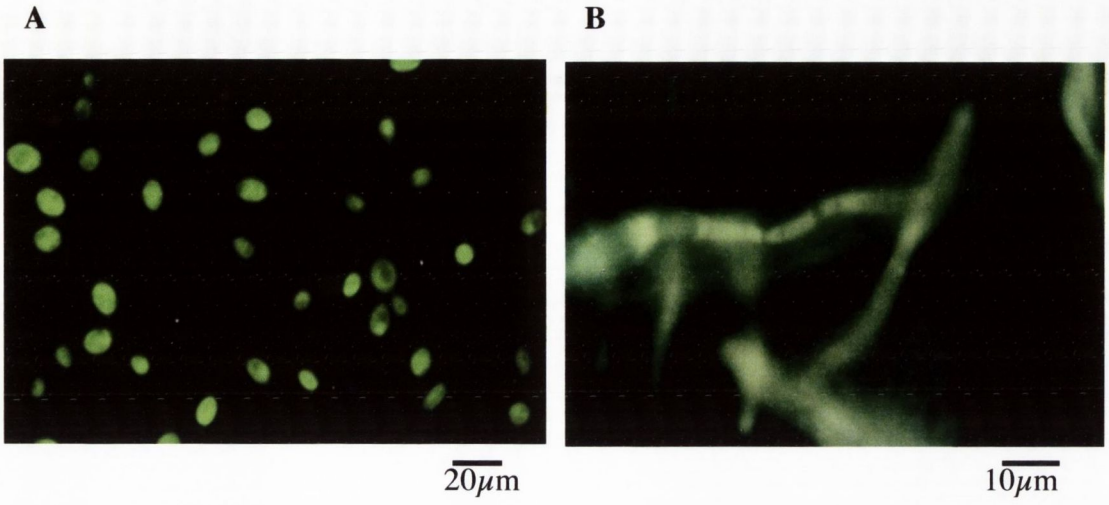

Figure 6.7. Fluorescent micrographs of cells from stomach smears of a mouse infected with (A) *C. dubliniensis* derivative CD36 (M/G) which shows the presence of only yeast cells and (B) *C. albicans* derivative SC5314 (M/G) which shows only hyphal cells.

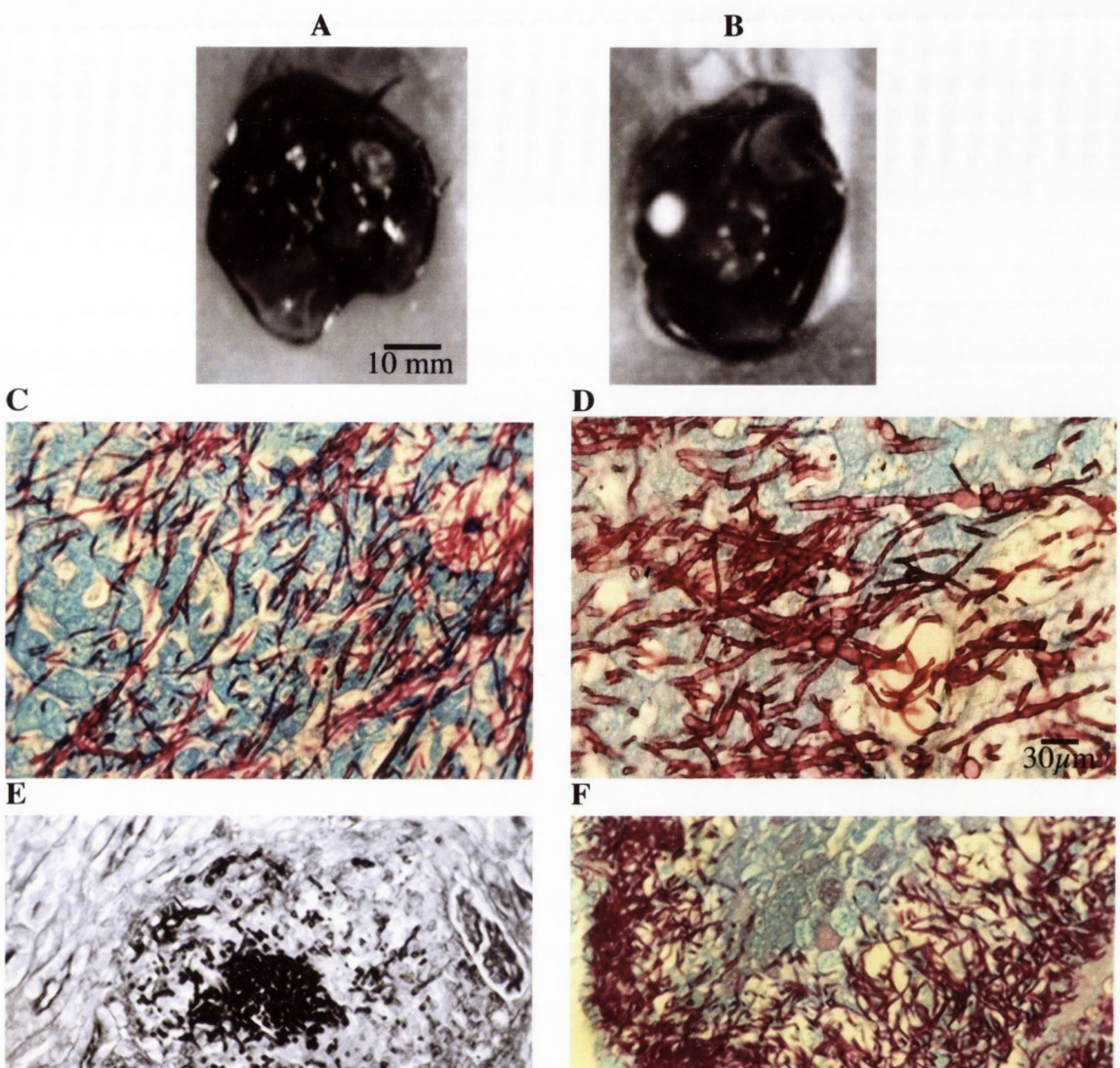

Figure 6.8. Photographs and light micrographs of PAS stained *Candida* abscesses in body organs of infected, immunocompromised mice 20 days post challenge. The presence of macro-abscesses are shown in the liver in (A) for *C. dubliniensis* strain CD36 and (B) for a mouse infected with *C. albicans* strain SC5314 and *C. dubliniensis* derivative CD36 (M/G) together in competition. Thin sections through abscessses in the liver, (C) for *C. dubliniensis* strains CD36 and (D) for *C. albicans* strain SC5314. Thin sections through abscesses in the kidney for (E) *C. dubliniensis* strain CD36 and (F) *C. albicans* strain SC5314. The scale bar of 10 mm is representative of photographs A and B, the scale bar of 30μm is representative of light micrographs C and D and the scale bar of 50μm is representative of light micrographs E and F.

6.4 Discussion

6.4.1 The ability of *C. albicans* and *C. dubliniensis* to colonise the intestine and stomach of the infant mouse model in single and co-infection studies

It has previously been shown that when mice were inoculated with *C. albicans* by the oral intragastric routes as infants (6-9 days old), they were found to develop gastointestinal (GI) candidiasis which persisted for at least 30-60 days post-challenge without the use of immunocompromising procedures (Cole *et al.*, 1989; Field *et al.*, 1981; Pope *et al.*, 1979). Organs of these non-immunocompromised mice were also shown to remain free of *C. albicans* for at least 20 days post inoculation, despite the presence of yeast in the GI tract (Cole *et al.*, 1989). It has previously been shown that immunocompromising treatments such as cyclophosphamide and cortisone acetate allow proliferation of *C. albicans* in the oesophagous, stomach and intestines, as well as hyphal invasion of the gastric mucousa in animals with persistent GI infection (Cole *et al.*, 1989; Odds, 1988; Suzuki *et al.*, 1988). This situation also occurs in neutropenic patients with systemic candidisais whereby the GI tract has been proposed as the principal portal of entry to the bloodstream and subsequently to organs such as the liver, spleen and kidneys (Narayanan *et al.*, 1991).

In the present study the infant mouse model provided a reliable model to investigate colonisation and dissemination of *Candida.* The results show that, as previously described, *C. albicans* is able to establish itself in the GI tract of infant mice and persist longer than 10 days. However, ten-fold less *C. dubliniensis* were present after 2, 4, and 6 days in the intestine and a hundred-fold less after 8 and 10 days (Fig. 6.1). Furthermore, no *C. dubliniensis* was detected in the stomach after 6 days indicating clearance of the yeast which most likely occurred in the faeces and would indicate a less stable attachment to the GI tract. The murine stomach is split into a glandular and stratified region with the cardial-atrium fold (CAF) representing the interface between the two regions (Fig. 6.4). The stratified and keratinised squamous epithelium of the oesophageal mucousa is continuous with a large portion of the gastric mucosa in the mouse. In the human stomach on the other hand the keratinised layer of the upper alimentary tract extends only to the cardiac sphincter. It is the cardial-atrium fold that has been identified as a major site of invasion of host tissue by *C. albicans* (Guentzel *et al.*, 1985; Myerowitz *et al.*, 1977; Odds, 1988; Suzuki *et al.*, 1988).

It has been shown previously by Kirkpatrick *et al*, when grown in competition *in vitro* that *C. albicans* is able to out-compete *C. dubliniensis* in liquid culture at 37°C over 96 h (Kirkpatrick *et al.*, 2000). In the present study, when *C. albicans* and *C. dubliniensis* were inoculated in equal proportions into the infant mouse model in competition with one another and the levels of each species in the stomach and intestine examined after 2, 4, 6, 8 and 10 days, C. *albicans* was present in greater numbers. The results reflect what was found in the single infection as no *C. dubliniensis* were detected after 6 days in the stomach, which indicates clearance of *C. dubliniensis* faster than *C. albicans*. *Candida albicans* was still present in large numbers 2.3×10^3 cfu/ml in the stomach and 2.2×10^3 cfu/ml in the intestine after 10 days (Table 6.2). These results when examined with the results from the single infection study suggest that *C. dubiniensis* is unable to colonise the gastrointestinal tract to the same levels as *C. albicans* or is cleared by the host much faster than *C. albicans* through shedding in the faeces.

The passage of *C. albicans* through the intestinal mucousa is recognised as being a significant source for systemic infection (Krause *et al.*, 1969). *Candida albicans* has been examined extensively in the infant mouse model and has been shown to associate readily with the gut wall most notably with the secreting and keratinised epithelium of the stomach and the epithelial surface of the intestine, and to pass through the mucousa to initiate systemic infection (Field *et al.*, 1981; Pope *et al.*, 1979). *Candida albicans* has also been shown to associate with the mucousa of antibiotic-treated mice by at least five distinct mechanisms, which include adhesion to epithelium, adhesion to mucous, co-adhesion to adherent fungi, co-adhesion to adherent bacteria and entrapment in the mucousal gel overlying the epithelium (Kennedy *et al.*, 1987). These results suggest that *C. dubliniensis* may have reduced adhesion or an inabilty to compete with host ligands in comparison with *C. albicans*

In conclusion, the results from this part of the present study suggest that the infant mouse model was very useful for comparing the ability of *C. albicans* and *C. dubliniensis* to colonise the GI tract. The results also suggest that *C. dubliniensis* is deficient in comparison with *C. albicans* in its ability to colonise and adhere to the GI tract. However, previous studies have shown that *C. dubliniensis* is capable of adhering as efficiently as *C. albicans* to purified small intestinal mucin (de Repentigny *et al.*, 2000). This was in contrast with other

Candida species, namely *C. parapsilosis* and *C. lusitaniae,* which showed only moderate adherence and *C. krusei* and *C. glabrata,* which showed only weak adherence. Further investigation is necessary to ascertain the reason for the rapid clearance of *C. dublininiensis* from the GI tract.

6.4.2 The ability of *C. albicans* and *C. dubliniensis* to disseminate from the stomach of the infant mouse model in both single and co-infection studies

From the data described in section 6.3.1, it is clear that *C. dubliniensis* has a reduced capacity to adhere to and colonise the infant mouse stomach. It was therefore decided to investigate the ability of each species to disseminate from the GIT to the liver and kidney. Following immunocompromising treatment with cyclophosphamide and cortisone acetate, dissemination of both *C. albicans* and *C. dubliniensis* was shown to occur in the infant mice. Cyclophosphamide is toxic to dividing cells which disrupts the integrity of the mucousal epithelium of the gut (Suzuki *et al.*, 1988). The presence of *Candida* in the liver is indicative of systemic spread from the GI tract. In this study (9/10) 90% of mice showed colonisation of the liver by *C. albicans* whereas only (5/10) 50% showed colonisation of the liver by *C. dubliniensis*. However, only (5/10) 50% of mice in this part of the present study showed colonisation of the stomach by *C. dubliniensis,* whereas (10/10) 100% of mice showed colonisation by *C. albicans.* The reason for the poor levels of dissemination of *C. dubliniensis* is more likely due to the poor levels of colonisation of the stomach. This also holds true for the co-infection study whereby 2.4 x 10^2 cfu/ml of *C. dubliniensis* derivative CD36 (M/G) was detected in the stomach in comparison with *C. albicans* strain SC5314 where 1.7 X 10^7 cfu/ml was detected. Furthermore in the liver 1.5 x 10 cfu/ml of *C. dubliniensis* derivative CD36 (M/G) was detected and 1.9 x 10^3 cfu/ml of *C. albicans* strain SC5314. These results indicate that *C. dubliniensis* is not significantly outcompeted by *C. albicans* when grown in competition and that *C. dubliniensis* without immunocompromising treatment is also unable to colonise as efficiently as *C. albicans* after immunocompromising treatment.

It has been suggested that deep mucousal tissue invasion and early haematogenous spread are thought to occur by transmigration (persorption) of yeast cells across the mucousal barrier (Cole *et al.*, 1993). Penetration of the mucin layer may also be facilitated by the *C.*

albicans secretory aspartyl proteinase sap2p, which has been shown to degrade murine small intestinal mucin *in vitro* (Colina *et al.*, 1996) and to be able to degrade secretory immunoglobulin A (IgA) (Goldman *et al.*, 1995; Ruchel, 1986). Phospholipase B has also been suggested as directly degrading the phospholipid constituents of cell membranes and the damage sustained to the protective cell membrane may provide rapid access to the cytoplasm (Mukherjee *et al.*, 2001). Morschhauser *et al.* have shown that induction of *C. albicans* proteinase caused digestion of soluble and immobilised extracellular matrix proteins produced by a human endothelial cell line, thus suggesting that Sap proteins may facilitate the dissemination of *C. albicans* via the circulatory system (Morschhauser *et al.*, 1997). The presence of secretory aspartyl proteinases have been detected for *C. dubliniensis*, however, phenotypic studies to determine the levels of proteinase production have yielded contradictory results (Borg-Von Zepelin *et al.*, 2002; Hannula *et al.*, 2000; McCullough *et al.*, 1995). Recently work by Moran *et al.*, indicated the presence of only one gene in *C. dubliniensis CdSAP4,* with strong homology to the *SAP4-6* subfamily in *C. albicans* (Moran *et al.*, 2004). The Sap4-6 subfamily has been shown to be the most important in this family in the establishment of murine intraperitoneal infection (Felk *et al.*, 2002; Sanglard *et al.*, 1997). This may partly explain why *C. dubliniensis* is less virulent than *C. albicans* in a murine model of systemic infection (Gilfillan *et al.*, 1998; Vilela *et al.*, 2002).

The results from this part of the present study indicate that tagging the strains with the molecular marker cassette had no deleterious or advantageous effect on the strains in the infant mouse model. These results were also reflected in a systemic mouse model whereby 6 BALB/c female mice were inoculated by the intravenous route with strains SC5314, SC5314 (M/G), CD36 and CD36 (M/G) separately. The mean survival times for each strain were calculated and were found to be 6.5 ± 2.3 days and 6.2 ± 0.8 days for *C. albicans* strain SC5314 and derivative SC5314 (M/G) and 28 ± 0.0 days and 21.7 ± 8.2 days for *C. dubliniensis* strain CD36 and derivative CD36 (M/G), respectively (personal communication Frank Odds). These results show that no significant difference was observed between tagged and untagged strains. These results also show that *C. dubliniensis* is significantly less virulent as mice survived four times as long as those infected with *C. albicans*. Based on these results 2 BALB/c female mice were injected with a co-culture of 25-fold more *C. dubliniensis* than *C. albicans* to see if the lower virulence of *C. dubliniensis* could be

overcome by a higher inoculum. After 4 days the animals were sacrificed and it was found that there was a 40-fold excess of *C. albicans* over *C. dubliniensis* in the kidneys while the number of both species were the same on examination of the spleen and brain.

In conclusion, the results from this study suggest that *C. dubliniensis*, like *C. albicans* is capable of dissemination to the liver from the GI tract after immunocompromising treatment in the infant mouse model. However, the levels present in the stomach and the liver are significantly lower in *C. dubliniensis*.

6.4.3 Histological analysis of organs after immunocompromising treatment

Animals were sacrificed 20-24 days post challenge by asphyxiation with carbon dioxide and dissected under aseptic conditions. The murine stomach is made up of a multitude of layers consisting of the outermost keratin layer, followed by epithelium, Lamina propria, Muscularis mucosa, Submucosa, Muscularis externa and Serosa (Fig. 6.5). The presence of both yeast and hyphae in the submucousa and gastric lumen for *C. albicans* strain SC5314 in Fig. 6.6 (B-D) indicates that penetration of these layers has occurred. On examination smears of stomachs of mice infected with *C. dubliniensis* derivative CD36 (M/G) and *C. albicans* derivative SC5314 (M/G) indicates that *C. dubliniensis* in contrast to *C. albicans* is present in the yeast phase and also that GFP is fully functional in both species (Fig. 6.7).

The presence of yeast and hyphal-containing focal abscesses in the liver, lungs and kidney have previously been demonstrated for *C. albicans* in the infant mouse model following immunocompromising treatment and are indicative of chronic systemic candidiasis (Cole *et al.*, 1989). The presence of micro- and macro-abscesses that contain *C. albicans* have been reported in granulocytopenic patients and have been suggested to arise as the result of dissemination of yeast via the hepatic portal vein from the foci of colonisation in the GI tract (Tashjian *et al.*, 1984). In the present study macro-abscesses were found on livers and kidneys in both *C. albicans* and *C. dubliniensis* (Fig. 6.8). The presence of *C. albicans* and *C. dubliniensis* in the liver suggests that the hepatic portal vein is involved in the dissemination of the pathogen from the GI tract after immunosuppression, which exposes the liver to high numbers of *Candida* cells. Previously Vilela *et al.*, showed that in a systemic mouse infection model, histopathological analysis of infected kidneys showed that *C.*

dubliniensis cells were predominantly in the yeast phase, whereas *C. albicans* produced far higher levels of hyphae and pseudohyphae (Vilela *et al.*, 2002). In the present study *C. dubliniensis* cells that were mainly in the yeast phase were detected on examination of an abscess in the kidney (Fig. 6.8 E). However, also in this study, the presence of both yeast and hyphal forms for *C. dubliniensis* strain CD36 was shown in the liver, which indicates that *C. dubliniensis* is capable of dissemination to the liver as previously shown using colony counts and does this through the production of both yeast and hyphal cells. This may indicate that colonisation of the kidney takes place in *C. dubliniensis* by production of yeast cells in contrast to hyphae.

