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Comparative Analysis of the filamentous growth regulators *EED1* in *Candida albicans* and *MDP1* in *Candida dubliniensis*

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

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Feburary 2009

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Declaration

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Maireann an cairdeas eadrainn

Summary

Candida albicans, the major fungal pathogen in humans is closely related to *Candida dubliniensis*. *C. dubliniensis* is less virulent than *C. albicans* in all models of infection. Comparative analysis of the *C. albicans* and *C. dubliniensis* genomes identified many ORFs whose nucleotide sequence has significantly diverged. One such *C. dubliniensis* ORF is syntenic with *C. albicans* IPF946/orf19.7561. This *C. albicans* gene had been identified previously as an essential regulator of filamentation (*EED1*). Following extensive database searches and protein alignments, a syntenic *C. dubliniensis* gene (Cd36_34980) was identified and also a syntenic *EED1* gene was identified in other yeast species. The proteins encoded by both the *C. albicans* and *C. dubliniensis* genes are similar in length, however they only share 22% identity. Pfam searches of these genes identified a Myb-like DNA binding domain in all these proteins with the exception of EED1p. Following the identification of this Myb-like DNA binding protein in *C. dubliniensis*, it was decided to refer to Cd36_34980 as *MDP1* (Myb domain protein).

Phenotypic analysis of both *EED1* and *MDP1* following gene disruption studies revealed that both mutants did not form hypha in 10 % serum. This demonstrates that both *EED1* and *MDP1* play a role in regulating filamentation. Preliminary quantitative real-time data analysis revealed a possible role for *C. albicans EED1* downstream of both *EFG1* and *CPH1*. Real-time analysis of *MDP1* revealed that *MDP1* is expressed at very low levels when compared to *C. albicans*. However overexpression of *MDP1* using the Tet-inducible gene expression system did result in increased filamentation when compared to non-induced cells following incubation at 10 % serum. However overexpression of *MDP1* did not increase filamentation under conditions that do not normally result in hypha formation suggesting that under these conditions other regulatory pathways are repressing filamentation.

The virulence models of infection demonstrated that *EED1* is required for escape and dissemination in the RHE model as reported in previous studies. In this study we also showed that *EED1* is required for escape in the macrophage model of infection. In *C. dubliniensis* both the wild-type and mutant exhibited attenuated virulence on the RHE, both grew exclusively in the yeast phase and only a small number of cells adhered to the epithelium. In the macrophage model of infection the

Δ*mdp1*/Δ*mdp1* mutant had a poorer survival rate when compared to the wild-type. The poor survival of the Δ*mdp1*/Δ*mdp1* mutant demonstrates that while *C. dubliniensis* is less filamentous than *C. albicans*, *MDP1* is still required for filamentation to occur.

In *C. albicans* gene expression is known to be regulated in response to pH. In the current study the role of *MDP1* in the pH response was investigated in *C. dubliniensis* using Lee's medium (a known inducer of the yeast to hypha transition). This medium was used to investigate the effect of a pH shift from pH 4.5 to pH 7.5 in both *C. albicans* and *C. dubliniensis*. The pH shift increased hypha formation in the *C. albicans* wild-type, however the pH shift only partially restored the ability of the Δ*eed1*/Δ*eed1* mutant to produce hypha. In *C. dubliniensis* hypha formation in both the wild-type and mutant increased to levels similar to a *C. albicans* wild-type. This result would indicate that the increased filamentation observed in *C. dubliniensis* following a pH shift is occurring independently of *EED1*. We hypothesized that the increase in filamentation observed following a pH shift in the *C. dubliniensis* wild-type and mutant could be a direct result of changes in *NRG1* expression. This hypothesis was investigated by examining the expression of *NRG1* in *C. dubliniensis* following a pH shift. Real-time analysis revealed that expression of *NRG1* was 4-fold lower following preculture in Lee's medium pH 4.5 when compared to preculture in Lee's medium pH 6.5. Thus the low levels of expression of *NRG1* in *C. dubliniensis* could be responsible for the increased filamentation observed in the *C. dubliniensis* wild-type and mutant. The pH shift also resulted in increased adherence in the RHE model of infection in both the *C. dubliniensis* wild-type and mutant. Complementation of Δ*mdp1*/Δ*mdp1* and the Δ*eed1*/Δ*eed1* mutants was attempted by cloning of the respective genes, however these methods proved unsuccessful. However a distinct function for *MDP1* cannot be determined until complementation of both *C. dubliniensis* and *C. albicans* has been completed.