In conclusion histopathological analysis of the organs confirms the data obtained from the colonisation and dissemination experiments whereby 90% of *C. albicans* and 50% of *C. dubliniensis* infected mice showed high levels of cell density in the liver. The results from this part of the present study show both species are capable of dissemination from the GI tract to the liver and kidney. Also both species were present in both the yeast and hyphal phase of growth in the liver, however, *C. dubliniensis* was present mainly in the yeast phase in the kidney and also in the stomach in comparison to *C. albicans* which, was found in both the yeast and hyphal cells. Overall the findings of this part of the study show that *C. albicans* is better able to colonise and establish itself in the gut of the infant mouse whereas *C. dubliniensis* is being cleared from the stomach and intestine most probably in the faeces.

Chapter 7
General Discussion

7.1 Discussion

Candida dubliniensis was first identified as a species distinct from *C. albicans* in 1995. Given the number of similarities between the two species, it would be expected that they would have a similar capacity to cause disease. However, epidemiological data clearly show that *C. albicans* is significantly more often associated with human disease. Recently it has been shown that *C. albicans* and *C. dubliniensis* were the most prevalent yeast isolates from HIV-positive women (Navazesh *et al.*, 2005). *Candida dubliniensis* is clearly able to cause both mucousal and systemic candidiasis, however, in comparison with *C. albicans* it is not as successful a pathogen. *Candida albicans* is the species most frequently recovered from patients presenting with candidiasis; consequently it is generally accepted that it possesses a significantly greater pathogenic potential than other members of the genus. Only a limited number of studies have been carried out comparing the virulence of *C. albicans* and *C. dubliniensis* and these have shown that *C. albicans* is significantly more pathogenic than *C. dubliniensis* using *in vivo* infection models (Gilfillan *et al.*, 1998; Vilela *et al.*, 2002).

The hypothesis on which the present study is based is that the increased capacity of *C. albicans* to cause disease in comparison with *C. dubliniensis* is due to an enhanced ability of *C. albicans* to thrive under many environmental conditions. In order to investigate this, the comparative growth of the two species was investigated under a wide range of experimental conditions, including liquid and solid media *in vitro* and by using two models of infection, the infant mouse model and reconstituted human epithelium (RHE). The ability of *C. albicans* and *C. dubliniensis* to grow alone and in competition with one another was also examined *in vitro* and *in vivo* in the infant mouse model.

The growth rate of the two species was compared in the present study using a well defined nutrient poor medium (YNB) and also a nutrient rich medium (SDB) at 37°C and it was found that the *C. albicans* strains tested had a higher growth rate in comparison with a range of *C. dubliniensis* strains (Table 3.4). The reason for the higher growth rate exhibited by *C. albicans* is unknown but may simply be that *C. albicans* is better adapted to growth at 37°C in comparison with *C. dubliniensis*. *Candida dubliniensis* may be less able to utilise the various sugars present in the media in comparison with *C. albicans,* or it may be due to

inefficient carbohydrate metabolism, which may cause this slower growth rate. The range of carbohydrates assimilated by *C. albicans* and *C. dubliniensis* has been shown to be significantly different (Boerlin *et al.*, 1995; Gales *et al.*, 1999; Jabra-Rizk *et al.*, 1999; Kirkpatrick *et al.*, 1998; Pincus *et al.*, 1999; Sullivan *et al.*, 1995; Tintelnot *et al.*, 2000). Further work comparing the growth rates of the two species over a variety of different temperatures would be interesting to determine whether at temperatures lower than 37°C *C. dubliniensis* may have a growth rate more comparable with *C. albicans*. In the present study the same experimental design used by Kirkpatrick *et al.* was used with reference strains of *C. albicans* and *C. dubliniensis,* but a different, more accurate method to differentiate between the two species was used (Kirkpatrick *et al.*, 2000). A molecular marker cassette was designed containing two markers MPA^R and GFP and inserted into strains of *C. albicans* and *C. dubliniensis* which allowed accurate differentiation to be performed between the two species on the basis of growth or lack of growth on MPA-containing media (Fig. 3.1). The results of this study showed that in each of the pairings between *C. albicans* and *C. dubliniensis* examined in both YNB medium over 48 h in the same flask and in SDB medium over 96 h with dilution into fresh culture every 24 h, *C. albicans* was present in significantly higher cell densities than *C. dubliniensis*. It is important to note that *C. dubliniensis* isolates from the oral cavity are most commonly recovered in mixed culture with other *Candida* species, especially *C. albicans,* which would be unlikely to occur if *C. albicans* had the ability to completely out-compete *C. dubliniensis* (Coleman *et al.*, 1997b). The reason for the competitive advantage shown by *C. albicans* is likely to be a result of its faster growth rate compared to *C. dubliniensis*.

The molecular marker cassette designed in the present study will allow comparative interactions to be performed with numerous *Candida* species to see if some species are able to out-compete others, which may be a reason why some species are more prevalent than others. It would also be interesting to add the antifungal azole drug fluconazole to the growth medium to examine whether this has any effect on the ability of one species to out-compete another as this may be what occurs in immunocompromised patients undergoing treatment with fluconazole as the emergence of other *Candida* species is thought to be due to the widespread therapeutic use of prophylactic antifungals as they have an inherent reduced

susceptibility relative to *C. albicans* (Chavanet *et al.*, 1994; De Bernardis *et al.*, 1996; Johnson *et al.*, 1995; Moran *et al.*, 1997; Powderly, 1992).

One of the most important virulence factors associated with *C. albicans* is its ability to form true unconstricted hyphae. A wide variety of experimental conditions have been shown to induce the production of hyphae in *C. albicans,* including conditions such as neutral pH and temperature of 37°C, the presence of serum, CO_2 concentrations and nutrient depletion. However, only one study has compared the ability of *C. albicans* and *C. dubliniensis* to produce hyphae under a limited set of induction conditions and found that hyphal production is less efficient in *C. dubliniensis* in comparison with *C. albicans* in response to shifts in pH/temperature or when incubated in serum (Gilfillan *et al.*, 1998). In the present study, *C. dubliniensis* was found to produce hyphae only under certain liquid environmental conditions. The only medium to consistently induce hyphae in *C. dubliniensis* was water with 10% serum whereby 73% (8/11) of strains tested were ≥ 50% in the hyphal phase of growth following 6 h incubation. In all of the liquid hyphal inducing media examined all strains of *C. albicans* (4/4) produced significant amounts (> 50%) of hyphae (Table 4.3), whereas in many cases only some strains of *C. dubliniensis* produced hyphae in each of the media examined and only very low percentages of strains (9-27%) were ≥ 50% in the hyphal phase of growth after 6 h.

The ability to produce hyphae on solid media was also examined in both *C. albicans* and *C. dubliniensis.* The data obtained show that hyphal induction was strain-dependent on Spider medium as some strains belonging to both species produced hyphae on this medium and no direct correlation could be made between the ability of either species to produce hyphae. Embedded conditions were also examined using a sandwich of yeast extract-peptone-sucrose (YPS), (Fig. 4.5). Eight out of eleven (73%) strains of *C. dubliniensis* and 4/4 (100%) strains of *C. albicans* tested produced hyphae when embedded in this medium. In contrast, when the two species were compared on Staib and Pal's agar *C. dubliniensis* strains (100%) produced abundant hyphae, pseudohyphae and chlamydospores in contrast to *C. albicans* strains which produced none of these which confirms the results of Al Mosaid *et al.* (Al Mosaid *et al.*, 2003; Al Mosaid *et al.*, 2001). These data suggest that there is likely to be a difference in the signalling and regulatory pathways governing the induction of hyphae

in the two species when grown on this medium. A difference in such a pathway has already been observed by Staib *et al.*, who recently demonstrated that the *C. albicans* and *C. dubliniensis NRG1* orthologues are differentially regulated. In *C. dubliniensis*, growth on Staib agar leads to repression of the *NRG1* gene and concommitant relief of the repression of filamentation. In *C. albicans*, however, the *NRG1* gene is constitutively expressed during growth on Staib agar and cells of *C. albicans* remain in the yeast phase on this medium (Staib & Morschhauser, 2005).

The present study also determined the presence of a homologue of the hyphal wall protein (Hwp1) in *C. dubliniensis* which is expressed on the surface of germ tubes and hyphae and is involved in adherence by forming tight covalent interactions with host cells. All of the published *C. dubliniensis* genes analysed to date share more than 90% identity at the nucleotide sequence level with the orthologous *C. albicans* genes. Surprisingly, *CdHWP1* and *CaHWP1* were only 45.3% homologous at the nucleotide level and 49.3% homologous at the amino acid level. The deduced amino acid sequence of the *C. dubliniensis* Hwp1p is only 421 amino acids long compared to 634 amino acids in *C. albicans* Hwp1p (Fig. 4.11). A large proportion of the region encoding the N-terminus is missing from the *CdHWP1* gene which is proposed to be the domain of the protein that is involved in adhesion and therefore *CdHWP1* may be defective and therefore result in reduced adhesion (Fig. 4.12). Although this study showed that the *HWP1* homologue in *C. dubliniensis* was expressed in the hyphal phase like *C. albicans,* it is still unknown whether the regulation of *HWP1* expression is the same in the two species. Sundstrom *et al.*, have shown that *C. albicans HWP1* mutants in immunodeficient mice fail to cause oral or oesophageal lesions, suggesting that expression of *HWP1* may be important for penetration of epithelial surfaces (Sundstrom, 2002). Interestingly, Moran *et al.*, have also shown the divergence of another hyphal expressed gene (*HYR1*) in *C. dubliniensis* in comparison with *C. albicans* and in the *SAP* genes which are expressed in the hyphal phase as only one gene in *C. dubliniensis* (*CdSAP4*) with similarity to *CaSAP4-6* was detected using comparative genomic hybridisation (CGH) with DNA microarrays (Moran *et al.*, 2004).

The recent availability of the *C. dubliniensis* genome sequence provided the opportunity to identify and compare homologues of the genes implicated in the regulation of hyphae in *C. albicans* with *C. dubliniensis*. The results from the present study show that *C.*

dubliniensis has homologous orthologues of many of the genes involved in the hyphal induction pathway in *C. albicans* (Table 4.6), however, little is known about the regulation of these genes in *C. dubliniensis*. Many of the *C. albicans* genes involved in the yeast-to-hyphae transition (Fig.4.20) are present in *C. dubliniensis*, but they may be regulated differently to *C. albicans* due to the different response of *C. dubliniensis* to the environmental conditions examined here. Further work is required to further investigate the regulation of the yeast-to-hyphal transition in *C. dubliniensis* in comparison with *C. albicans*. This could be achieved using microarray analysis on the two species under a variety of conditions used in this study which induce hyphae. This analysis will allow a comparison of the behaviour of large numbers of genes in different tissues and under different environmental conditions to identify virulence genes, gene pathways, and regulatory networks. This would allow the identification of the genes involved in hyphal formation under the different environmental conditions in the two species and highlight differences in their expression and regulation in order to attempt to explain the difference in virulence of the two species. Gene disruption studies would also be useful to determine the role of some key regulators of filamentaion in *C. dubliniensis*, in particular genes orthologous to *EFG1*, *CPH1*, *TUP1* and *NRG1*. In particular further investigation of the role of *NRG1* needs to be performed due to its differential regulation in *C. dubliniensis* on Staib agar.

Previous studies have relied upon the use of animal infection models to investigate the virulence of *Candida* strains and specific mutants, in particular the systemic mouse infection model (Gilfillan *et al.*, 1998; Vilela *et al.*, 2002). However, this model is difficult to standardise and there are increasing difficulties in performing animal based studies. For these reasons, researchers have turned to other models and have recently begun to investigate the use of *ex vivo* infection models to study virulence (Korting *et al.*, 1998; Korting *et al.*, 1999; Korting *et al.*, 2003; Schaller *et al.*, 1998a; Schaller *et al.*, 2004). One of the most commonly used *ex vivo* models is Reconstituted Human Epithelium (RHE) which is commercially available. The present study was the first to examine the ability of *C. dubliniensis* to attach to and penetrate RHE and compare this with what occurs with *C. albicans*. When the two species were examined in the present study using RHE, *C. dubliniensis* showed an inability to adhere to the surface of the epithelium and the cells, which did adhere, failed to produce evident hyphae. In comparison, *C. albicans* showed

large numbers of cells attaching to the surface of the epithelium and hyphal cells penetrating down through the tissue. These findings clearly indicate that there are very likely differences in regulation of hyphal expression and in the ability of the two species to adhere to and colonise the RHE model (Fig. 5.2). The lack of colonisation of *C. dubliniensis* is similar to what was observed by Dietrich *et al.* in *C. albicans* efg1/efg1 mutants. In this *C. albicans* strain the inability to form hyphae was correlated with the inability to adhere to or penetrate a reconstituted epithelium model of infection (Dietrich *et al.*, 2002). The importance of hyphal expressed proteinases in the ability of *C. albicans* to damage RHE cells has also been shown (Schaller *et al.*, 1998a; Schaller *et al.*, 1998b; Schaller *et al.*, 2000; Schaller *et al.*, 1999a; Schaller *et al.*, 1999b; Schaller *et al.*, 2003).

In the RHE model lactate dehydrogenase (LDH) activity, which is a marker of cell injury, was examined from each species every twelve hours over 72 h. It was found over early timepoints, namely t =12-36 h, that there was a significant difference between the ability of the two species to cause damage to RHE integrity, with *C. albicans* showing significantly higher levels of tissue damage. This may be because there was no obvious breakdown or damage to the integrity of the tissue at earlier timepoints in *C. dubliniensis* in comparison with *C. albicans* which showed disruption of the integrity of the tissue and large numbers of cells adhering to the RHE. After 36 h disruption of the tissue was visible in *C. dubliniensis* and LDH activity increased despite the lack of cells attaching to the epithelium, suggesting that secreted factors, such as secreted aspartyl proteinases or lipases, produced by *C. dubliniensis* cells were responsible for the tissue damage. The obvious drawback of the RHE model is that it lacks the intrinsic immune response of normal mucousa, however the model did prove valuable in highlighting the differences in the ability of the two species to invade, both in terms of depth and pattern of invasion. To further our knowledge with regard to adherence between *C. albicans* and *C. dubliniensis* an in-depth analysis of adherence in *C. dubliniensis* in comparison with *C. albicans* is needed. One informative approach to this would be to perform microarray analysis on the RHE model to determine what genes are being expressed in this model as it resembles the human oral epithelium and to determine the difference in regulation of genes involved in adhesion and in genes that are regulated in the transition from yeast to hyphal cells in *C. albicans* and *C. dubliniensis.*

Previous studies have compared the ability of the two species to adhere to buccal epithelial cells (BECs) and vaginal epithelial cells (VEC) and found conflicting results (Gilfillan *et al.*, 1998; Jabra-Rizk *et al.*, 2001; Vidotto *et al.*, 2003). Furthermore, Borg-von Zepelin *et al.* have shown that *C. dubliniensis* isolates adhered better to an epithelial cell line (Vero) which was used to mimick mucousal infections in the presence of fluconazole in comparison with controls without fluconazole (Borg-Von Zepelin *et al.*, 2002). This was in contrast to *C. albicans* which showed a decreased adherence in the presence of fluconazole independently of their *in vitro* susceptibility to this drug (Borg-Von Zepelin *et al.*, 2002). This increased adherence in the presence of fluconazole may have been a significant contributory factor in the emergence of *C. dubliniensis* relative to *C. albicans* in HIV and AIDS patients in recent decades.

As Hwp1 is known to be involved in adherence and because this study has shown there is a significant difference in the protein sequence between the two species, Hwp1 may be a contributory factor to the differences in adherence observed between the two species. Other genes which are known to be involved in adherence are the agglutinin-like sequence (ALS) gene family, of which eight have been identified in *C. albicans* (Hoyer, 2001). Hoyer *et al.* have also shown the presence of three distinct *ALS*-like sequences in *C. dubliniensis* (*ALSD1-3*) (Hoyer *et al.*, 2001). These genes however, show significant divergence in comparison with *C. albicans* most notably at the 3' ends (Hoyer *et al.*, 2001). Hoyer *et al.* also suggested that in *C. dubliniensis* there was a difference in regulation of the *ALS* gene family and in production of cell wall proteins (Hoyer *et al.*, 2001). The *ALS* gene family may thus contribute to the differences observed between the two species in attaching to the epithelial surfaces in the RHE model.

In addition cell surface hydrophobicity (CSH) of *Candida* cells has been proposed to play a role in candidal adhesion. Interestingly *C. dubliniensis* is hydrophobic at 37°C, unlike *C. albicans*, which is hydrophilic at this temperature. The molecular basis of this difference has not been established, however, this may also contribute to the difference in adhesion in *C. albicans* and *C. dubliniensis* (Hazen, 2004). It has also been shown that mice challenged with hydrophobic yeast cells died faster than those challenged with hydrophilic cells (Hazen & Hazen, 1988). Furthermore, hydrophobic cells germinated more rapidly than hydrophilic cells after engulfment by polymorphonuclear neutrophils, providing them with an escape

mechanism and making them less susceptible to killing by phagocytes (Glee *et al.*, 1995; Hazen *et al.*, 1991). Cell surface hydrophobicity may therefore be another reason for the differences in the adherence and pathogenic ability of the two species.

Previous studies have compared the virulence of *C. albicans* and *C. dubliniensis* using a systemic mouse model and have shown that animals infected with *C. dubliniensis* survive longer than those infected with *C. albicans* (Gilfillan *et al.*, 1998; Vilela *et al.*, 2002). The vast majority of invasive infections are caused by endogenous *Candida* strains that belong to the patient's normal flora. Indeed, several studies have highlighted the correlation between previous gastrointestinal colonisation by *Candida* species and candidemia (Kullberg & Filler, 2002; Pittet *et al.*, 1994). In the present study a murine animal model was chosen to examine the comparative growth of *C. albicans* and *C. dubliniensis* when grown alone and in co-infection *in vivo*. The two species were examined in the infant mouse model, which has been developed as a model for intestinal colonisation and subsequent dissemination of *Candida* in immunocompromised patients. The two species were inoculated alone and in co-culture and the cfu/ml determined in both the stomach and the intestine. When mice were infected with either *C. albicans* or *C. dubliniensis* alone there was ten-fold less *C. dubliniensis* present in the stomach after 2 and 4 days and similar levels at day 6. However, at days 8 and 10 no *C. dubliniensis* were detected and 2.0×10^3 and 1.5×10^3 cfu/ml of *C. albicans* were detected on days 8 and 10, respectively (Table 6.1). Similar results were found in the intestine (Fig. 6.1). When the two species were inoculated together in competition with one another, after day 8 densities of *C. albicans* were a thousand-fold higher in the stomach and a hundred-fold higher in the intestine and after 10 days it was a thousand-fold higher in the stomach and intestine, in comparison with *C. dubliniensis* which showed no colonisation (Fig. 6.1).

The ability of *C. albicans* to disseminate from the stomach to other organs such as the liver, kidney and brain has been examined in *C. albicans* in previous studies (Cole *et al.*, 1990a; Cole *et al.*, 1989). The aim of this part of the present study was to examine the ability of *C. dubliniensis,* both alone and in co-infection with *C. albicans,* to disseminate from the stomach to other organs after immunocompromising treatment. When the mice were inoculated with a single species only (5/10) 50% of mice in this part of the present study showed colonisation of the stomach by *C. dubliniensis,* whereas (10/10) 100% of mice

showed colonisation by *C. albicans*. Similar results were found in the liver. When co-inoculated the levels of the *C. dubliniensis* derivative CD36 (M/G) found in the stomach were 2.4 x 10^2 cfu/ml in comparison with *C. albicans* strain SC5314, which were 1.7 X 10^7 cfu/ml. This difference was significant with a *P* value < 0.001 (Table 6.4). The difference in the levels of *Candida* found in the liver was also found to be significant between the two species with a *P* value of 0.042. These results suggest an inability of *C. dubliniensis* to colonise and establish itself in the gastrointestinal tract in comparison with *C. albicans*.

Upon histological analysis it was found that in all the organs examined, stomach, liver and kidney, *C. albicans* was present in both the yeast and hyphal phase in contrast to *C. dubliniensis* which was only present in the yeast phase in the stomach and liver, however hyphal cells were observed in the kidney. This may be due to the environment and an inability to produce hyphae or simply that early colonisation of organs occurs with the yeast phase and then deeper penetration then occurs with production of hyphae. It is predictable that the conditions that stimulate hyphal formation vary inside the human host because of different micro-environments (osmolarity, anaerobiosis, pH and/or chemicals) within the human body and therefore, the invasive capacity of the fungus may change during the course of infection. Previously Vilela *et al.,* showed that in a systemic mouse infection model, histopathological analysis of infected kidneys showed that *C. dubliniensis* cells were predominantly in the yeast phase, whereas *C. albicans* produced far higher levels of hyphae and pseudohyphae (Vilela *et al.*, 2002). The results from the present study may also suggest that the mechanism of lower virulence of *C. dubliniensis* may relate in part to the finding that in the infant mouse model examined in the present study *C. dubliniensis* generally remains in the yeast form. Manifesting a lower or slower rate of mycelial change in the infected organs probably is a significant factor in the reduced virulence of *C. dubliniensis*. This may be because yeast cells are less invasive. It has also been shown that yeast cells are more susceptible to macrophage killing. Lo *et al.* have shown that the non filamentous efg1/cph1 double mutant was avirulent in a mouse model and was killed more effectively by macrophages (Lo *et al.*, 1997).

Vilela *et al.*, have shown a relatively strong inflammatory reaction of the host to *C. dubliniensis* in comparison with *C. albicans*. *Candida dubliniensis* may therefore be lacking some virulence factor that suppress the immunological reaction of the host (Vilela *et al.*,

2002). Greater susceptibility of *C. dubliniensis* to polymorphonuclear leukocytes (PMN) suggests lower virulence and may be due to the high susceptibility of this species to hydrogen peroxide (Vilela *et al.*, 2002). Recently van der Graaf *et al.* have shown there is a different immunological response to yeast cells and hyphae; *C. albicans* yeast cells stimulate both toll-like receptor 2 (TLR2) and toll-like receptor 4 (TLR4). TLR4 is important for interferon γ (IFN-γ) which is a key promoter of the protective immunity against this pathogen (van der Graaf *et al.*, 2005). In contrast, hyphae were not recognised by TLR4 and therefore were not able to stimulate IFN-γ release. Therefore, the transition form yeast to hyphal phase results in a change from pro-inflammatory cytokine release to an anti-inflammatory cytokine release with stimulation of IL-10 by TLR2 which may be used as an escape mechanism by the host (van der Graaf *et al.*, 2005). This may also indicate a difference in host response to *C. albicans* and *C. dubliniensis* due to the inability of *C. dubliniensis* to produce hyphae as readily as *C. albicans* in a wide variety of environmental conditions already outlined in this study.

Despite the similar phenotypic and genotypic properties of *C. albicans* and *C. dubliniensis*, *C. dubliniensis* is only rarely associated with systemic infections and is responsible for as few as 2% of cases of candidemia which is in contrast to *C. albicans* which accounts for approximately 65% of the *Candida* isolates recovered from blood samples (Sullivan *et al.*, 2004). In all of the aspects examined in this study *C. dubliniensis* was found to be far less successful than *C. albicans*. *Candida dubliniensis* is less efficient at growth in both the yeast and hyphal forms and is easily out-grown by *C. albicans* when the two species are cultured together *in vitro* and *in vivo*. It is not surprising therefore that *C. albicans* is also far more successful at colonisation and infection in a range of infection models. As well as the slower growth of *C. dubliniensis*, this species also appears to be significantly less equipped to adhere to host cells and shows an inability to produce hyphae. Since these are two of the most important virulence attributes of *Candida* spp. it is not surprising that despite the very close relationship between the two species *C. albicans* is more pathogenic than *C. dubliniensis*. The molecular basis of this dichotomy in virulence of the two species has yet to be determined. However, it is hoped that the nearly completed *C. dubliniensis* genome sequence will prove an excellent resource in answering this conundrum.

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Publications

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Comparative genomics using *Candida albicans* DNA microarrays reveals absence and divergence of virulence-associated genes in *Candida dubliniensis*

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Candida dubliniensis is a pathogenic yeast species closely related to *Candida albicans*. However, it is less frequently associated with human disease and displays reduced virulence in animal models of infection. Here comparative genomic hybridization was used in order to assess why *C. dubliniensis* is apparently less virulent than *C. albicans*. In these experiments the genomes of the two species were compared by co-hybridizing *C. albicans* microarrays with fluorescently labelled *C. albicans* and *C. dubliniensis* genomic DNA. *C. dubliniensis* genomic DNA was found to hybridize reproducibly to 95·6 % of *C. albicans* gene-specific sequences, indicating a significant degree of nucleotide sequence homology (>60 %) in these sequences. The remaining 4·4 % of sequences (representing 247 genes) gave *C. albicans*/*C. dubliniensis* normalized fluorescent signal ratios that indicated significant sequence divergence (<60 % homology) or absence in *C. dubliniensis*. Sequence divergence was identified in several genes (confirmed by Southern blot analysis and sequence analysis of PCR products) with putative virulence functions, including the gene encoding the hypha-specific human transglutaminase substrate Hwp1p. Poor hybridization of *C. dubliniensis* genomic DNA to the array sequences for the secreted aspartyl proteinase-encoding gene *SAP5* also led to the finding that *SAP5* was absent in *C. dubliniensis* and that this species possesses only one gene homologous to *SAP4* and *SAP6* of *C. albicans*. In addition, divergence and absence of sequences in several gene families was identified, including a family of *HYR1*-like GPI-anchored proteins, a family of genes homologous to a putative transcriptional activator (*CTA2*) and several *ALS* genes. This study has confirmed the close relatedness of *C. albicans* and *C. dubliniensis* and has identified a subset of unique *C. albicans* genes that may contribute to the increased prevalence and virulence of this species.

INTRODUCTION

Candida dubliniensis is associated with oral candidosis in the HIV-infected population in which this species was first identified in 1995 (Jabra-Rizk *et al.*, 2001; Sullivan *et al.*, 1995, 2004). Recently, several studies have also identified *C. dubliniensis* as a cause of oral disease in diabetic and cancer patients (Sebti *et al.*, 2001; Willis *et al.*, 2000). However, the closely related species *Candida albicans* appears to be more successful than *C. dubliniensis* as a commensal of the human oral cavity in healthy individuals, as determined by standard oral swab sampling methods (Coleman *et al.*, 1997). In addition, the incidence of *C. dubliniensis* isolation from blood cultures is extremely low compared to *C. albicans* (Kibbler *et al.*, 2003; Pfaller & Dieker, 2004). In a recent study of *Candida* species recovered from blood cultures in six sentinel hospitals in England and Wales between 1997 and 1999, *C. dubliniensis* was isolated from only 2 % of samples compared to 65 % for *C. albicans*. Two studies have also demonstrated that *C. dubliniensis* is less virulent than *C. albicans* in a murine model of systemic candidosis (Gilfillan *et al.*, 1998; Vilela *et al.*, 2002). The reason for the apparent difference in virulence between the two species is unknown as they are

Abbreviations: CGH, comparative genomic hybridization; GPI, glycosylphosphatidylinositol.

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are AJ634664–AJ634672, AJ634675–AJ634676 and AJ634382.

phenotypically very similar and seem to share many of the traits traditionally associated with virulence in *C. albicans*. In particular both species have the ability to form true hyphae, to adhere to human epithelium and to produce secreted aspartyl proteinases (Gilfillan *et al.*, 1998; Hannula *et al.*, 2000; Vilela *et al.*, 2002). However, *C. dubliniensis* does not form hyphae as rapidly as *C. albicans* in response to shifts in pH/temperature or when incubated in serum (Gilfillan *et al.*, 1998). In contrast, when cultured on Staib agar or Pal's agar *C. dubliniensis* forms abundant hyphae, pseudohyphae and chlamydospores, whereas *C. albicans* remains in the yeast phase (Al Mosaid *et al.*, 2001, 2003). *C. dubliniensis* also seems to be more sensitive to environmental stress, such as elevated temperature and NaCl concentration (Alves *et al.*, 2002; Pinjon *et al.*, 1998).

Comparative genomic hybridization (CGH) studies with DNA microarrays provide a rapid and cost-effective method for obtaining informative data about uncharacterized genomes and have been used extensively to compare gene content in prokaryotic and eukaryotic micro-organisms (Daran-Lapujade *et al.*, 2003; Dong *et al.*, 2001; Murray *et al.*, 2001). The completion of the *C. albicans* genome project and the availability of *C. albicans* DNA microarrays now enables genomes of different strains of *C. albicans* and closely related species, such as *C. dubliniensis*, to be compared. In the present study, CGH was performed between *C. albicans* and *C. dubliniensis* using *C. albicans* DNA microarrays in order to identify genomic differences that might account for the difference in virulence between *C. albicans* and *C. dubliniensis*. This approach was deemed feasible as all *C. dubliniensis* genes analysed to date share more than 90 % identity at the nucleotide sequence level with the orthologous *C. albicans* genes. Total genomic DNA from *C. albicans* and *C. dubliniensis* was co-hybridized to *C. albicans* DNA microarrays and the relative hybridization efficiency of *C. dubliniensis* and *C. albicans* DNA to each gene-specific spot was compared. This approach allowed us to identify the presence of thousands of *C. albicans*-homologous genes in *C. dubliniensis* without the need for sequence analysis and has guided us towards genes which are highly divergent or even absent from *C. dubliniensis*. We anticipate that this collection of *C. albicans*-specific sequences may contain genes that contribute to the observed differences in virulence and epidemiology between these two organisms.

METHODS

***Candida* strains and culture conditions.** *C. albicans* strain SC5314 was used as a control in all CGH experiments using Eurogentec *C. albicans* DNA microarrays. *C. dubliniensis* strains used in this study included the *C. dubliniensis* type strain CD36 (American Type Culture Collection reference MYA-178, British National Collection of Pathogenic Fungi reference NCPF3949), which is a representative of *C. dubliniensis* Cd25 fingerprint group I (genotype I), and *C. dubliniensis* strain CD514, a strain representative of Cd25 fingerprint group II (genotype 3) (Gee *et al.*, 2002). Strains were routinely grown on potato dextrose agar (PDA; Oxoid) medium, pH 5·6, at 37 °C. For liquid culture, cells were grown in yeast extract-peptone-dextrose (YEPD) broth, also at 37 °C (Gallagher *et al.*, 1992).

Chemicals, enzymes and radioisotopes. All chemicals used were of molecular biology grade and were purchased from Sigma-Aldrich. Molecular biology enzymes and kits were purchased from Promega or New England Biolabs unless otherwise indicated. Cy5 and Cy3 dUTP were purchased from Amersham Biosciences Europe. Supplies of [α-^{32}P]dATP (6000 Ci mmol^{-1}, 220 TBq mmol^{-1}) were purchased from NEN Life Sciences.

DNA microarrays. *C. albicans* DNA microarrays used in this study were constructed by Eurogentec based on the Galar Fungail consortium's annotation of the *C. albicans* SC5314 genome sequence in the CandidaDB (http://www.pasteur.fr/Galar_Fungail/CandidaDB/). This annotation was produced based on the genome sequence released by the Stanford Genome Technology Center. Each glass slide microarray contained sequences corresponding to 6039 ORFs (98 % of annotated genes) that were approximately 300 bp in length and spotted in duplicate.

Genomic DNA preparation. High-molecular-mass total genomic DNA was recovered from *Candida* strains by organic extraction following digestion of the cell wall with Zymolyase 20T (Seikagu) and proteinase K treatment (Roche Diagnostics) as described by Gallagher *et al.* (1992).

Genomic DNA labelling and microarray hybridization. For DNA labelling experiments with Cy5 dUTP and Cy3 dUTP, total genomic DNA (2 μg) was first fragmented by either restriction endonuclease digestion or sonication. For restriction endonuclease digests, two 1 μg aliquots of DNA were separately digested with *Tru*1I or *Rsa*I (Fermentas). These digests were then heat inactivated, extracted once with a mixture of phenol/chloroform/isoamyl alcohol (25 : 24 : 1, by vol.) and ethanol precipitated. The two separate aliquots of digested DNA were combined to give a mixture of *Tru*1I and *Rsa*I fragments (50–4000 bp) for labelling. Alternatively, separate DNA samples were prepared for labelling by sonication using a Sonoplus HD70 sonicator (Bandelin Electronic) at 75 % power for 30 cycles producing DNA fragments ranging from 500 to 5000 bp.

Each labelling reaction was carried out with 2 μg of either sheared or digested genomic DNA using the RadPrime random priming labelling system (Invitrogen) incorporating Cy5 dUTP or Cy3 dUTP into *C. albicans* SC5314 or *C. dubliniensis* DNA fragments. After labelling, reaction products were purified with Nucleospin PCR clean up columns (Macherey-Nagel) and concentrated to a final volume of <5 μl with a Microcon YM-30 column (Millipore). Cy5-labelled and Cy3-labelled reactions were mixed together in DIG EasyHyb buffer (Roche Diagnostics) to a final volume of 60 μl for hybridization. The mixture was denatured at 98 °C for 5 min then chilled on ice. Microarray slides (Eurogentec) were placed in a hybridization chamber (Corning), covered with a glass LifterSlip (Erie Scientific Company) and the labelling reaction was carefully applied at the edges of the slide. The chamber was sealed and incubated in the dark at 42 °C for 16–18 h. Slides were washed at high stringency at room temperature as follows: (i) 5 min in 1 × SSC, 0·03 % (w/v) SDS, (ii) 5 min in 0·2 × SSC and (iii) 5 min in 0·05 × SSC. Following washing, slides were dried thoroughly by centrifugation at low speed for 5 min in a 50 ml disposable plastic tube (Greiner Bio-One) and scanned immediately.

Each of the hybridizations performed using digested DNA and sheared DNA was carried out on two separate occasions. A third sheared DNA hybridization was performed with the Cy3 and Cy5

dyes swapped. One additional hybridization was performed between sheared Cy5-labelled *C. albicans* DNA and sheared Cy3-labelled *C. dubliniensis* CD514 DNA.

Data analysis. DNA microarray slides were scanned with the GenePix 4000B scanner (Axon Instruments). Data were extracted from scanned images using the GenePix Pro 4.1.1.4 software package (Axon Instruments). Data normalization and subsequent analysis was carried out with the GeneSpring 6.1 software package (Silicon Genetics). Hybridization data from each DNA 'spot' on the slide were only included for analysis if the control (*C. albicans*) channel signal was above local background plus two standard deviations. Signal intensities in both channels were background corrected. Measurements were normalized across the whole chip by dividing each measurement by the median of all measurements taken for that chip. A normalized fluorescence ratio value was determined for each spot by dividing the *C. albicans* control channel normalized signal by the *C. dubliniensis* normalized signal values. The $\log_2$ value of each ratio was determined and the $\log_2$ ratios of duplicate spots were averaged. The significance of normalized ratios of <1 was determined in replicate experiments using Student's t test. The raw data from all of the experiments has been submitted in a MIAME-compliant format to the ArrayExpress database at the European Bioinformatics Institute (accession code E-MEXP-99).

The relationship between $\log_2$ ratio values and nucleotide sequence homology was determined by linear regression analysis using Prism 4.0 (GraphPad Software). For this analysis, nucleotide sequence homology between the array printed *C. albicans* sequences ($\sim$300 bp) and the corresponding region of available homologous *C. dubliniensis* sequences was determined using DNA Strider 3.1 software. Sequences used in this analysis included the available *C. dubliniensis* gene sequences from GenBank and PCR-amplified sequences described here (Table 1). Sequences were included for analysis only when a minimum of 100 bp of uninterrupted sequence could be aligned. Gaps of over 50 bp in length were excluded from homology calculations. On the basis of this analysis, we chose genes with normalized ratios of $<0{\cdot}5$ ($P<0{\cdot}05$) in replicate dye-swap experiments with sheared genomic DNA for further study.

Larger sequence alignments (>500 bp) described in the results were carried out using the CLUSTAL_W software package (Higgins & Sharp, 1988).

Southern hybridization. Southern hybridization analysis was carried out as described previously (Moran *et al.*, 1998; Southern, 1975) using DNA sequences labelled with [α-^{32}P]dATP by random primer labelling (Prime-a-Gene system; Promega) or using DIG-labelled probes incorporating DIG-11-dUTP (Roche Diagnostics) during PCR amplification as described by the manufacturers. In order to identify homologues of individual ORFs in *C. dubliniensis*, the entire *C. albicans* ORF was used in labelling reactions. In the case of gene families (e.g. *CTA2*, *IFA*, *IFF*), probes were designed by identifying the most conserved region of the ORFs in CLUSTAL_W alignments. These gene family probes represented >35% of the entire ORF and constituted a different region of the ORF to that present on the microarray. The post-hybridization washes were performed at low stringency (60 °C with 0·5× SSC, 0·1%, w/v, SDS) unless otherwise indicated.

PCR amplification of *C. dubliniensis* genome sequences. PCR amplification from the *C. dubliniensis* genomic DNA template

Table 1. Percentage nucleotide sequence homology of *C. dubliniensis* genes to corresponding *C. albicans* Eurogentec microarray sequences

Gene	Percentage homology	Normalized ratio*	GenBank accession no.	Reference
ACT1	98·8	>1·5	AJ236897	Donnelly *et al.* (1999)
URA3	93·0	>1·5	AJ302032	Staib *et al.* (2001)
MDR1	92·0	1·4	AJ227752	Moran *et al.* (1998)
CDR1	91·9	1·16	AJ439073	Moran *et al.* (2002)
CDR2	91·0	>1·5	AJ439075	Moran *et al.* (2002)
ERG3	90·1	1·17	AJ421248	Pinjon *et al.* (2003)
ERG11	90·4	>1·5	AY034876	Perea *et al.* (2002)
PHR2	90·5	1·40	AF184908	Kurzai *et al.* (1999)
PHR1	88·0	>1·5	AF184907	Kurzai *et al.* (1999)
SAP4	86·0	0·55	AJ634382	This study
SAP2	83·6	1·18	AJ634672	This study
CZF1	80·0	0·53	AJ634475	This study
IPF8147	79·6	0·29	AJ634476	This study
IPF1873	75·0	0·24	AJ634664	This study
RVS161	75·0	0·30	AJ634665	This study
IPF16104	74·0	0·25	AJ634666	This study
IFA8	70·4	0·36	AJ634667	This study
IPF9787	70·0	0·26	AJ634668	This study
IPF3448	69·6	0·42	AJ634669	This study
SSN6	68·0	0·30	AJ634670	This study
IPF20057	59·0	0·17	AJ634671	This study

*Mean ratio of normalized fluorescence values of *C. albicans* and *C. dubliniensis* hybridizing DNA at each gene-specific spot. Values above 1·5 are quoted as >1·5 for the purposes of regression analysis (Fig. 1).