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Abbreviations

A_{595}, A_{600}	absorption at 600_{nm}
ACT	Artemis comparison tool
ACT1	Encoding beta-actin
AIDS	Acquired Immunodeficiency Syndrome
ATCC	American type tissue culture collection
ATP	adenosine 5'-triphosphate
BLAST	Basic local alignment tool
bp	base pairs
Bq	becquerel
BSA	bovine serum albumin
c.f.u	colony forming units
CGD	*Candida* genome database
CGH	Comparative genomic hybridisation
CGOB	*Candida* gene order browser
Ci	curie
cm	centimetres
CO_2	Carbon dioxide
C_t	Cycle threshold
DEPC	diethylenePyrocarbonate
DMEM	Dulbeccos modified eagles medium
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	dideoxynucleoside
Dox	Doxycycline
dTT	dithiothreitol
e.g.	for example
EDT1	*EFG*-dependent transcript
EDTA	ethylenediamine tetraacetic acid
EED1	Epithelial escape and dissemination
ESCRT	Endosomal sorting complexes required for transport
et al.	and others
EtOH	ethanol
g	gram
g	gravity
h	hour(s)
HIV	human immunodeficiency virus

HWP	Hyphal wall protein
i.e.	that is
INT	Tetrazolium salt
IPTG	isopropyl-β-D-thiogalactopyransoide
kb	kilobase pairs
kDa	kildalton
kg	kilogram
l	litre
LA	Luria-Bertani agar
LDH	Lactate dehydrogenase
log	logarithm (common)
M	molar
Mb	megabase
MDP	Myb-domain protein
mg	milligram
MIC	Mean inhibitory concentration
min	minute
ml	millilitre
MOIs	Multiplicities of infection
mol wt	molecular weight
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
ng	nanograms
nm	nanometer
no.	number
OD	optical density
OPC	oropharyngeal candidiasis
ORF	open reading frame
PAS	Periodic Acid Schiff
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDA	Potatoe Dextrose Agar
PMA	Plasma membrane ATPase pump
mRNA	messenger ribonucleic acid
r.p.m.	revolutions per minute
RAT	Rice agar tween
RFLP	restriction fragment length polymorphism

RHE	Reconstituted human oral epithelial
RNA	ribonucleic acid
RNase	ribonuclease
s	seconds
SD	Synthetic minimal medium
SDS	sodium dodecyl sulphate
SLD	Synthetic low dextrose
SNPs	Single nucleotide polymorphisms
SSC	Salt sodium citrate
T	Thymidine
TBE	Tris-borate EDTA
TE	Tris-EDTA
TEF	Transcript elongation factor
Tet	Tetracycline
tetO	*tet* operator
TetR	Tetracycline repressor protein
Tris	tris (hydroxymethyl) aminoethane
UK	United Kingdom
USA	United States of America
UV	ultraviolet
µg	microgram
µl	microlitre
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 grams of solution or mixture
X-gal	5-bromo-4-chloro-indoyl-β-D-galactoside
XTT	2,3-bis[2-Methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide inner salt
YNB	Yeast nitrogen base
YPD	Yeast extract peptone dextrose
YPM	Yeast peptone maltose
YPS	Yeast extract peptone sucrose

~	approximately
>	greater than
<	less than

Chapter 1

General Introduction

1.1 *Candida* species and Candidosis

The overall incidence of fungal infections has increased dramatically over the last 20 years, as have the variety of infecting fungal agents. Infections due to *Candida* species accounts for approximately 49 % of all major systemic fungal infections (2008). This dramatic increase coincides with the increase in the number of individuals with deficient cell-mediated immunity, in particular those infected with human immunodeficiency virus (HIV) and those receiving cytotoxic therapy with prolonged neutropenia, immunosuppressive therapies, use of broad spectrum antibiotics, indwelling invasive devices and intensive care support (Yang & Lo, 2001)