Table 2. Sequences of oligonucleotide primers used in this study

Primer name*	Sequence (5′–3′)	Nucleotide coordinates†
CRH12F	ACTGGAATGGGAAACTGAAC	+1159 to +1178
CRH12R	CACAACACTACTGAAAGATG	+1476 to +1457
1760F	CAGAAATCTGGTATTGACAC	+712 to +731
1760R	TCTATATCCATATCGCATTC	+1121 to +1102
11560F	CAATCTAAACCTCCATCAGG	+451 to +470
11560R	AGGGGAATTACTAATGACTC	+821 to +812
8147F	CCCTACTACTTCATCATCAC	+405 to +424
8147R	AATTAAATCCTTCAGAATAC	+769 to +750
CHS5F	AGGAAACAGATATTGTTGAG	+1055 to +1074
CHS5R	TTCTGTAGATGTTGGCTCAG	+1476 to +1457
IPF3448F	GGAGTATTTGGAGACCCAAG	+492 to +511
1PF3448R	TGGCATTGTTCTTCACCAAC	+868 to +849
BIO3F	CAGGAACATGCTGGTATCTG	+791 to +809
BIO3R	ACCCAAACACCTAAATCGAC	+1187 to +1168
HIK1.3F	AAAAGTCTAACCCAATTGAC	+443 to +462
HIK1.3R	TTCACTAATTGTAGTGATCG	+843 to +824
HIK1.5F	AAAACGTTAGCCGTCAAAGC	+1753 to +1772
HIK1.5R	TCTAATATTAGATGGCGACA	+2202 to +2183
HIT1F	TGTCCTAAATGTTCAATTGC	+49 to +58
HIT1R	ATTCATCAATTCAAGACATC	+444 to +425
HNM4F	TAGGTGGAGAACCAATTGTG	+887 to +906
HNM4R	AAAGCACACCCAAAGGACAG	+1350 to +1331
HNM4A	ATGTCTTATCAAATTACAGA	+1 to +20
HNM4B	ATCAATATTATGATCATAGC	+1497 to +1478
CZF1F	ATCTCAACCTTTGTATTCTG	+657 to +676
CZF1R	TTTCGTCATCCTTTTGATCC	+1087 to +1068
8627F	AATCAACAACTGTTTAATCG	+1249 to +1268
8627R	TATTGATAAATTACCATGAG	+1671 to +1652
15920F	CTACCACAACTAAACAACAG	+1433 to +1452
15920R	ACTTTGTTGTAATGATTGAG	+1875 to +1856
2057F	ACTACTGTTCCTGCTGCTAC	+967 to +986
2057R	CCCTTGATATCAACAATGTC	+1385 to +1366
2971F	AATTGCTAAACAAGGAGATC	+927 to +956
2971R	TATCTTTTCCTTGTTCTAAC	+1372 to +1352
DAL52F	TGTTGTTGGATGTATTATCC	+1119 to +1138
DAL52R	CATCTTCACTATTACGTGCT	+1558 to +1539
FUR4F	AGGGTTCATTCTGCTAATTG	+1281 to +1300
FUR4R	GATACCATCATGCACTTCAT	+1662 to +1643
FUR4A	AAAAGTTGGTTGTCTCCATG	+26 to +45
FUR4B	TCTTCGAACTGACAGATTCC	+1702 to +1683
HNM3F	ATTTTGACTGGTATCGTTTG	+976 to +995
HNM3R	GATACATAATTCATGTTGGT	+1409 to +1390
HNM3A	ATGGATACCAAGTATGAAAAC	+1 to +21
HNM3B	ATTCAGTCTTATTGGCAATG	+1495 to +1476
BIO4F	TGGAAGCCCATTCAAACAGG	+91 to +110
BIO4R	CCACGGTTCTTCAAATGCTC	+467 to +448
OPT1F	GGTAAAGTTTTCTTCAATGC	+1843 to +1862
OPT1R	AGTCGGTGTTTGTTAAACTC	+2239 to +2220
OPTA	AAAATAAGGGCAGTAATTAG	+7 to +26
OPTB	TCAATATGTGTCTGATATTG	+2317 to +2298
IFF2F	AATGGTTCTGGTAATGGATC	+3340 to +3359
IFF2R	CAAGAAAACAACAACCATTG	+3728 to +3747
4805F	AAAACTCTTATGCTTTTGAG	+4069 to +4088
4805R	AATTCAATACCTAACATACC	+4418 to +4399

Table 2. cont.

Primer name*	Sequence (5′–3′)	Nucleotide coordinates†
IPF257F	TACCAAACTCATGTTTCACG	+58 to +78
IPF257R	AATGACTGATTTCATAGTA	+402 to +383
1873F	GAATTTGCTAAACGAATCGG	+433 to +452
1873R	AGATGACTGTTTTAATCGAG	+886 to +869
RSV161F	ACAATCGCTCTACTCGAATG	+246 to +265
RSV161R	CAAGTACTGTTGGATCTGTG	+690 to +671
RRN3F	CGCCGCATTTCAGGCATTGC	+1248 to +1267
RRN3R	CTATATATCGTCTTCACTATC	+1671 to +1651
13135F	TAATTGTTTTGATAGCAATG	+218 to +238
13135R	GATATTGATGAATCATTAGC	+590 to +571
9787F	AAGAACAGTTGGATTCAGAG	+989 to +1008
9787R	AATTGATCATTTCTTGGACG	+1349 to +1330
SSN6F	ACCAGTTAACCAACCTGTTG	+2817 to +2836
SSN6R	TCATCATAATTTTCATCTTC	+3233 to +3116
16104F	AACTCTAAACAATTGTTGAC	+1741 to +1760
16104R	TCATACAAGATTCATTTTGG	+2172 to +2153
IFA1F	GCGAATTCAGTATGGACTTTATGTATC	+1279 to +1297
IFA1R	GCGAATTCCACTTCCATTATCCCGATC	+1969 to +1951
HYR1F	GAATTCAAGGTTTCCATGGTGAT	+95 to +117
HYR1R	GAATTCAGCAGTGGAAGATGATTG	+1200 to +1183
IFF1F	GCGAATTCTGCTTAATTCTGTCTTAGC	+1 to +20
IFF1R	GCGAATTCATAAACTAGGATTATAACC	+844 to +826
SAP4-6F	GCGAATTCATATCTTGAGTGTTCTTGC	+14 to +32
SAP4-6R	GCGAATTCACTTGGCCTTGTCAATACC	+763 to +745
InvSAPF	GCGAATTCACGCAACAGCAAGAACACTC	+40 to +21
InvSAPR	GCGAATTCCTACTGAGTCTATGTATGAC	+623 to +642
CdSAP4F	CGTCTAGAGGAGGAACTCTTGACGATGT	−226 to −207
CdSAP4R	CGCTCGAGCTTTCATTTCTAGGCATATG	+1463 to +1444
RBTF2	GCGAATTCGAAGAATTAAGTAACGATGGT	+694 to +714
RBTR2	GCTCTAGAAGAAGTGACTGAAGTAGAATC	+1410 to +1390
HWP1F	CGGAATTCCGATGAGATTATCAACTGCT	−14 to +5
HWP1R	CGGAATTCCGAATTAGATCAAGAATGCAG	+1908 to +1889
HWPR2	GGAATTCTAGGATTGTCACAAGG	+223 to +210
APL6F	CGGAATTCCGAATACAAGATGTTTC	+1678 to +1693
CTA2F	GCGAATTCATGCCAGAAAACCTCCAAAC	+1 to +20
CTA26R	GCGAATTCCTTCGTTTACGTGGTTGGTG	+781 to +751

*Primer names refer to gene annotations in CandidaDB (http://genolist.pasteur.fr/CandidaDB/).
†Nucleotide coordinates are given for each gene, where +1 refers to the first base of the ATG start codon. All coordinates are for *C. albicans* genes with the exception of InvSAPF/R and CdSAPF/R, which refer to the coordinates of the *CdSAP4* gene, and HWPR2, which refer to the *CdHWP1* gene.

was carried out as described previously (Moran *et al.*, 1998, 2002). Oligonucleotide primers used in this study were synthesized by Sigma-Genosys (Table 2). PCR-amplified DNA fragments were sequenced where indicated using the dideoxy chain-termination method by Lark Technologies.

RESULTS

Comparative genomic microarray hybridization

To label *Candida* chromosomal DNA efficiently by random priming with Cy3 or Cy5 dUTP, it was first necessary to fragment the chromosomal DNA. Two DNA fragmentation methods, sonication and restriction endonuclease digestion, were compared in order to determine if either labelling method introduced artifacts into the microarray results. In replicate experiments involving *C. albicans* SC5314 genomic DNA labelled with Cy3 dUTP following restriction endonuclease or sonication, over 98 % of spots gave fluorescent signals that were two standard deviations above background. Using the same criteria, *C. dubliniensis* CD36 genomic DNA prepared by either sonication or restriction endonuclease digestion hybridized to at least 95 % of gene-specific spots in replicate hybridizations. The dataset chosen

for analysis here was generated with sonicated genomic DNA from *C. albicans* and *C. dubliniensis* labelled with either Cy5 dUTP or Cy3 dUTP in dye-swapped replicate experiments. In these hybridizations, 5931 duplicate spots (98 %) exhibited signals in the *C. albicans* channel two standard deviations above background and were included for analysis.

Relative hybridization efficiency of co-hybridized *C. albicans* SC5314 and *C. dubliniensis* CD36 genomic DNA to the microarrays was assessed by determining a normalized ratio of *C. albicans* and *C. dubliniensis* signal intensities at each spot included for analysis (Cy5/Cy3 normalized ratios). In dye-swapped replicate experiments, 96 % of these normalized ratios differed by less than twofold.

In order to investigate whether a relationship existed between the strength of hybridization of *C. dubliniensis* DNA to the array and the degree of nucleotide homology between the corresponding *C. albicans* and *C. dubliniensis* sequences, we plotted $\log_2$ ratio values versus percentage nucleotide sequence homology. We determined the nucleotide sequence homology between the *C. albicans* probe sequences present on the array and the corresponding sequences of 11 *C. dubliniensis* genes sequences available in GenBank (Table 1). We also attempted to PCR amplify sequences from *C. dubliniensis* using *C. albicans*-specific oligonucleotide primers (Table 2) corresponding to 35 genes which hybridized poorly with *C. albicans* genomic DNA (normalized ratios ranging from 0·17 to 0·53) on the microarray. Under low-stringency conditions, 10 of these PCR primer sets yielded PCR amplification products with sequences homologous to the corresponding *C. albicans* gene (nucleotide sequence homology range 59–80 %, Table 1). We plotted the percentage nucleotide sequence homology between the 11 GenBank and 10 PCR-amplified sequences and their *C. albicans* homologues versus the $\log_2$ ratio values from the 21 DNA spots representing these genes on the array (Fig. 1). Linear regression analysis was used to generate a best-fit-line from the dataset, which demonstrated a relationship between nucleotide sequence homology and $\log_2$ ratio ($r^2 = 0{\cdot}80$, $P < 0{\cdot}0001$). The 12 most homologous nucleotide sequences (80–98·8 % identity), including housekeeping genes such as *ACT1*, *URA3* and *ERG11*, all had normalized ratio values of $> 0{\cdot}5$. The remaining nine genes formed a second group with intermediate sequence homologies of 59–79 %. These nine sequences possessed normalized ratios ranging from 0·17 to 0·46. Based on this analysis we categorized genes into three homology groups based on normalized ratio values: (I) a high-homology group (normalized ratio $> 0{\cdot}5$; > 80 % nucleotide sequence homology), (II) a medium-homology group (normalized ratio 0·25–0·5; 60–80 % nucleotide sequence homology) and (III) sequences that possessed low homology or were possibly absent in *C. dubliniensis* (normalized ratio $< 0{\cdot}25$; < 60 % nucleotide sequence homology).

In total, 677 sequence-specific spots exhibited normalized ratios below 0·5 ($P < 0{\cdot}05$) in replicate dye-swap array experiments with sheared genomic DNA. From this group, 418 sequences were classified as likely to possess intermediate nucleotide sequence homology (60–80 %). The remaining 259 sequences (representing 4·4 % of the spots analysed) gave normalized ratios of $< 0{\cdot}25$ and were predicted to possess low nucleotide sequence homology (< 60 %) or were possibly absent in *C. dubliniensis*.

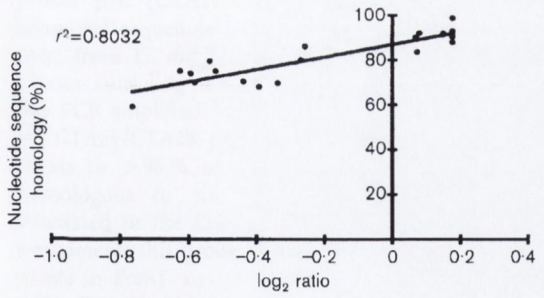

Fig. 1. Standard curve used to determine the relationship between percentage nucleotide sequence homology of *C. albicans* SC5314 and *C. dubliniensis* CD36 sequences and normalized fluorescence ratio. GenBank sequences for 11 *C. dubliniensis* genes of known homology and 10 novel PCR-amplified sequences were included. The mean of the $\log_2$ ratio values (Table 1) for each gene was plotted against percentage nucleotide sequence homology. Linear regression analysis was used to predict the best-fitting line. The r^2 value was calculated using Prism 4.0.

Categorization of divergent genes

We decided to examine the group of sequences predicted to possess low nucleotide sequence homology in more detail as these genes were most likely to be functionally different or possibly even absent in *C. dubliniensis*. This group of 259 sequences was found to correspond to 247 genes (Table 3) since 12 genes were found to be represented on the array by two different duplicate spots. However, 134 (54 %) of these were hypothetical genes with no homology to genes of known function. Within this group, 43 genes could be classified as conserved hypothetical genes due to significant homology to other hypothetical genes in GenBank or CandidaDB (Table 4). Twenty-one sequences (8·5 %) were found to have homology to genes encoding *C. albicans* retrotransposon elements, including transposases and reverse transcriptases. Two sequences identified, IPF17727 and HOK, are contained in the *C. albicans*-specific fingerprinting probes 27A and Ca3 (Soll, 2000). IPF17727 is a region of the *C. albicans* RPS region and the HOK gene is found within the *C. albicans*-specific fingerprinting probe Ca3 that has previously been shown to hybridize very poorly to *C. dubliniensis* genomic DNA (Sullivan *et al.*, 1995).

Table 3. Functional categories of *C. albicans* genes predicted to be of low nucleotide sequence homology or absent in *C. dubliniensis* (normalized ratio < 0·25)

Functional category	No. of genes	Percentage of total
Hypothetical genes	134	54·3
Retrotransposon elements	21	8·5
Putative transcriptional regulator	19	7·6
Leucine-rich repeat family (*IFA*)	18	7·3
GPI-anchored proteins	17	6·9
Transporters	8	3·2
Protein processing/modification	7	2·8
Cell metabolism/biosynthesis	6	2·4
mRNA processing	4	1·6
Cell division	3	1·2
Chromatin/DNA-binding	3	1·2
Morphogenesis related	3	1·2
Cytoskeletal	2	0·8
Mitochondrial	2	0·8
Total	**247**	**100**

(i) Putative transcriptional regulators. A group of 19 genes were identified that possessed homology to putative transcriptional regulators. Eight of these regulators had strong homology to genes encoding transcriptional activators in *Saccharomyces cerevisiae* with zinc-finger DNA-binding motifs. A further eight corresponded to a family of genes encoding proteins with homology to a putative *C. albicans* transcriptional activator, *CTA2* (Kaiser *et al.*, 1999). All eight *CTA2*-like genes included for analysis exhibited normalized ratios of <0·25. A CLUSTAL_W-generated alignment of the nucleotide sequences of *CTA21*, *CTA22*, *CTA25* and *CTA26* from *C. albicans* revealed that these sequences were 89 % identical. A PCR primer pair (CTA2F/CTA26R, Table 2) homologous to conserved sequences in these ORFs did not yield amplimers from *C. dubliniensis* genomic DNA template at a primer annealing temperature of 50 °C. A *CTA2* probe was PCR amplified from *C. albicans* template DNA using the CTA2F/CTA2R primer pair. This probe which corresponds to >90 % of the *CTA2* ORF was at least 90 % homologous to six *C. albicans CTA2*-like sequences annotated in the CandidaDB. In Southern hybridization experiments this probe revealed multiple hybridizing fragments in *Eco*RI- or *Hin*dIII-digested *C. albicans* genomic DNA (Fig. 2). Hybridization of the same sequence to *C. dubliniensis* CD36 genomic DNA digested with *Eco*RI or *Hin*dIII at low stringency did not reveal any hybridizing fragments (Fig. 2). In addition, this *CTA2* probe did not hybridize to genomic DNA from seven additional epidemiologically unrelated *C. dubliniensis* isolates in Southern hybridization experiments (data not shown). These findings are in agreement with the array data that members of this gene family are significantly divergent (i.e. share low nucleotide sequence homology) or are absent in *C. dubliniensis*.

(ii) Putative membrane transporters. Eight genes encoding proteins with strong homology to transporters of nutrients or other small molecules were also found to hybridize poorly with *C. dubliniensis* genomic DNA. These included the genes encoding the oligopeptide transporter *OPT1*, the choline transporters *HNM3* and *HNM4*, the uracil permease *FUR4* and the allantoin permease *DAL52*. The absence of homologous sequences for this group of genes was confirmed in *C. dubliniensis* by Southern hybridization analysis with probes corresponding to the complete *C. albicans* ORF (Fig. 3).

(iii) A leucine-rich repeat family of proteins. A large family of genes encoding proteins with leucine-rich repeats (termed the *IFA* family, Pasteur CandidaDB) in *C. albicans* was also identified. Of 26 *IFA* sequences included for analysis, 18 gave normalized ratios of <0·25 following hybridization of *C. dubliniensis* genomic DNA. Four *IFA* sequences gave intermediate ratios (0·25–0·5) and four sequences (*IFA3*, *IFA13*, *IFA20* and *IFA21*) gave ratios above 0·5. A CLUSTAL W-generated alignment of four *IFA* sequences with ratios of <0·25 (*IFA1*, *IFA2*, *IFA4* and *IFA5*) revealed that the 3′ half of these ORFs exhibited the highest levels of conservation. In order to detect sequences homologous to *IFA* genes in *C. dubliniensis* we amplified a conserved region of 690 bp representing 34 % of the *IFA1* ORF using the primer pair IFA1F/IFA1R (Table 2). Southern hybridization experiments with this conserved *C. albicans* sequence revealed the presence of a single hybridizing 1·5 kb *Eco*RI fragment in CD36 and in seven additional epidemiologically unrelated *C. dubliniensis* isolates (data not shown). We applied the IFA1F/IFA1R primer pair to CD36 template DNA in low-stringency PCR reactions (50 °C annealing temperature) and successfully amplified a 728 bp fragment with 70 % homology to the *C. albicans IFA8* ORF, in keeping with its ratio value between 0·25 and 0·5.

(iv) Genes encoding GPI-anchored proteins. Genes encoding GPI-anchored proteins, including the hypha-specific protein Hyr1 (Bailey *et al.*, 1996), were also identified in our CGH analysis. Two sequences, representing the 3′ end and an internal fragment of the *HYR1* gene, were present on the array. Both sequences yielded normalized ratios of <0·3, and no homologous sequence could be identified in *C. dubliniensis* by low-stringency Southern blot analysis of *C. dubliniensis* CD36 genomic DNA with PCR-amplified *C. albicans HYR1* gene sequences (nucleotides +95 to +1183, representing 40 % of the ORF, chosen because the domain bears most homology to other GPI-anchored protein-encoding genes in *C. albicans*) (Fig. 4a). This was confirmed in Southern hybridization experiments with seven additional epidemiologically unrelated *C. dubliniensis* isolates (data not shown).

Several genes encoding Hyr1-related proteins (termed the *IFF* gene family, Pasteur CandidaDB) were also present on the array. Of the nine *IFF* sequences included for analysis

Table 4. *C. albicans* SC5314 genes predicted to be of low homology (<60% nucleotide sequence identity) or absent in *C. dubliniensis* CD36

Gene identifier*	Assembly 19 orf	Putative or known function†
Unknown function (134)		
IPF3468	orf19.4055	Homology to IPF708
IPF2690.3f/IPF2690.5f	orf19.6614	Contains DEAD helicase box
IPF17640	orf19.3906	Homology to IPF15492
IPF4137.3f	orf19.5370	Homology to *Sc*YBR075w
IPF7945	orf19.6604	NH
IPF7010	orf19.4241	Homology to IPF324.3
IPF17417	orf19.4691	Homology to IPF11506
IPF708	orf19.5370	Homology to *Sc*YBR075w
IPF6387.3f	orf19.584.3	NH
IPF12498.3f/IPF12498.53f	orf19.1677	NH
IPF13810.3	orf19.1825	NH
IPF5661	orf19.7125	NH
IPF2702	orf19.2163	Homology to *Sc*YBR074w
IPF17661	orf19.1348	Homology to *Sc*YBR075w
IPF17272.3f	orf19.3522	NH
IPF13072	orf19.2905	NH
IPF3105	orf19.4439	NH
IPF17488.3f/IPF17488.5f	orf19.1818	Homology to IPF13810
IPF7940	orf19.6608	NH
IPF6266	orf19.6311	Homology to *Sc*YPR036w
IPF11508	orf19.3904	NH
IPF14254	orf19.4768	NH
IPF19766	Not found	NH
IPF11506	orf19.3903	Homology to IPF17417
IPF15772	orf19.4883.3	NH
IPF19377	orf19.3336	NH
IPF10280	orf19.1266	Homology to IPF3748
IPF7804.5f	orf19.3134	NH
IPF19720.3eoc	orf19.4643	NH
IPF4504	orf19.7002	Homology to *An*AN3284.2
IPF2815	orf19.6777	NH
IPF17131	orf19.69	NH
IPF9655	orf19.3988	NH
IPF2754	orf19.2585	Homology to *Sc*YER181c
IPF9401	orf19.938	NH
IPF4751	orf19.1999	NH
IPF6325	orf19.1116	NH
IPF13290	orf19.5314	NH
IPF9400	orf19.937	NH
IPF17727	orf19.5190	Part of RPS region
IPF17322.3f	orf19.4068	Homology to IPF13810
IPF2195	orf19.6920	NH
IPF11936.3f	orf19.6277	NH
IPF17794	orf19.6469	NH
IPF2617	orf19.6687	NH
IPF17991	orf19.6465	NH
IPF12093	orf19.4786	NH
IPF14587.3	Not found	NH
IPF20134	orf19.994	NH
IPF11051	orf19.4321	NH
IPF12399	orf19.3210	NH
IPF324.3	orf19.7545	Homology to IPF13810

Table 4. cont.

Gene identifier*	Assembly 19 orf	Putative or known function†
IPF3444.5f	orf19.6184	NH
IPF635	orf19.7614	NH
IFB1	orf19.6703	NH
IPF18833.3f	orf19.1042.1	NH
IPF10231.exon	orf19.426	NH
IPF9211.5f	orf19.3713	NH
IPF15506	orf19.2213	Homology to *Sc*YGR025w
IPF19812	orf19.7304	NH
IPF5373	orf19.5555	NH
IPF13724	orf19.259	Local homology to *Sc*Rsa1p
IPF8642	Not found	Homology to IPF10761
IPF243	orf19.3254	NH
IPF3301	orf19.4394	NH
IPF5730	Not found	Homology to *Sc*YNL211c
IPF7578	orf19.4366	Homology to *An*AN4487.2
IPF10168.3	orf19.643	NH
IPF14618	orf19.6079	NH
IPF9057	orf19.1189	NH
IPF7539	orf19.4886	NH
IPF5978	orf19.7311	NH
IPF5217	orf19.7270	NH
IPF931	orf19.7567	Homology to *Sc*YDR124w
IPF15255	orf19.4884	Homology to *Sc*YEL007W
IPF2057	orf19.5269	NH
IPF14107	orf19.2449	NH
IPF11756	orf19.7006	NH
IPF15824	orf19.6030	Local homology to *Sc*YKR023w
IPF7338	orf19.3762	Homology to IPF13810
IPF122	orf19.5933	NH
IPF15335	orf19.4970	NH
IPF8741.5f	orf19.4275	NH
IPF1709	orf19.3076	Homology to *Sc*Tvpp15 and *An*ANO175.2
IPF16368.3f	orf19.254	NH
IPF8942	orf19.204	Local homology to *Sc*Rim2p
IPF7848	orf19.2558	NH
IPF1742.3f.eoc	orf19.3091	NH
IPF19554.3f	orf19.3371	Homology to *An*AN2129.2
IPF13231	orf19.3877	NH
IPF19807	orf19.7032	Homology to *Sc*YLR173
IPF16231	orf19.1913	NH
IPF17542	orf19.5578	NH
IPF2082	orf19.4085	NH
IPF19542.5f	orf19.371	NH
IPF7644	orf19.862	NH
IPF15601	orf19.2433	NH
IPF5453	orf19.5692	Homology to *An*AN0905.2
IPF14827	orf19.1611	NH
IPF3733	orf19.5287	NH
IPF11118	orf19.6967	NH
IPF9325	orf19.6288	NH
IPF6070	orf19.4921	Homology to *An*AN4487.2
IPF10761	orf19.6021	Homology to IPF8642
IPF7023.3	orf19.134	NH
IPF7201	orf19.2476	NH

Table 4. cont.

Gene identifier*	Assembly 19 orf	Putative or known function†
IPF5248	orf19.7254	NH
IPF14614	orf19.6080.1	NH
IFJ3	orf19.3924	NH
IPF19671	orf19.5854.1	NH
IPF5330	orf19.3448	NH
IPF15540	orf19.258	NH
IPF11347	orf19.4340.1	NH
IPF1548	orf19.951	NH
IPF12829	orf19.721	NH
IPF5217	orf19.7272	NH
IPF7097	orf19.480	Homology to *Sc*YJR056c
IPF15781	orf19.716	NH
IPF4750	orf19.1998	NH
IPF4563.5f	orf19.911	Local homology to *ScTOM1*
IPF7166	orf19.1202	NH
IPF1717	orf19.3080	Homology to *Sc*YDR180w
IPF2822	or19.6781	Homology to IPF9894
IPF17064	orf19.6342	NH
IPF6794	orf19.5596	NH
IPF1873	orf19.5902	NH
IPF2856	orf19.3914	Homology to *Sc*YOL139c
IPF9206	orf19.3687	NH
IPF19588	orf19.2318.1	Homology to *Sc*YDR367w
IPF1805	orf19.5003	Homology to *Sc*YGL164c
IPF8420	orf19.929	Contains F-box domain
IPF2195	orf19.6920	NH
IPF171	orf19.3228	Homology to *Sc*YER113c
HOK	orf19.7004	Part of Ca3 fingerprinting probe
Retrotransposon-encoded sequences (21)		
Zorro1b.5f/Zorro1b.3f	orf19.7274	Reverse transcriptase
Zorro2a.3f	orf19.3387	Reverse transcriptase
Zorro2b.5f	orf19.7275	Reverse transcriptase
IPF14825	orf19.1610	Homology to reverse transcriptases
IPF19295.5eoc	orf19.6468	Homology to reverse transcriptases
IPF17652.3	orf19.6078	Homology to reverse transcriptases
Cirt	orf19.4919	Transposase
Cirt1a	orf19.3492	Transposase
Cirt2	orf19.3820	Transposase
Cirt3	orf19.4918	Transposase
Cirt4a	orf19.2839	Transposase
Cirt4b	orf19.2839	Transposase
IPF6235	orf19.5372	*C. albicans* Tca2 retrotransposon
POL.3	orf19.2372	Pol polyprotein, reverse transcriptase
POL0	orf19.5373	Pol part of pCal retrotransposon
IPF6238	orf19.2374	GAG protein of retrotransposon pCAL
Tca5a	orf19.2427	Polyprotein of Tca5 retrotransposon
IPF2535	Not found	Homology to Tca5 polyprotein (*POL*) gene
IPF19295.3f	orf19.6469	Homology to Pol polyprotein of *A. thaliana*
IPF4450	orf19.6078	Pol polyprotein, reverse transcriptase
IPF13885.repeat/IPF13885	orf19.5503	Homology to GAG
Putative transcriptional regulators (19)		
CTA21	orf19.6112	Homology to *C. albicans CTA2*
CTA22	orf19.3074	Homology to *C. albicans CTA2*
CTA241.exon1/CTA241.exon2	orf19.5700	Homology to *C. albicans CTA2*

Table 4. cont.

Gene identifier*	Assembly 19 orf	Putative or known function†
CTA24.3/CTA24	orf19.4054	Homology to *C. albicans CTA2*
CTA2.5.3f/CTA25	orf19.362	Homology to *C. albicans CTA2*
CTA26	orf19.7680	Homology to *C. albicans CTA2*
CTA27	orf19.631	Homology to *C. albicans CTA2*
CTA29.exon2/CTA29.exon1	orf19.7544	Homology to *C. albicans CTA2*
RRN3	orf19.1923	*ScRRN3*, for transcription of rDNA
IPF9315	orf19.4647	Homology to *ScHAP3* activator
IPF4708	orf19.5276	Involved in transcriptional elongation
IPF14255	orf19.4767	Zinc-finger protein, GAL4 domain
IPF15920	orf19.4972	Zinc-finger protein
IPF10533.exon1/IPF10533.exon2	orf19.1255	Zinc-finger protein, homology to *AnAFLR*
IPF16067	orf19.3190	Zinc-finger protein, homology to *Sc*Hal9p
IPF9191.3f	orf19.3188	Zinc-finger protein, homology to *Sc*Hal9p
IPF8627	orf19.3969	Zinc-finger protein, homology to *ScMGA1*
IPF19540	orf19.723	Zinc-finger protein
IPF13021	orf19.2647	Zinc-finger protein, homology to *ScHAP1*
Leucine-rich repeat family (18)		
IFA1	orf19.156	Leucine-rich repeat protein
IFA2	orf19.195	Leucine-rich repeat protein
IFA4	orf19.4510	Leucine-rich repeat protein
IFA5	orf19.6353	Leucine-rich repeat protein
IFA6	orf19.5177	Leucine-rich repeat protein
IFA7	orf19.1326	Leucine-rich repeat protein
IFA9	orf19.2663	Leucine-rich repeat protein
IFA10	orf19.4549	Leucine-rich repeat protein
IFA11	orf19.1596	Leucine-rich repeat protein
IFA12	orf19.5619	Leucine-rich repeat protein
IFA15	orf19.996	Leucine-rich repeat protein
IFA17.5f/IFA17.3f	orf19.4511	Leucine-rich repeat protein
IFA18.3	orf19.4507	Leucine-rich repeat protein
IFA19	orf19.5141	Leucine-rich repeat protein
IFA22	orf19.1002	Leucine-rich repeat protein
IFA24.3	orf19.1596	Leucine-rich repeat protein
IFA25	orf19.5140	Leucine-rich repeat protein
IPF3540	orf19.2814	*C. albicans IFA* family homologue
GPI-anchored (17)		
PGA15	orf19.2878	Predicted GPI-anchored protein
PGA28	orf19.5144	Predicted GPI-anchored protein
PGA42	orf19.2907	Predicted GPI-anchored protein
PGA44	orf19.1714	Predicted GPI-anchored protein
PGA50	orf19.1824	Predicted GPI-anchored protein
ALS1.3eoc	orf19.5741	GPI-anchored protein, putative adhesin
ALS5	orf19.5736	GPI-anchored protein, putative adhesin
ALS6	orf19.7414	GPI-anchored protein, putative adhesin
ALS7	orf19.7400	GPI-anchored protein, putative adhesin
ALS10	orf19.2355	GPI-anchored protein
ALS11.3f	orf19.5742	GPI-anchored protein
HYR1	orf19.4975	GPI-anchored protein
IFF2	orf19.575	GPI-anchored protein
IFF4	orf19.7472	GPI-anchored protein
IFF7	orf19.3279	GPI-anchored protein
IFF8	orf19.570	GPI-anchored protein
CRH12	orf19.3966	GPI-anchored protein

Table 4. cont.

Gene identifier*	Assembly 19 orf	Putative or known function†
Putative membrane transporters (8)		
OPT1	orf19.2602	Oligopeptide transporter
DAL52	orf19.3208	Putative allantoin permease
IPF11550.3f/IPF11560.5f	orf19.2552	Homology to Ca^{2+}-transporting ATPases
FUR4	orf19.313	Uracil permease
HNM3	orf19.2587	Choline permease
HNM4	orf19.2946	Choline permease
IPF1992	orf19.7336	Homology to *ScAZR1*, drug efflux pump
PTH2	orf19.4231	Proline transport helper
Protein processing/modification (7)		
IPF4710	orf19.5275	Homology to *ScVTA1*
CTM1	orf19.7612	Homology to *ScCTM1*
IPF4195	orf19.5441	Homology to *Sc*Ulp2p
UBR11.3	orf19.2695	Homology to *ScUBR1*
PBN1	orf19.3447	Homology to protease B
IPF2997	orf19.6983	Homology to *Sc*Reg1p, protein phosphatase
IPF11977	orf19.853	Homology to aspartic proteinases
Cell metabolism/biosynthesis (6)		
IPF19538	orf19.4826	Putative isocitrate dehydrogenase
IPF5239	orf19.7260	Putative aldose reductase
PPX1	orf19.4107	Putative exopolyphosphatase
BIO4	orf19.2590	Dethiobiotin synthetase
IPF4940	orf19.7667	Putative isoamyl acetate esterase
CHS5	orf19.807	Chitin biosynthesis protein
mRNA processing (4)		
IPF4706	orf19.5277	Homology to *Sc*Upf3p; mRNA decay
IPF1911	orf19.7139	Homology to *Sc*Syf2p; mRNA splicing
IPF6444	orf19.1900	Homology to *Sc*Tgs1p
CUS1	orf19.7581	Spliceosome-associated protein
Chromatin/DNA-binding (3)		
IPF10490	orf19.419	Homology to *ScESC1*
IPF4805	orf19.3348	Homology to *ScNFI1/SIZ1*
IPF670	orf19.7598	Homology to *ScAHC1*
Morphogenesis related (3)		
IPF13247	orf19.3376	Homology to *ECE1*
IPF13252	orf19.13252	Homology to *ECE1*
IPF946	orf19.7561	*EFG1*-dependent transcript *EDT1*
Cell division (3)		
IPF2971	orf19.4284	*Schiz. pombe* cyclin c homologue
IPF1760.3f	orf19.4984	Homology to endochitinases
IPF1759.53f	Not found	Homology to endochitinases
Mitochondrial proteins (2)		
IPF5224	orf19.7267	Homology to *Sc*YHR083w
IPF11802	orf19.2797	Homology to *Sc*YDR332w
Cytoskeletal (2)		
IPF11222	orf19.5840	Homology to dynein light-chain proteins
YKE2.3	Not found	Actin-binding protein

*Gene identifiers refer to those in the CandidaDB (http://genolist.pasteur.fr/CandidaDB/).

†Gene functions as assigned in the CandidaDB, except where significant homology was independently detected by searches of GenBank or *Saccharomyces* Genome Database (SGD). *An* indicates homology to *Aspergillus nidulans* genes, *Sc* indicates homology to *Saccharomyces cerevisiae* genes, NH indicates that no homology was detected.

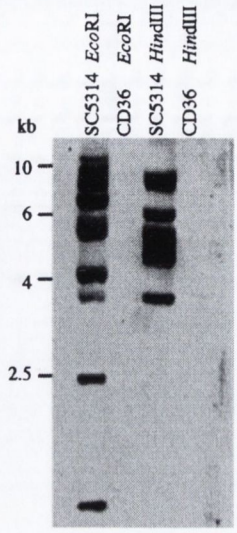

Fig. 2. Southern hybridization analysis of *C. albicans* and *C. dubliniensis* DNA with a DIG-11-dUTP-labelled probe homologous to nucleotides +1 to +781 of *CTA26*. The blot contains *C. dubliniensis* CD36 genomic DNA and *C. albicans* SC5314 genomic DNA digested with *Eco*RI and *Hin*dIII, as indicated. Molecular size markers are indicated on the left. Washes were performed at reduced stringency (60 °C in 0·5 × SSC).

(*IFF2* to *IFF11*) only *IFF10.5* and *IFF11* gave ratios above 0·5. Sequence alignments of *C. albicans IFF* genes generated with CLUSTAL_W identified *IFF1* as the most likely ancestral gene based on its homology to other members, with the most conserved sequences present in the 5′ region. As sequences homologous to this region of *IFF1* were not included on the Eurogentec array, we amplified 844 bp at the 5′ end of *IFF1* (~50 % of the ORF) from *C. albicans* SC5314 with the primer pair IFF1F/IFF1R (Table 2) and hybridized it to *C. dubliniensis* CD36 genomic DNA to reveal a single hybridizing band (Fig. 4b). We could detect this hybridizing fragment in the genomic DNA of seven additional unrelated *C. dubliniensis* isolates in Southern hybridization experiments. We used the IFF1F/IFF1R primer pair in PCR amplification reactions using *C. dubliniensis* CD36 template DNA and successfully generated a 750 bp amplimer with 86 % homology to *C. albicans IFF1*.

Other sequences with characterized gene products or functions that could be inferred from homology searches included genes involved in biotin synthesis (*BIO3*, *BIO4*) and several unrelated genes encoding metabolic enzymes (Table 4).

Putative virulence factors

The dataset of genes with normalized ratios of <0·5 was searched to identify genes which had previously been associated with *C. albicans* virulence.