Candida species can cause infections in a wide range of human tissues and organs including the skin, oral cavity, oesophagus, gastrointestinal tract, vagina and the vascular system. Infections of the skin and mucous membranes may occur in either immunocompromised or healthy individuals, this is in contrast to systemic candidosis which is only seen in severely immunocompromised patients. The superficial forms of infection, referred to as oropharyngeal candidiasis (OPC) is the most frequent form encountered in HIV-infected individuals, however oral *Candida* infections can occur in neonates, the elderly, diabetics and individuals using topical steroids (Challacombe, 1994). Angular cheilitis, an inflammatory lesion at the angle of the mouth and denture stomatitis are also associated with *Candida* species. *Candida* species are also a common cause of vaginal infection, which presents as vulvovaginal candidiasis (VVC) in apparently normal healthy women. However, life threatening invasive candidiasis may also occur when *Candida* species enter the blood, causing bloodstream infections which then spread throughout the body to infect a wide range of organs including the liver, kidney, spleen, and heart. *Candida* species are the fourth most commonly isolated organism in bloodstream infections and *Candida* accounts for approximately 15 % all nosocomial infections (Eggimann *et al.*, 2003). *Candida* cells colonise the gastrointestinal tract mucosal surfaces of many individuals and this is believed to be the main portal of entry, however direct inoculation into the blood can also occur. It has been shown that *Candida* bloodstream infections have an attributable mortality rate of 40 % (Odds, 1988; Pfaller *et al.*, 1998). *Candida albicans* is generally acknowledged to be the most pathogenic of the *Candida* species and the species most frequently associated invasive and disseminated candidiasis, however the incidence of infection caused by non-*Candida albicans* species including *Candida krusei*, *Candida tropicalis*

and *Candida glabrata* has increased dramatically during the last decade (Moran *et al.*, 1998). 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 compared to *C. albicans* (Moran *et al.*, 1997; Ponton *et al.*, 2000; Sullivan *et al.*, 2004)

1.2 Identification and characterisation of *Candida dubliniensis*

1.2.1 Emergence of *Candida dubliniensis*

The identification and classification of fungal species has depended on the analysis of a limited number of morphological and physiological traits, such as the ability to produce true hyphae, chlamydospores and by their nitrogen and carbohydrate assimilation patterns (Sullivan *et al.*, 1996). This method of identification has been unreliable due to the variability of phenotypic properties between isolates of the same species. Therefore coinciding with the dramatic increase in fungal opportunistic infections in the immunocompromised patient came reports of the isolation of unusual *Candida* species that were referred to as "atypical *C. albicans*"

In the early 1990's a number of authors reported the recovery of unusual or atypical isolates of *C. albicans* from HIV-infected individuals in Australia, Ireland, Switzerland and the UK (Schmid *et al.*, 1992; Sullivan *et al.*, 1993). Schmid *et al.* (1992) identified two isolates that yielded different DNA fingerprinting patterns, placing them outside the *C. albicans* species group, using the *C. albicans* specific probe 27A and Ca3. Sullivan *et al.* (1993) also used the Ca3 and 27A DNA fingerprinting probe on genomic DNA from samples of non-*C. albicans Candida* species which were recovered from cases of recurrent oral candidosis in HIV-positive and AIDS patients. This study found that the atypical *Candida* isolates all produced germ tubes and chlamydospores. The study concluded with the possibility that these atypical isolates were either an unusual type of *C. albicans* or perhaps an undescribed species of *Candida*. A study by McCullough *et al.* (1995) also reported *Candida* strains which showed many phenotypical traits of *C. albicans* but were genetically different. Results showed that strains from the study were similar to those isolated by Sullivan *et al.* (1993). A further study by Boerlin *et al.* (1995), found several atypical *Candida* strains which were isolated from the oropharynxes of asymptomatic HIV-positive drug users.

These strains showed abnormal sugar assimilation patterns. Subsequently, Sullivan *et al.* (1995) carried out an extensive study of the phenotypic and genotypic characteristics of atypical isolates recovered from HIV-infected patients in Ireland and Australia. Additionally Sullivan *et al.* carried out phylogenic analysis of the V3 region of the large rRNA subunit, on the basis of this and the phenotypic and genotypic analysis, Sullivan *et al.* concluded that these atypical isolates constitute a novel, distinct taxon within the genus of *Candida.* This novel species of *Candida* was termed *Candida dubliniensis.*

1.2.2 Phenotypic characteristics of *Candida dubliniensis*

The ability to produce germ tubes and chlamydospores was originally used to identify *C. albicans* strains. However, *C. dubliniensis* also possess the ability to produce chlamydospores on Rice Agar Tween (RAT) and cornmeal agar. *C. dubliniensis* chlamydospores were found attached to short pseudohyphae and were present in an abundance. This is in contrast to *C. albicans* strains which produced very few chlamydospores and the chlamydospores were found permanently attached to the pseudohyphae (Sullivan *et al.*, 1995). However this feature in not true of every *C. dubliniensis* isolate (Kirkpatrick *et al.*, 1998). However in contrast to *C. albicans* a large majority of *C. dubliniensis* isolates produced chlamydospores on Staib agar (Al Mosaid *et al.*, 2001; Staib & Morschhauser, 1999). *C. dubliniensis* also produced germ tubes when incubated in serum (Sullivan *et al.*, 1995). However *C. dubliniensis* produced hyphae less rapidly than *C. albicans*. In media containing N-acetylglucosamine, *C. dubliniensis* isolates failed to produce hyphae.