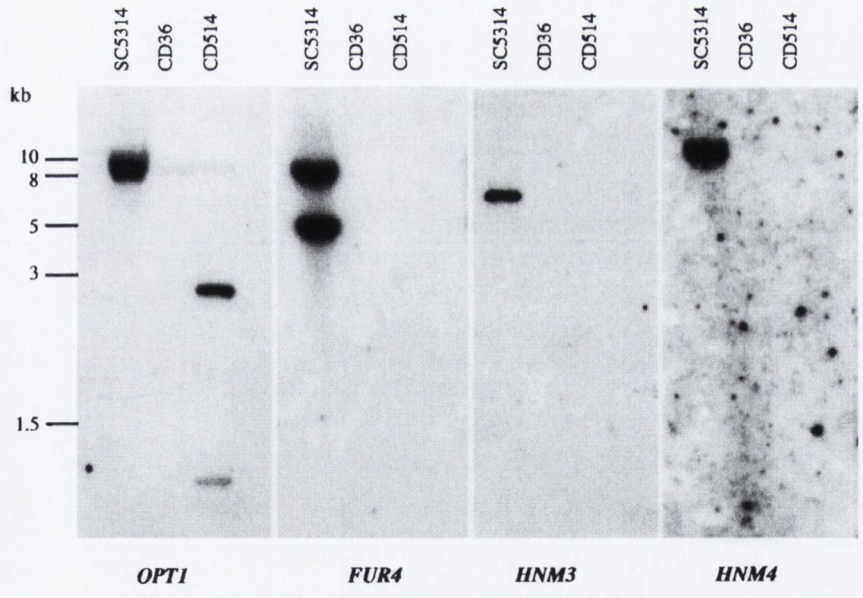

Fig. 3. Southern hybridization analysis of *C. albicans* and *C. dubliniensis* DNA with [α-^{32}P]dATP-labelled probes corresponding to the complete ORF sequences of the *C. albicans* genes *OPT1*, *FUR4*, *HNM3* and *HNM4*. The ORFs were amplified from *C. albicans* genomic DNA with the primer sets OPTA/B, FUR4A/B, HNM3A/B and HNM4A/B (Table 2). Each blot contains *Eco*RI-digested genomic DNA from *C. albicans* SC5314, *C. dubliniensis* CD36 and *C. dubliniensis* CD514. Molecular size markers are indicated on the left. Washes were performed at reduced stringency (60 °C in 0·5 × SSC).

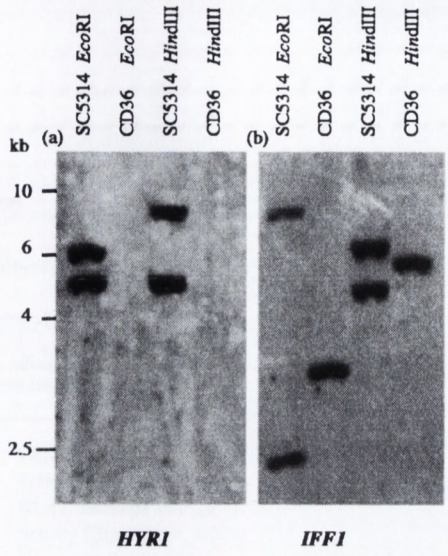

Fig. 4. Southern hybridization analysis of *C. albicans* and *C. dubliniensis* genomic DNA with sequences corresponding to highly conserved regions of *C. albicans* GPI-anchored protein encoding genes. Panel (a) was hybridized with an [α-^{32}P]dATP-labelled probe corresponding to nucleotides +95 to +1183 of the *C. albicans HYR1* gene. Panel (b) was hybridized with an [α-^{32}P]dATP-labelled probe corresponding to nucleotides +1 to +844 of *IFF1*. Each blot contains *C. dubliniensis* CD36 genomic DNA and *C. albicans* SC5314 genomic DNA digested with *Eco*RI and *Hin*dIII, as indicated. Molecular size markers are indicated on the left. Washes were performed at low stringency (60 °C in 0·5 × SSC).

(i) Genes encoding putative adhesins. Of the eight sequences with homology to members of the *ALS* gene family of GPI-anchored proteins (encoding putative adhesins) included for analysis, all gave normalized ratios of <0·5, with sequences specific for *ALS1*, *ALS5*, *ALS6* and *ALS7* yielding normalized ratios of <0·25 (Hoyer, 2001). Spots homologous to *ALS2*, *ALS3* and *ALS9* were excluded from our analysis due to poor hybridization with *C. albicans* genomic DNA.

We also investigated whether sequences encoding another group of GPI-anchored proteins were present, namely *HWP1*, which encodes a hyphal adhesin, and related sequences, *RBT1* and *IPF14331* (Braun *et al.*, 2000; Staab *et al.*, 1999). *HWP1* has been associated with virulence in *C. albicans* by mediating adhesion to epithelial cells (Staab *et al.*, 1999). *HWP1* and *RBT1* both yielded normalized ratios of <0·5 (0·48 and 0·37, respectively) in all experiments with *C. dubliniensis* CD36 genomic DNA. In order to identify a homologue of *HWP1*, a primer set designed based on the *C. albicans HWP1* sequence (HWP1F/HWP1R, Table 2) was used to amplify a 1·3 kb region of *C. dubliniensis* genomic DNA. The putative 5′ upstream region was also amplified with primers designed based on the sequence of the corresponding *C. albicans* region (APL6F and HWPR2, Table 2). An ORF of 1266 bp with homology to *C. albicans HWP1* was identified in these sequences. However, the ORF shared only 49% identity with the nucleotide sequence of *C. albicans HWP1* due to the presence of several large deletions within the coding sequence (GenBank accession no. AJ632273). The overlapping 5′ region amplified from *C. dubliniensis* contained upstream sequences homologous to the *C. albicans APL6* gene. This synteny between *HWP1* and *APL6* is conserved in *C. albicans*, and provides further evidence that this gene is a *C. dubliniensis HWP1* homologue. However, the predicted protein encoded by the *C. dubliniensis* gene was 421 amino acids in length, 213 residues shorter than the *C. albicans* protein. The first 50 residues of each protein were highly homologous, both containing the KR signature of the KEX2 cleavage site (Fig. 5a). However, the remainder of the N-terminal half of CdHwp1p contained several large deletions compared to the *C. albicans* protein, including most of the region rich in proline, glutamine and aspartate residues (Fig. 5a) (Sundstrom, 2002). Two of these deletions (of 89 bp and 119 bp) spanned the region homologous to the microarray probe and were likely to be responsible for the low signal detected with *C. dubliniensis* genomic DNA. Further deletions were found in the serine-threonine-rich region, although the ω-site for GPI-anchor addition was conserved.

Similarly, a *C. dubliniensis* sequence 513 bp in length was PCR-amplified using the primers RBTF2/RBTR2 designed based on the *C. albicans RBT1* sequence (Table 2). This sequence was 45% identical to base pairs +580 to +1410 of the *C. albicans RBT1* ORF, the region encoding the internal serine-threonine-rich domain of the *C. albicans* protein. Like the *CdHWP1* gene, this sequence also contained deletions (of 52 bp and 82 bp), when aligned to the homologous *C. albicans* sequence, that were responsible for the poor hybridization of *C. dubliniensis* DNA to the *CaRBT1* array spots.

Southern blot analysis was performed to determine whether single or multiple *HWP1* and *RBT1* homologues could be detected in *C. dubliniensis*. Hybridization of the *C. dubliniensis HWP1* amplified sequence to *Eco*RI-digested *C. albicans* genomic DNA revealed a single hybridizing fragment of 4 kb (Fig. 5b). In *C. dubliniensis* genomic DNA, a strongly hybridizing fragment of 9 kb was detected and a second, weakly hybridizing fragment of 5 kb in *Eco*RI-digested DNA (Fig. 5b). This second fragment was identical in size to the fragment detected in Southern blots of *C. dubliniensis* DNA with sequences corresponding to *CaRBT1* (Fig. 5c), indicating that this second hybridizing fragment was likely to correspond to *CdRBT1*.

(ii) Secreted aspartyl proteinases. Sequences homologous to the 10 *C. albicans* secreted aspartyl proteinase (*SAP*)-encoding genes (*SAP1* to *SAP10*) were included on

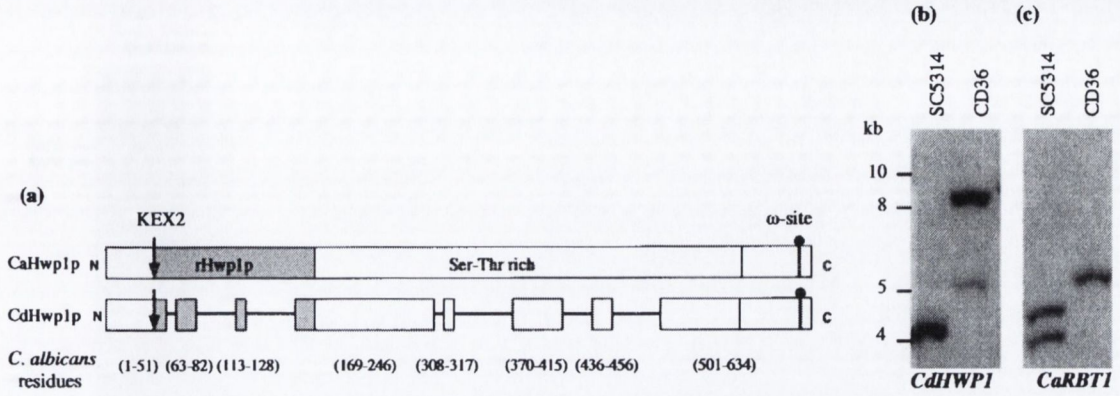

Fig. 5. (a) Diagram illustrating regions of homology to *C. albicans* CaHwp1p and the extent of deletions in the predicted CdHwp1p protein sequence. The upper rectangular box represents the CaHwp1p protein and shows the position of the KEX2 cleavage site (arrow), the recombinant rHwp1p domain (shaded area) shown to possess transglutaminase substrate activity (Sundstrom, 2002), the serine-threonine-rich region (Ser-Thr rich) and the carboxy-terminal ω-site. The lower boxes represent the homologous regions of the predicted *C. dubliniensis* CdHwp1p protein. The numbers in parentheses indicate the positions of the homologous *C. dubliniensis* protein domains relative to the corresponding *C. albicans* amino acid residues. (b, c) Southern hybridization analysis of *C. albicans* SC5314 and *C. dubliniensis* CD36 genomic DNA with sequences corresponding to (b) *HWP1* and (c) *RBT1*. DNA in (b) was hybridized with an [α-^{32}P]dATP-labelled probe corresponding to the entire *C. dubliniensis HWP1* ORF. DNA in (c) was hybridized with an [α-^{32}P]dATP-labelled probe corresponding to nucleotides +694 to +1410 of *RBT1* amplified from *C. albicans* genomic DNA. Lanes 1 and 2 in both panels contain *Eco*RI-digested genomic DNA from *C. albicans* and *C. dubliniensis*, respectively. Molecular size markers are indicated on the left. Washes were performed at reduced stringency (60 °C in 0·5× SSC).

the arrays. All of the *SAP* genes with the exception of *SAP4*, *SAP5* and *SAP6* gave normalized ratios of >0·6. *SAP5* gave a mean ratio of 0·38 (among genes with intermediate homology) in all *C. dubliniensis* CD36 experiments. We probed the *C. dubliniensis* genome for homologues of *SAP4–6* using PCR primers homologous to conserved regions of these genes (SAP4-6F/SAP4-6R, Table 2). Amplification using *C. dubliniensis* genomic DNA as the template yielded a PCR product of 750 bp that shared 86% identity with *SAP4* and *SAP6*. Using an inverse PCR strategy (primers InvSAPF/InvSAPR, Table 2) we amplified flanking sequences from *C. dubliniensis* genomic DNA to obtain the complete ORF (GenBank accession no. AJ634382). This *C. dubliniensis* gene was found to lie upstream of the *C. dubliniensis* homologue of the *C. albicans SAP1* gene and was equally homologous to *SAP4* and *SAP6* (~85%). This ORF was designated *CdSAP4* as the synteny at this locus with *SAP1* is identical to that at the *SAP4* locus in *C. albicans*. We used the entire *C. dubliniensis SAP4* gene as a probe in Southern blots with *C. albicans* and *C. dubliniensis* genomic DNA in order to identify *SAP5* and *SAP6* homologues in *C. dubliniensis*. The *SAP4–6* genes in *C. albicans* are highly homologous (89% nucleotide sequence identity), so we anticipated that the *C. dubliniensis SAP4* gene should hybridize strongly to any *C. dubliniensis SAP5* or *SAP6* homologues. Indeed, the *C. dubliniensis SAP4* gene hybridized to four separate *Kpn*I fragments in *C. albicans* genomic DNA. Three of these fragments corresponded in length to the predicted sizes of *Kpn*I fragments expected to contain homologues of *SAP4*, *SAP5* and *SAP6* based on the *C. albicans* genome sequences at the Stanford Genome Technology Center. The *SAP4*-hybridizing fragment was less intense than the *SAP5*- and *SAP6*- containing fragments. However, a fourth hybridizing fragment of similar intensity at 7 kb was also detected and could contain a second *SAP4* allele due to an RFLP (Fig. 6). The *C. dubliniensis SAP4* gene was hybridized to *C. dubliniensis* genomic DNA digested with *Kpn*I, *Hin*dIII and several restriction endonucleases that do not cleave within the *CdSAP4* ORF (*Bgl*II, *Spe*I, *Sal*I, *Xba*I); this revealed only one significantly hybridizing band in *C. dubliniensis* genomic DNA (Fig. 6). Furthermore, hybridization of the *C. albicans SAP5* and *SAP6* genes to *C. dubliniensis* DNA resulted in hybridization to the same restriction fragment harbouring the *C. dubliniensis SAP4* gene (data not shown). These findings were confirmed by Southern blot analysis on eight epidemiologically unrelated isolates of *C. albicans* and *C. dubliniensis* (data not shown).

Hybridization of a second *C. dubliniensis* strain to microarrays

In order to confirm the above data obtained with *C. dubliniensis* CD36 and to investigate the levels of intra-species variation between unrelated *C. dubliniensis* strains, we hybridized genomic DNA from a second *C. dubliniensis*

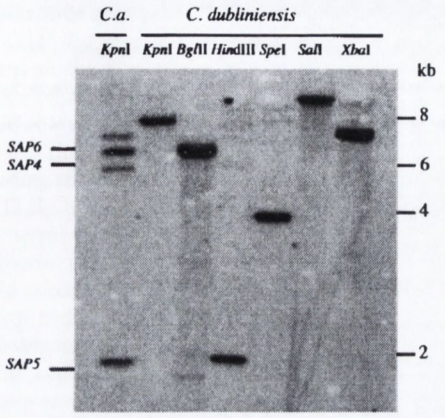

Fig. 6. Southern hybridization analysis of *C. albicans* and *C. dubliniensis* genomic DNA with sequences corresponding to the *C. dubliniensis CdSAP4* gene. The blot was hybridized with an [α-^{32}P]dATP-labelled probe of the entire *CdSAP4* ORF. The blot contains genomic DNA from *C. albicans* SC5314 digested with *Kpn*I and genomic DNA from *C. dubliniensis* CD36 digested with *Kpn*I, *Bgl*I, *Hin*dIII, *Spe*I, *Sal*I or *Xba*I, as indicated. The markers on the left side of the panel indicate the predicted positions of the *SAP4*, *SAP5* and *SAP6* genes in *C. albicans* SC5314. Molecular size markers are indicated on the right. Washes were performed at reduced stringency (60 °C in 0·5 × SSC).

isolate, CD514, to these arrays. We chose this strain as it has been shown to be genetically unrelated to *C. dubliniensis* CD36 based on the DNA fingerprint pattern obtained with the *C. dubliniensis* fingerprint probe Cd25. Sheared genomic DNA from *C. dubliniensis* CD514 was labelled with Cy3 and co-hybridized with Cy5-labelled DNA from *C. albicans* SC5314. We compared the dataset from CD514 with that generated from CD36 in order to identify genes unique to each strain. Only three additional genes were discovered that hybridized significantly to CD514 DNA (ratio > 0·59) and that were deemed absent in CD36 (normalized ratio < 0·2, $P < 0{\cdot}035$). These genes were IPF4450 and IPF17652.3, with homology to an integrase and a reverse transcriptase, respectively, and the oligopeptide transporter encoding gene *OPT1*. The presence of the *OPT1* sequence in CD514 and its absence in CD36 was confirmed by Southern hybridization with the *C. albicans OPT1* sequence (Fig. 2). Conversely, only one sequence, encoding *GIT1* (glycerophosphoinositol transporter), was identified which failed to hybridize with CD514 DNA (ratio 0·112, *P* value 0·008) and gave significant signals with CD36 DNA (ratio 1·1).

DISCUSSION

Phylogenetic analysis of rRNA sequences has confirmed that *C. dubliniensis* and *C. albicans* are the two most closely related *Candida* species of clinical importance in humans (Sullivan *et al.*, 1995, 2004). However, whereas *C. albicans* is the most significant yeast pathogen, responsible for superficial and deep-seated infections, *C. dubliniensis* is of lesser clinical importance in mucosal infections in non-HIV-infected patients, and is relatively insignificant in the case of bloodstream infection (Kibbler *et al.*, 2003; Meis *et al.*, 2000). This apparent lower virulence of *C. dubliniensis* is also evident in data from animal model infection studies. However, the exact reasons why *C. albicans* is more virulent are not clear. In this study we have utilized recently available *C. albicans* whole-genome DNA microarrays to investigate and identify genomic differences between *C. albicans* and *C. dubliniensis* that could account, at least in part, for the enhanced virulence potential of *C. albicans* relative to *C. dubliniensis* and for the differences in epidemiology between the two species.

The findings presented in this study obtained by CGH reinforce the phylogenetic data that originally implied the close relatedness of the two organisms (Gilfillan *et al.*, 1998; Sullivan *et al.*, 1995). Our data show that only 4·4 % of *C. albicans* sequences analysed in our studies (normalized ratio < 0·25) were likely to be absent or highly divergent (< 60 % homologous at the nucleotide sequence level) in *C. dubliniensis*. That the vast majority of *C. albicans* genes are highly conserved in *C. dubliniensis* indicates that the two species have probably only diverged relatively recently and thus are likely to inhabit similar environments in the human body. Thus only a small subset of *C. albicans* genes seem to be unique to this species and could possibly be important contributory factors to the greater success of *C. albicans* as a commensal on the human mucosal epithelium and as a pathogen in compromised hosts. Surprisingly, some spots exhibited reproducibly higher fluorescent signals with *C. dubliniensis* genomic DNA. There are several possible reasons for these findings, including cross-hybridization of paralogous *C. dubliniensis* sequences to the same spot, increases in gene copy number or differences in ploidy.

Of the 247 *C. albicans* genes identified that were predicted to have less than 60 % homology at the nucleotide sequence level or even possibly be absent in *C. dubliniensis*, 134 were hypothetical genes of unknown function (Table 3). However, 43 of these hypothetical ORFs were conserved, with homology to genes in *S. cerevisiae*, *Aspergillus nidulans* or paralogous sequences in the *C. albicans* genome. Of the 113 genes with a confirmed or hypothetical function, few were identified that corresponded to housekeeping genes involved in central metabolism, cell structure, or molecular biosynthesis. However, several transporter-encoding genes involved in nutrient uptake were identified as being absent, including two of the four genes encoding choline permeases in *C. albicans* (*HNM3*, *HNM4*), a uracil permease (*FUR4*) and an allantoin permease (*DAL52*). The only confirmed intrastrain difference between the two Cd25 fingerprint group *C. dubliniensis* strains analysed here was the presence of sequences homologous to the

oligopeptide transporter *OPT1* (Lubkowitz *et al.*, 1997) in the Cd25 fingerprint group II strain CD514 which were absent in the fingerprint group I isolate CD36. It is not known whether the absence of these genes could affect the ability of *C. dubliniensis* to grow relative to *C. albicans in vivo*, since, for example, our data suggest other genes encoding choline (*HNM2*) and allantoin permeases (*DAL51*) are likely to be present in *C. dubliniensis*. *C. dubliniensis* also seems to lack sequences involved in the biosynthesis of biotin (*BIO3* encoding DAPA aminotransferase and *BIO4* encoding dethiobiotin synthetase). Although biotin is required for growth, *C. albicans* and *C. dubliniensis* probably acquire sufficient biotin from exogenous sources in the oral cavity, most likely from commensal bacteria (Phalip *et al.*, 1999).