Isolates of *C. dubliniensis* grow well at 30°C and 37°C on culture media routinely used to grow *C. albicans*. However *C. dubliniensis* isolates grew poorly or not at all at 42 °C, this is a feature shared with the type I *C. stellatoidea* (Sullivan *et al.*, 1995). At 37°C *in vitro C. dubliniensis* grows well following growth in rich culture conditions, however it has been shown that the growth rate is less of *C. albicans*. Also in mixed cultures *C. albicans* outgrows *C. dubliniensis* (Kirkpatrick *et al.*, 2000; Vilela *et al.*, 2002). Similarly under conditions of environmental stress, elevated temperature (> 42°C), osmotic stress (> 0.6 M NaCl), or oxidative stress (10 mM H_2O_2), it has been shown that the growth rate of *C. dubliniensis* is significantly less than that of *C. albicans* (Alves *et al.*, 2002; Pinjon *et al.*, 1998; Vilela *et al.*, 2002).

The ability of individual *Candida* isolates to assimilate a range of carbohydrate compounds as the sole source of carbon or nitrogen has been used extensively for species identification. Commercially available yeast identification systems such as the bioMériux API ID 32C and API 20C Aux systems are routinely used. The range of carbohydrates assimilated by *C. albicans* and *C. dubliniensis* has been shown to be significantly different. From these studies it has been demonstrated that *C. dubliniensis* isolates are unable to assimilate methyl-αD-glucoside, lactate or xylose (Boerlin *et al.*, 1995; Kirkpatrick *et al.*, 1998; Pincus *et al.*, 1999; Salkin *et al.*, 1998; Sullivan *et al.*, 1995).

A number of differential media used to distinguish between *C. dubliniensis* from *C. albicans* has been described, however many of these are described as being unreliable. The commercially available CHROMagar® Candida medium can be used for the preliminary identification of *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.*, 1997; Schoofs *et al.*, 1997; Sullivan & Coleman, 1997). However, *C. dubliniensis* isolates can lose their distinctive dark green colouration following prolonged storage or subculture (Schoofs *et al.*, 1997). Studies have also shown that *C. dubliniensis* isolates form rough colonies when incubated on Staib agar at 30°C, whereas *C. albicans* do not (Al Mosaid *et al.*, 2001; Staib & Morschhauser, 1999). A study by Al Mosaid *et al.* (2001) found that 14.6 % of *C. dubliniensis* isolates did not produce hyphae and Chlamydospores on Staib medium and concluded that differentiation between *C. dubliniensis* and *C. albicans* on Staib agar should be based on colony morphology rather than hyphae and chlamydospores production.

Many of the phenotypic properties described have been used for the identification of *C. dubliniensis* from *C. albicans*, however the majority of these properties are not definitive for *C. dubliniensis* and therefore in order to achieve absolute identification of *C. dubliniensis* molecular methods based on genotypic characteristics should be utilised. PCR based techniques have been developed to facilitate the identification of *C. dubliniensis*. Donnelly *et al.* (1999) 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).

1.3 Epidemiology of *Candida dubliniensis*

1.3.1 The epidemiology of *C. dubliniensis*

Candida dubliniensis was first identified in 1995 from oral samples from HIV-infected individuals and AIDS patients with recurrent oral candidosis (Sullivan *et al.*, 1995). Since its identification *C. dubliniensis* has been isolated from many geographical locations around the world and has been found in a wide range of anatomical sites and clinical samples (Al Mosaid *et al.*, 2001; Kirkpatrick *et al.*, 1998; Odds *et al.*, 1998; Pinjon *et al.*, 1998; Ponton *et al.*, 2000; Pujol *et al.*, 2004; Schoofs *et al.*, 1997; Sullivan *et al.*, 1997; Sullivan *et al.*, 2004; Willis *et al.*, 2000). The vast majority of *C. dubliniensis* isolates have been recovered from the oral cultures of HIV-infected individuals and patients with AIDS. *C. dubliniensis* is commonly found with *C. albicans* and other *Candida* species (Coleman *et al.*, 1997; Sullivan *et al.*, 1997). However *C. dubliniensis* has also been implicated as an agent of oral candidosis in HIV-negative individuals, including healthy individuals and diabetics. A small number of *C. dubliniensis* isolates have also been recovered from the vaginal tracts of HIV-negative women with vaginitis. *C. dubliniensis* has also been recovered in other specimens such as, faeces, sputum, urine, wounds and the respiratory tract (Odds, 1988; Polacheck *et al.*, 2000)