Twenty-one sequences corresponded to genes present in retrotransposons of *C. dubliniensis*, indicating that since their divergence the genomes of the two species may have acquired different mobile genetic elements.

Seventeen sequences homologous to genes encoding various GPI-anchored proteins were identified in our analysis as being absent or of low homology in *C. dubliniensis* by a combination of array hybridization data, PCR analysis and Southern blot analysis (Sundstrom, 2002). Poor *C. dubliniensis* hybridization signals were detected from sequences homologous to the *C. albicans* hyphal specific *HYR1* gene (mean ratio 0·13), and no homologous gene was identified in *C. dubliniensis* following Southern hybridization with conserved *C. albicans HYR1* sequences (Bailey *et al.*, 1996). Sequences corresponding to several *HYR1*-related GPI-anchored proteins in *C. albicans* also exhibited poor hybridization signals with *C. dubliniensis* genomic DNA (*IFF* family genes). Although specific functions have not been assigned to proteins encoded by these genes, their likely location on the cell surface indicates possible roles in maintaining cell wall integrity, environmental signalling or adhesion to host surfaces.

Interestingly, subsequent analysis (Southern blotting) with sequences homologous to the *C. albicans IFF1* gene (absent from Eurogentec microarrays) identified a homologous gene in *C. dubliniensis* for which sequences were later identified by PCR. These data suggest that at least one *IFF*-like gene is present in the *C. dubliniensis* genome. This may represent an ancestral *IFF*-related gene; however, additional *IFF*-related genes may be present in the *C. dubliniensis* genome but may be difficult to detect by CGH as they could have diverged more extensively than essential housekeeping genes with greater sequence-based constraints on protein function. A similar conclusion could be reached with regard to sequences homologous to genes encoding proteins of the α-agglutinin-like ALS family of adhesins. The *ALS* probes on the Eurogentec arrays used in this study consist of sequences from the 3′ region of the ORFs, which within the *ALS* family are the least conserved regions (Hoyer *et al.*, 2001). By Southern hybridization analysis Hoyer and co-workers noted that the 3′ regions of the *C. albicans ALS* genes are poorly conserved in *C. dubliniensis*. We observed low hybridization ratios (<0·25) for several members of this family including *ALS1*, *ALS5*, *ALS6* and *ALS7*. However, Hoyer and co-workers identified partial 5′ nucleotide sequences for three *ALS* homologues in *C. dubliniensis* (*ALSD1*, *ALSD2* and *ALSD3*). Their study demonstrated that *ALSD1* is closely related to *ALS6* and *ALSD3* is closely related to *ALS4*. The present study confirms their findings that the 3′ regions of the *ALS* genes are poorly conserved in *C. dubliniensis*, but does not provide further evidence for the existence of other *C. dubliniensis ALS* homologues. Since the microarray DNA spots correspond to 300–400 bp regions of each gene, our data reflect differences present in these regions only.

As the CGH data obtained for the *ALS* gene family demonstrate, data indicating the absence or divergence of a particular gene require confirmation as these regions may encompass non-conserved regions of the gene. Conversely, there may be divergent regions in many genes that remain undetected as they lie outside the regions compared here. Similarly, minor genetic differences (e.g. point mutations) and differences in non-translated regions that cannot be detected using these methods could also influence virulence and epidemiology. In addition, phenotype can also be influenced by post-transcriptional events unrelated to DNA sequence.

Low hybridization ratios were also observed for the *HWP1* gene and the related sequences *RBT1* and *IPF14331* (Braun & Johnson, 1997; Staab *et al.*, 1999; Sundstrom, 2002). The *HWP1*-encoded protein has been identified as a hypha-specific substrate for host transglutaminases involved in covalent adhesion to host cells. However, the functions of the other two gene products are as yet uncharacterized. In this study we identified the *C. dubliniensis HWP1* homologue. The *C. dubliniensis HWP1* gene hybridized poorly to the *C. albicans* array *HWP1* sequences due to the presence of large deletions in the *C. dubliniensis* ORF. The predicted translated protein encoded by *CdHWP1* contains several large deletions compared to the *C. albicans* protein (Fig. 5a). These deletions lie within the N-terminal glutamine- and proline-rich repeat domain containing the transglutaminase substrate activity and the internal serine- and threonine-rich domain. It will be of interest to determine whether the transglutaminase substrate activity of the *C. dubliniensis* homologue is affected by the presence of deletions in glutamine-rich regions of the N-terminus. A defect in the ability of *C. dubliniensis* to form stable attachments to oral epithelium may partly explain its reduced prevalence in the oral cavities of healthy individuals and patients with oral disease.

One of the most intensely studied virulence attributes of *C. albicans* is the ability to secrete aspartyl proteinases, encoded by 10 separate genes (Naglik *et al.*, 2003). Sequences from all 10 *SAP* genes were present on the array.

Sequences from only one of these genes, *SAP5*, gave consistently low signals from *C. dubliniensis* hybridizing DNA. *SAP5* is a member of the *SAP4–6* subfamily of proteinases, which are all highly homologous at the nucleotide sequence level and preferentially expressed by hyphae (Hube *et al.*, 1994). In our efforts to determine if *SAP5* was present in *C. dubliniensis*, we identified a gene that is most homologous to *SAP4* and *SAP6*, which we designated *CdSAP4* as the ORF was located upstream of *CdSAP1*, identical to the synteny observed in *C. albicans* (Miyasaki *et al.*, 1994). Southern hybridization analysis with this *CdSAP4* sequence revealed that it could hybridize to multiple fragments of *C. albicans* restriction-endonuclease-digested DNA corresponding to sequences of *SAP4*, *SAP5* and *SAP6*. Such cross-hybridization is likely to be responsible for the strong signal detected from spots representing *SAP6* on the *C. albicans* microarray. However, the *CdSAP4* sequence consistently hybridized to only one single band (between ~2 and 10 kb) in Southern hybridization experiments with *C. dubliniensis* genomic DNA. These data indicate that only one gene with strong homology to the *SAP4–6* subfamily exists in *C. dubliniensis*. Attempts to identify the corresponding genomic loci of putative *SAP5* and *SAP6* genes in *C. dubliniensis* by low-stringency PCR were unsuccessful (data not shown).

Together, the *SAP4–6* subfamily has been shown to play an important role in the establishment of *C. albicans* systemic infections in mice and *SAP6* has been shown to be the most important gene within this family in the establishment of murine intraperitoneal infection (Felk *et al.*, 2002; Sanglard *et al.*, 1997). As *C. dubliniensis* only possesses one gene with homology to *SAP4–6*, this could partly explain why *C. dubliniensis* is less able than *C. albicans* to establish systemic infection. *In vivo* virulence studies and epidemiological data support this hypothesis, since *C. dubliniensis* is less virulent than *C. albicans* in a murine systemic model of infection and the incidence of recovery of this organism from human blood cultures is extremely low (Gilfillan *et al.*, 1998; Kibbler *et al.*, 2003). The absence of these hypha-specific proteinases in *C. dubliniensis* may affect the ability of its hyphae to penetrate host tissues, acquire nutrients or evade killing by macrophages. We are currently testing the role of *C. dubliniensis* Sap proteins in infection models.

Differences in gene regulation have not been explored in any great detail in *C. dubliniensis* to date. The array CGH data indicates the presence of genes homologous to many of the transcription factors involved in regulating hypha formation in *C. albicans* (e.g. *EFG1*, *CPH1*, *TUP1*). However, poor hybridization signals were detected from several other genes encoding putative transcriptional regulators that could affect regulatory circuits in *C. dubliniensis*. Eight of these sequences had homology to genes encoding proteins with zinc-finger DNA-binding motifs in *S. cerevisiae*. Another group of genes with homology to the putative *C. albicans* transcriptional activator encoding gene *CTA2* were also identified. *CTA2* (GenBank ID AJ006637)was identified by Kaiser *et al.* (1999) in a one-hybrid screen in *S. cerevisiae* for *C. albicans* proteins with transcriptional activating properties. A family of possibly up to 10 genes with strong homology to *CTA2* has been identified in *C. albicans*. Twelve sequences homologous to these genes were included in our analysis and all gave normalized ratios of $<0{\cdot}25$. Southern hybridization also failed to identify any sequences with significant homology to these genes in *C. dubliniensis*. Although the function of these proteins has yet to be confirmed, the absence or divergence of a large family of transcriptional activators in *C. dubliniensis* could have major implications for the growth and virulence of this fungus.

In the present study, *C. albicans* DNA microarrays facilitated a whole-genome comparison between *C. albicans* and its close relative *C. dubliniensis*. Our experiments have revealed the absence and divergence of several genes and gene families in *C. dubliniensis*. These include putative virulence factors and many genes specific for or preferentially expressed in the hyphal phase, such as *SAP5*, *SAP6*, *HWP1* and *HYR1*. *C. dubliniensis* is generally less efficient than *C. albicans* at forming hyphae in response to serum and the absence of these hypha-regulated genes may also indicate that *C. dubliniensis* hyphae are less specialized as virulence-promoting structures (Gilfillan *et al.*, 1998). We have endeavoured to confirm the absence or divergence of genes directly involved in virulence (e.g. *HWP1*, *SAP5*); however, conclusive confirmation of this data will have to await the completion of the *C. dubliniensis* genome sequencing project which is currently under way (http://www.sanger.ac.uk/Projects/C_dubliniensis/). When complete this project will facilitate more thorough comparative genomic analysis and will allow the identification of *C. dubliniensis*-specific genes that could be responsible for phenotypic differences. Until then, this dataset represents a framework for further investigations into genetic and phenotypic differences between *C. albicans* and *C. dubliniensis*.

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MiniReview

Comparison of the epidemiology, drug resistance mechanisms, and virulence of *Candida dubliniensis* and *Candida albicans*

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Abstract

Candida dubliniensis is a pathogenic yeast species that was first identified as a distinct taxon in 1995. Epidemiological studies have shown that *C. dubliniensis* is prevalent throughout the world and that it is primarily associated with oral carriage and oropharyngeal infections in human immunodeficiency virus (HIV)-infected and acquired immune deficiency syndrome (AIDS) patients. However, unlike *Candida albicans*, *C. dubliniensis* is rarely found in the oral microflora of normal healthy individuals and is responsible for as few as 2% of cases of candidemia (compared to approximately 65% for *C. albicans*). The vast majority of *C. dubliniensis* isolates identified to date are susceptible to all of the commonly used antifungal agents, however, reduced susceptibility to azole drugs has been observed in clinical isolates and can be readily induced in vitro. The primary mechanism of fluconazole resistance in *C. dubliniensis* has been shown to be overexpression of the major facilitator efflux pump Mdr1p. It has also been observed that a large number of *C. dubliniensis* strains express a non-functional truncated form of Cdr1p, and it has been demonstrated that this protein does not play a significant role in fluconazole resistance in the majority of strains examined to date. Data from a limited number of infection models reflect findings from epidemiological studies and suggest that *C. dubliniensis* is less pathogenic than *C. albicans*. The reasons for the reduced virulence of *C. dubliniensis* are not clear as it has been shown that the two species express a similar range of virulence factors. However, although *C. dubliniensis* produces hyphae, it appears that the conditions and dynamics of induction may differ from those in *C. albicans*. In addition, *C. dubliniensis* is less tolerant of environmental stresses such as elevated temperature and NaCl and H_2O_2 concentration, suggesting that *C. albicans* may have a competitive advantage when colonising and causing infection in the human body. It is our hypothesis that a genomic comparison between these two closely-related species will help to identify virulence factors responsible for the far greater virulence of *C. albicans* and possibly identify factors that are specifically implicated in either superficial or systemic candidal infections.
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Keywords: *Candida dubliniensis*; *Candida albicans*; Epidemiology; Virulence; Resistance

1. Introduction

Fungi are important agents of human disease. Amongst the most important fungal pathogens are yeast species belonging to the genus *Candida*. These species can cause a wide range of human diseases ranging from superficial mucosal infections, such as vulvovaginal (VVC) and oropharyngeal candidosis (OPC), to life-threatening invasive infections. In the majority of cases OPC and systemic infections occur only in individuals who are severely ill and/or immunocompromised. In particular, oropharyngeal infections are very commonly diagnosed in human immunodeficiency virus (HIV)-infected individuals and individuals with acquired immune deficiency syndrome (AIDS), while deep-seated systemic infections are frequently associated with patients with neutropenia, for example as a result of antineoplastic therapy or immunosuppressive therapy associated with organ transplantation. The most common cause of candidosis is the polymorphic species *Candida albicans*, which can grow as yeast cells, pseudohyphae and hyphae. It also produces chlamydospores which are refractile spore-like structures that are mainly produced at the termini of hyphae under specific environmental condi-

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tions in vitro. However, other *Candida* species, including *Candida glabrata* and *Candida parapsilosis*, are currently also significant human pathogens. Indeed, it has been suggested that the incidence of infections caused by non-*C. albicans Candida* species is increasing [1].

While performing an in-depth epidemiological analysis of the *Candida* species associated with oral candidal infections in the Irish HIV-infected and AIDS population in the early 1990s, we identified an unusual group of isolates [2]. These were originally identified as *C. albicans* because they were germ tube- and chlamydospore-positive (two traits previously recognised as being specific for this species only), however, when they were fingerprinted using the *C. albicans*-specific DNA fingerprinting probe 27A, the fingerprint patterns obtained were observed to be atypical and clearly distinct from those of known *C. albicans* strains. Following a thorough comparison of the phenotypes and genotypes of these atypical isolates and representative strains of *C. albicans* (and *Candida stellatoidea*), it became increasingly clear that there were many differences between these organisms. This was confirmed by performing a phylogenetic analysis comparing the nucleotide sequences of the V3 variable region of the large subunit ribosomal RNA gene from the atypical isolates and from *C. albicans*. The results of this led us to suggest that the atypical organisms belonged to a novel taxon, which we called *Candida dubliniensis* (after the city and University of Dublin) [2]. The phylogenetic relationship between *C. dubliniensis* and the rest of the *Candida* genus has since been established on the basis of comparisons between the nucleotide sequences of a wide range of other genes, including the small ribosomal RNA gene [3], *ACT1* [4], *MDR1* [5], *CDR1* [6] and *ERG3* [7].

2. Clinical significance of *C. dubliniensis*

In order to determine the clinical importance of *C. dubliniensis* and to determine its role in human disease it is essential to be able to accurately identify the species in clinical samples. However, due to the phenotypic similarities between *C. dubliniensis* and *C. albicans* this can be problematic. Since its identification, a large number of phenotypic and genotypic tests have been developed with a view to investigating the prevalence of *C. dubliniensis* in the human population. Many of these tests have been described in detail in earlier reviews [8–10] and are summarised in Table 1. Very few of these phenotype-based identification tests are 100% accurate. However, it has recently been reported that all 128 *C. dubliniensis* isolates tested produced hyphal fringes when incubated at 30°C for 48–72 h on Pal's agar (a medium containing bird seed extract), whereas all of the 124 *C. albicans* isolates tested produced smooth colonies when grown under the same culture conditions (Fig. 1), thus indicating that this is a very accurate and reliable differentiation test [11]. However, the most definitive methods of identifying *C. dubliniensis* are based on differential amplification of species-specific sequences using the polymerase chain reaction (PCR) and *real-time* PCR [4,12–16]. Ideally, in the clinical laboratory more than one method should be used to definitively identify *C. dubliniensis* isolates.

C. dubliniensis was originally identified because DNA from this species hybridised very poorly to *C. albicans* species-specific DNA fingerprinting probes and had unusual karyotype patterns, suggesting that the genomes of the two species have very distinctive differences [2]. This suggestion has since been confirmed by a study that identified *C. dubliniensis*-specific semi-repetitive sequences [17]. These sequences (i.e. Cd1, Cd24 and Cd25), have been fully characterised and developed for use as DNA fingerprinting probes in the analysis of *C. dubliniensis* populations. The interesting finding from this study and from an extended follow-up study that compared fingerprints and sequences derived from the ITS region of the rRNA operon is that *C. dubliniensis* is comprised of four separate genotypes [18]. The significance of these genotypes has yet to be established. However, isolates belonging to the predominant clade (genotype 1) are primarily associated with carriage and infection in HIV-infected individuals. It is also apparent that the *C. dubliniensis* genome undergoes microevolutionary genomic rearrangements at a far higher rate than that of *C. albicans* [18]. In a thorough investigation of this phenomenon, Joly et al. [19] have determined that the *C. dubliniensis* genome contains more than twice the number of RPS sequences than that of *C. albicans*, and that these could act as recombination hotspots leading to non-homologous recombination between chromosomes. This might explain the unusual karyotypes with small supernumary chromosomes that are typical of *C. dubliniensis*. What effects this increased level of mitotic recombination might have on *C. dubliniensis* is not known. It could have a beneficial effect by facilitating adaptation to changing environments by modulating phenotypic traits, such as drug resistance. However, it could also have a detrimental effect by leading to a loss of heterozygosity.

The development of improved methods for identifying *C. dubliniensis* during the past five years has resulted in a large volume of published data describing the epidemiology of this species. It has been identified in studies from every continent and has been found in a wide range of anatomical sites and clinical samples [20–23]. Interestingly, despite the phenotypic similarities between *C. dubliniensis* and *C. albicans*, the former appears to be only a minor constituent of the normal oral and vaginal microbial flora. In a study on an Irish population of normal healthy individuals only 3.5% of individuals were found to carry *C. dubliniensis* in the oral cavity while the prevalence of this species in the vagina was found to be even lower [24]. *C. dubliniensis* is most commonly associated with OPC in HIV-infected and AIDS patients. In a study on oral can-

Table 1
Comparison of specific phenotypic traits of *C. albicans* and *C. dubliniensis*

Trait	*C. albicans*	*C. dubliniensis*	Ref.
Production of germ tubes	++	+	[2]
Production of chlamydospores	+	++	[2]
Growth at 37°C[a]	++	++	[2]
Growth at 42°C[a]	++	−	[2]
Growth at 45°C[a]	+	−	[59]
Growth in broth containing 6.5% (W/V) NaCl	+	−	[60]
Growth on xylose[b]	+	−	[62]
Growth on lactate[b]	+	−	[62]
Growth on α-methyl-D-glucoside[b]	+	−	[62]
Growth on trehalose[b]	+	±	[62]
Colony colour on CHROMagar Candida	light blue/green	dark green	[63]
Colony morphology on Pal's agar	smooth	rough+hyphal fringe	[11]

[a]Strains grown on potato dextrose agar.
[b]Based on data obtained using the ID32C yeast identification system (bioMérieux, France)

didosis in our own laboratory, we have shown that in an Irish cohort, 26% of HIV-infected and 32% of AIDS patients with symptoms of OPC harboured *C. dubliniensis*, while in patients without symptoms of OPC the levels were 18 and 25%, respectively [24]. *C. dubliniensis* was usually found in combination with other yeast species, especially *C. albicans*. However, in up to 10% of cases it was the only *Candida* species detectable, indicating that *C. dubliniensis* was very likely to be responsible for the symptoms of disease observed in these cases. *C. dubliniensis* carriage and infection were also particularly prevalent in recurrent cases of infection, following prior treatment with azole antifungal drugs, such as fluconazole. A high prevalence of *C. dubliniensis* in the oral cavities of HIV-infected and AIDS patients has also been reported in studies from Germany [25] and the USA [26,27]. However, in a number of other studies, the levels of *C. dubliniensis* found were significantly lower [28,29]. The reasons for the disparity in the prevalence of *C. dubliniensis* in these studies are not clear. However, it is possible that the sampling and identification methods used, the geographical locale and the cohort of HIV-infected individuals examined (e.g. intravenous drug user, homosexual, etc.) could have contributed to the differences in the epidemiological data. In addition, a relatively high prevalence of *C. dubliniensis* has also been observed in the oral cavities of patients with denture stomatitis [24], diabetes [30] and cystic fibrosis [31].

C. dubliniensis has also been recovered from blood samples obtained from cases of invasive disease, particularly in patients who were neutropenic following bone marrow or solid organ transplantation [32–35]. However, despite the high prevalence of *C. dubliniensis* in cases of OPC in HIV-infected individuals, this species is only rarely associated with systemic infections. In two recent studies, *C. dubliniensis* accounted for approximately 2% of cases of candidemia in the UK [36] and in the USA [34]; this is in stark contrast with *C. albicans*, which accounts for approximately two thirds of all cases of candidemia. It should also be noted that in many cases *C. dubliniensis* isolates were recovered from blood samples that also yielded bacterial species such as *Staphylococcus aureus* and *Escherichia coli*, thus in these cases it is impossible to attribute the symptoms of disease to *C. dubliniensis* alone. These epidemiological data suggest that despite the phenotypic similarities between the two species, *C. albicans* has a competitive advantage in colonising and infecting humans.

In summary, *C. dubliniensis* is a rare constituent of the human normal microbial flora, and this is reflected in the low prevalence of *C. dubliniensis* in invasive infections. However, *C. dubliniensis* is a very significant contributor to cases of OPC in HIV-infected and AIDS patients and is clearly pathogenic and present at high levels in these patients. The reasons for the puzzling disparity between the levels of *C. dubliniensis* in HIV- and non-HIV-infected individuals are not understood. One possible explanation is that the methods currently used for routinely isolating oral yeasts (e.g. swabs and rinsing) do not adequately sample all of the microniches that *C. dubliniensis* might inhabit in the oral cavity (e.g. the gingivae). Another possibility is that *C. dubliniensis* strains could be acquired from exogenous sources following sufficient depletion of an individual's T cell count as HIV infection proceeds. Another interesting question concerns whether *C. dubliniensis* has only recently emerged as a human pathogen or whether it has always been associated with human colonisation and infection. From the published epidemiological studies it is clear that the vast majority of *C. dubliniensis* strains identified to date have been found in collections from HIV-infected patients dating from the early 1990s onwards, possibly correlating with the introduction of fluconazole for the treatment of oral candidosis in these patients. However, in several studies of archival strain collections a small number of *C. dubliniensis* strains have been identified that predate the emergence of HIV [37,38], with one strain dating as far back as the 1950s [9]. This suggests that *C. dubliniensis* has been associated with human colonisation (and possibly infection), albeit at a

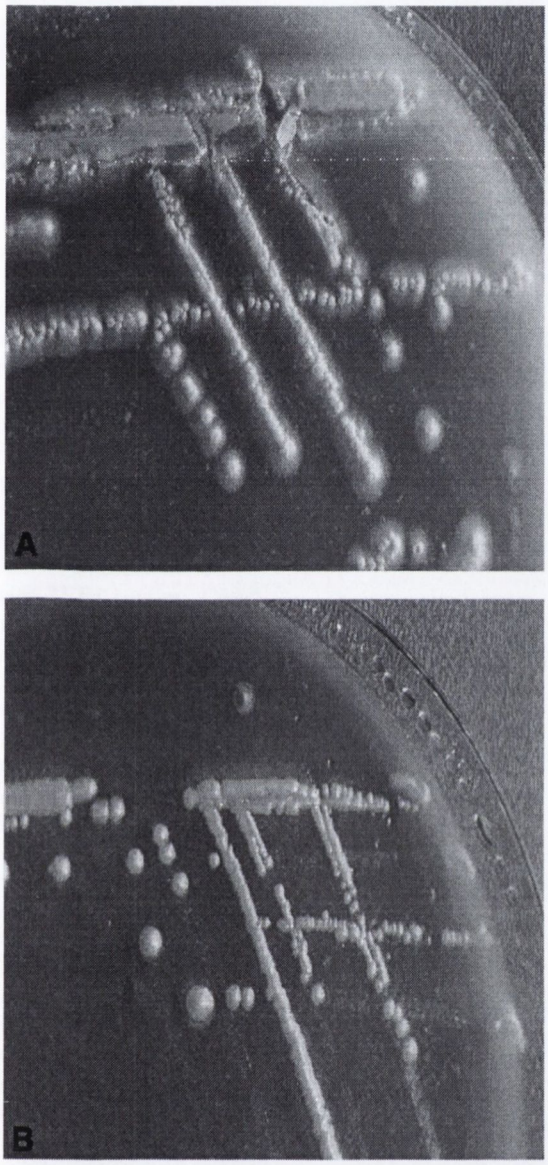

Fig. 1. Photograph of colonies of *C. dubliniensis* (A) and *C. albicans* (B) grown on Pal's agar at 30°C for 48–72 h. The *C. dubliniensis* colonies are rough and are surrounded by a hyphal fringe, whereas the *C. albicans* colonies are smooth and have no hyphal fringe.

lower level than *C. albicans*, for a long time and has very likely been misidentified as *C. albicans*. However, the HIV pandemic and antifungal therapy appears to have led to its selection and ultimately to its identification as a novel species in 1995.

3. Antifungal drug resistance in *C. dubliniensis*

As *C. dubliniensis* was initially isolated from the oral cavities of HIV-infected patients with recurrent oral candidosis, many of whom had previously received azole antifungal drug therapy, it was originally suggested that the apparent emergence of *C. dubliniensis* during the early 1990s may have been due to positive selection as a result of the introduction of novel therapeutic strategies, in a manner similar to that suggested for the emergence of *C. glabrata*. However, this hypothesis may not be correct as several studies have since shown that the great majority of *C. dubliniensis* isolates are inherently susceptible to azole, polyene and echinocandin antifungals [37,39–42]. Despite this, it is worth noting that one study has reported that the geometric mean minimal inhibitory concentration (MIC) for fluconazole in a group of *C. dubliniensis* isolates was significantly higher than that of a matched cohort of *C. albicans* isolates [37].

Resistance to fluconazole in *C. dubliniensis* was first described by Moran et al. in a group of isolates recovered from HIV-infected and AIDS patients [40]. In addition, this study showed that fluconazole-resistant derivatives could be generated from susceptible isolates following exposure to fluconazole in vitro, indicating that this species can rapidly develop resistance. Resistance to fluconazole in clinical isolates of *C. dubliniensis* has been reported by others, and as with *C. albicans*, resistance is primarily associated with isolates recovered from HIV-infected patients [39,42,43]. Exposure of *C. dubliniensis* isolates to fluconazole in vitro not only results in the selection of derivatives with reduced susceptibility, but also increases the adherence of *C. dubliniensis* to epithelial cells and results in increased levels of proteinase secretion [44]. Thus, it is possible that fluconazole therapy could provide a selective pressure that favours the growth of *C. dubliniensis* over *C. albicans* under some conditions in the oral cavity. In one longitudinal study, Martinez et al. [39] described the replacement of *C. albicans* with *C. dubliniensis* in HIV-infected patients receiving fluconazole therapy. All 42 patients included in the study harboured *C. albicans* at the outset, 12 of whom went on to develop infections with fluconazole-resistant *C. albicans*. However, by the end of the study, in another eight patients *C. albicans* was replaced by *C. dubliniensis* in the oral cavity. Surprisingly, in only two of these eight cases were the *C. dubliniensis* isolates resistant to fluconazole in vitro. These results suggest that factors in addition to antifungal drug resistance might play a role in the positive selection of *C. dubliniensis* in the oral cavities of HIV-infected individuals. Clearly, the effects of fluconazole on oral *Candida* population dynamics are complex and further epidemiological analysis and in vitro studies on the potential virulence-modulating effects of fluconazole are required before definitive conclusions can be reached.

The molecular mechanisms of azole resistance in *C. dubliniensis* have been investigated in a number of studies [5,6,40,42,43]. Homologues of the genes encoding *C. albicans* drug efflux pumps, *CDR1* and *MDR1*, have been described in *C. dubliniensis* (termed *CdCDR1* and

CdMDR1, respectively) and have been implicated in the development of resistance to azole drugs. Moran et al. [5] analysed the resistance mechanisms in a group of fluconazole-resistant isolates of *C. dubliniensis* and in vitro generated fluconazole-resistant derivatives and found that in each case, fluconazole resistance was associated with up-regulation of *CdMDR1*. Similarly, Perea et al. [42] described increased *CdMDR1* expression in fluconazole-resistant clinical isolates of *C. dubliniensis* recovered from two separate AIDS patients. The importance of *CdMDR1* in the development of fluconazole resistance in clinical isolates of *C. dubliniensis* was confirmed by gene deletion studies in the fluconazole-resistant strain CM2, which was rendered fluconazole-susceptible following deletion of both alleles of *CdMDR1* [45].

In contrast to *C. dubliniensis*, where *CdMDR1* is the principle fluconazole efflux mechanism identified so far, in most isolates of *C. albicans* increased expression of *CaCDR1* is more commonly identified as the main mediator of fluconazole resistance. Unlike the fluconazole-specific Mdr1 pumps, Cdr1 pumps can transport a broad range of azole drugs, including ketoconazole and itraconazole. Cross-resistance to these azoles is relatively rare in fluconazole-resistant *C. dubliniensis* isolates, and this may reflect the high prevalence of *MDR1*-mediated drug resistance in *C. dubliniensis*. The reason(s) for the apparent reduced contribution of *CdCDR1* overexpression to fluconazole resistance in *C. dubliniensis* is not clear. However, Moran et al. [6] recently provided a likely explanation for the lack of *CdCDR1* expression seen in many fluconazole-resistant isolates. Approximately 58% of genotype 1 *C. dubliniensis* isolates (genotype 1 is the predominant genotype recovered from HIV-infected patients) were found to harbour a nonsense mutation in the *CdCDR1* gene, resulting in the translation of a truncated, non-functional protein. But fluconazole-resistant isolates with functional *CdCDR1* alleles may still exhibit increased *CdCDR1* mRNA expression in conjunction with *CdMDR1*. Deletion of both *CdCDR1* copies in an isolate coexpressing both pumps resulted in increased susceptibility to ketoconazole and itraconazole. However, no effect on fluconazole MIC was observed. This was most likely due to the high levels of *CdMDR1* still expressed in this mutant [6].

Point mutations in the *ERG11* gene which result in specific amino acid substitutions in the enzyme 14α-lanosterol demethylase have also been shown to be important in the development of fluconazole resistance in *C. albicans*. To date, only one study, by Perea et al. [42], has described mutations in the *C. dubliniensis CdERG11* gene associated with fluconazole resistance. Two of the mutations described are identical to mutations previously shown to be involved in fluconazole resistance in *C. albicans*, namely G307A and G464S. It has yet to be experimentally verified whether the remaining *CdERG11* mutations described affect fluconazole susceptibility. These mutations occurred in isolates which also exhibited increased drug efflux pump expression, indicating that fluconazole resistance in *C. dubliniensis*, as in *C. albicans*, is multifactorial.

Resistance to itraconazole has not yet been described in clinical isolates of *C. dubliniensis*, but itraconazole-resistant derivatives (that are also cross-resistant to other azoles) can be generated in vitro following serial subculture of susceptible isolates on agar medium containing increasing concentrations of drug [7]. Itraconazole-resistant derivatives were found to have altered membrane permeabilities compared to susceptible isolates. Analysis of their membrane sterol contents revealed profiles lacking ergosterol which is consistent with a mutation in the sterol C5,6-desaturase enzyme encoded by *CdERG3*. Although increased expression of *CdCDR1* and *CdERG11* was also noted in these derivatives, loss-of-function mutations in *CdERG3* were found to be responsible for the high levels of azole cross-resistance observed. Mutations in the *ERG3* gene have also been associated with azole resistance in *C. albicans* [46], however, the contribution of this resistance mechanism to azole drug resistance in *Candida* species has not been fully investigated.

In summary, as in *C. albicans*, the majority of *C. dubliniensis* isolates are susceptible to a wide range of antifungal agents, thus it seems unlikely that the emergence of *C. dubliniensis* in HIV-infected patients has been due to selection by antifungal therapy. When resistance does emerge the molecular mechanisms are broadly similar in the two species. However, there appear to be differences in the relative roles of the efflux proteins Cdr1p and Mdr1p, probably due to the fact that in a large number of *C. dubliniensis* isolates the *CdCDR1* gene is defective.

4. Virulence of *C. dubliniensis*

The epidemiological data on the prevalence of *C. dubliniensis* described above clearly show that this species is only relatively rarely encountered in the normal oral flora of immunocompetent individuals, but is apparently enriched selectively in the oral cavities of immunocompromised patients, particularly HIV-infected and AIDS patients who have received antifungal therapy with fluconazole. It is also clear that while *C. dubliniensis* may in some cases cause systemic infections, it does so far less frequently than *C. albicans*. Confirmation of this apparent reduced virulence of *C. dubliniensis* has been obtained in comparative studies using a mouse model of systemic infection [3,47]. Therefore, despite the significant phenotypic and genotypic similarities shared between *C. albicans* and *C. dubliniensis*, current evidence from epidemiological and virulence studies indicates that *C. albicans* is better adapted to colonise and cause disease in vivo. This begs the question, what differences are there between the two species that render *C. albicans* more pathogenic?

Candida infections involve a very complex interaction between a wide range of host factors and yeast virulence

determinants that may be differentially expressed depending on the prevailing environmental conditions. The identification and determination of the contribution of specific virulence factors to various *C. albicans* infections is currently a topic of major interest to medical mycologists. One of the most important virulence factors of *C. albicans* is its ability to adhere to certain human tissues. However, given the range of host tissues which *Candida* species can colonise and infect this suggests that these organisms possess a large number of surface adhesins, very few of which have actually been characterised. There have been several studies to date which have compared the adherence of *C. albicans* and *C. dubliniensis* to a range of human cells. However, it is difficult to meaningfully compare the results of these studies as different cells and culture conditions were used in each case. Depending on the conditions used, *C. dubliniensis* was sometimes found to be more adherent than *C. albicans* [3,48,49], while using different assay conditions the opposite was also found [3,44]. Interestingly, in one study, inclusion of fluconazole in the assay medium led to an increase in adherence of *C. dubliniensis* to Vero cells, suggesting that this might contribute to the high prevalence of *C. dubliniensis* in HIV-infected and AIDS patients receiving fluconazole therapy [44]. It has also been shown that *C. dubliniensis*, unlike *C. albicans*, is hydrophobic at 37°C and that *C. dubliniensis* expresses greatly reduced levels of acid-labile mannans in cell surface *N*-glycans [49,50]. Cell surface hydrophobicity has previously been associated with enhanced adherence and resistance to phagocytosis [51], however, this hydrophobicity does not appear to confer resistance to phagocytosis on *C. dubliniensis* [52]. The only study that investigated the adherence of *C. dubliniensis* to a specific host protein compared the adherence of a range of *Candida* species to mucin, to which *C. dubliniensis* was found to adhere as strongly as *C. albicans* [53]. A gene family encoding proteins homologous to the ALS family of surface adhesion glycoproteins in *C. albicans* has also been identified in *C. dubliniensis*, but it appears that these genes are regulated differently in the two species [54].

Another trait of *C. albicans* that has been proposed as an important virulence factor is its ability to grow either in yeast or in hyphal form (i.e. dimorphism). *C. dubliniensis* is the only *Candida* species other than *C. albicans* able to produce germ tubes and true hyphae. However, the dynamics of production of hyphae in both species differ depending on the culture conditions used; in most cases induction of hyphae is more efficient in *C. albicans* (e.g. in the presence of *N*-acetyl-D-glucosamine [3]), but conversely, under certain circumstances *C. dubliniensis* produces hyphae, while *C. albicans* does not (e.g. on Pal's agar [11]) (Fig. 1). There also appears to be a disparity in the production of hyphae in vivo, as histopathological analysis of infected kidneys in a systemic mouse infection model showed that the *C. dubliniensis* cells were predominantly in the yeast phase, while the *C. albicans* strains produced far higher levels of hyphae and pseudohyphae [47]. These data suggest that there are clearly differences in the regulation and dynamics of induction of hyphae in the two species. Phenotypic switching is another important *C. albicans* virulence factor, however, it has been reported that *C. dubliniensis* can undergo phenotypic switching more frequently than *C. albicans* [55]. The production of a range of extracellular hydrolases, such as the secretory aspartyl proteinases (Saps), has been implicated in the pathogenicity of *C. albicans*. Southern hybridisation analysis has shown that *C. dubliniensis* encodes a similar range of genes as the *C. albicans SAP* family. However, phenotypic studies designed to determine the levels of proteinase produced by *C. dubliniensis* have yielded contradictory results [44,48, 55]. Interestingly, analysis of phospholipase production in the two species suggests that *C. dubliniensis* may produce lower levels than *C. albicans* [55,56].

While *C. dubliniensis* grows well in vitro at 37°C and produces biofilm under specific conditions [57], it has been reported that its growth rate is less than that of *C. albicans* [47,58], and that in mixed cultures, *C. albicans* out-grows *C. dubliniensis* [58]. This suggests that *C. albicans* has a competitive growth advantage under the conditions tested. The different growth characteristics of the two species are even more pronounced under conditions of environmental stress and it has been shown that *C. dubliniensis* is significantly less tolerant of elevated temperature (e.g. >42°C [59]), osmotic pressure (e.g. 6.5% (w/v) NaCl [60]) and oxidative stress (e.g. 10 mM H_2O_2 [47]).

The comparative virulence of *C. dubliniensis* and *C. albicans* is clearly a very complex topic. While some differences in virulence factors have been identified there are also numerous contradictory data. A full understanding of the reasons for the greater capacity of *C. albicans* to cause infection will require a concerted effort to compare the genomes and phenotypes of the two species. In particular, there is an urgent need to compare the effects of a greater number of strains belonging to each of the two species in a wider range of animal models of infection. The results of these studies should provide valuable information concerning the molecular mechanisms of how *Candida* species cause disease.

5. Conclusions

C. dubliniensis is now firmly recognised as a significant human pathogen. Since the introduction of highly active anti-retroviral therapy (HAART) the incidence of oral candidosis in HIV-infected and AIDS patients has decreased dramatically [61]. Consequently, the incidence of *C. dubliniensis* in OPC has decreased since it first emerged in the early 1990s. However, the prevalence of this species should continue to be monitored in case of changes in the epidemiology of AIDS (e.g. due to emerging resistance to HAART or to lack of compliance) and due to the intro-

duction of novel antifungal agents such as new azole derivatives and the echinocandins. In addition, comparative genomic analyses using microarrays and genome sequence data will aid the identification of genetic differences between the two species and contribute to the elucidation of the reasons for the differences in the capacity of these species to colonise and infect humans, thus improving our understanding of candidal pathogenicity.

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