In a study of the Irish population of normal healthy individuals, only 3.5 % were found to carry *C. dubliniensis* in the oral cavity and the occurrence in the vagina was even lower (Ponton *et al.*, 2000). A study by Sullivan *et al.* (2004) showed that in an Irish cohort, 26 % of the 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 (Table 1.1) (Ponton *et al.*, 2000). Other studies, predominantly from the USA, found the prevalence of oral *C. dubliniensis* in HIV-infected individuals to be between 11 % and 25 % (Brown *et al.*, 2000; Jabra-Rizk *et al.*, 2001; Kirkpatrick *et al.*, 1998; Meiller *et al.*, 1999). Interestingly, *C. dubliniensis* has been isolated from the oral cavities of patients with denture stomatitis, diabetes and cycstic fibrosis (Table 1.1) (Peltroche-Llacsahuanga *et al.*, 2002; Ponton *et al.*, 2000;

Table 1.1 Epidemiology of *C. dubliniensis* from different subject cohorts in Ireland[a]

Group	Number of subject's[b]	% of subjects yielding *C. dubliniensis* and other *Candida* spp.	% of subjects yielding *C. dubliniensis* only
HIV	185 [+]	26	6
	216 [-]	18	3
AIDS	82 [+]	32	10
	36 [-]	25	8
Denture Stomatitis	72 [+]	14	4
Diabetic	318 [-/+]	18	4
Normal Healthy	202 [-]	3.5	0.5
Vaginitis	110 [+]	3	1

[a] Modified from Ponton *et al.* (2000)
[b] [+] patient presented with clinical symptoms of oral candidosis, [-] patient presented without clinical symptoms of oral candidosis.

Willis *et al.*, 2000). In contrast, other investigators have found significantly lower levels of *C. dubliniensis* in the oral cavities of HIV-positive patients (Vargas & Joly, 2002). However as Sullivan *et al.* (2004) reported the sampling and identification methods used, geographical location, and the cohort of HIV-infected individuals examined, could be a contributing factor to the differences in the epidemiological data.

There have been few reports of systemic infection caused by *C. dubliniensis* (Meis *et al.*, 1999). The most comprehensive review of the incidence of *C. dubliniensis* in systemic bloodstream infections were carried out by Kibbler *et al.* (2003). This study examined the incidence of bloodstream infections due to *Candida* species in England and Wales over a twentyeight month period. This study revealed that *C. albicans* was responsible for 64.7 % of the cases studied with *C. dubliniensis* being responsible for only 2.2 % of cases studies (Table 1.2)

A study by Nunn *et al.* (2007) obtained fungal strains from *Ixodes uriae* ticks at a seabird breeding colony on Great Saltee Island, in Ireland. The ticks were taken from cracks in the cliffs used by common guillemots (*Uria aalge*). Tissue cultures of tick homogenates were occasionally contaminated with yeast-like fungi. Twenty-two isolates were obtained, all the isolated were germ-tube positive and produced chlamydospores at 37°C on Corn Meal Tween 80 agar. In addition none of the isolates grew at 43 °C, which gave an indication that these isolates may be *C. dubliniensis*. This theory was confirmed by sequencing the 5.8S RNA gene which definitively identified the isolates as *C. dubliniensis*. In addition as Nunn *et al.* (2007) determined that *C. dubliniensis* was found predominantly adhering to the surface of the tick, they concluded that the most likely source of the fungal isolates was from bird excrement, thus providing evidence that *C. dubliniensis* is not confined to humans and that *C. dubliniensis* may inhabit the digestive tract of marine birds (Nunn *et al.*, 2007).

1.3.2 Antifungal drug resistance in *Candida dubliniensis*

An increase in the number and spectrum of fungal infections, boosted by the AIDS pandemic and advances in anticancer and transplantation medicine has attracted a new interest into the development of new compounds with antifungal activity. As *C. dubliniensis* was first isolated from HIV-infected individuals with recurrent oral candidosis, many of whom who had received azole antifungal therapy it was was initially thought the emergence of *C. dubliniensis* was as a result of novel therapeutic

strategies. However, these studies have revealed that the majority of *C. dubliniensis* isolates are susceptible to commonly used antifungals drugs (Moran *et al.*, 1997; Odds *et al.*, 1998; Pfaller *et al.*, 1999; Ruhnke, 2000). However, the study by Odds *et al.* (1998) reported that the 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. Other studies have also found resistance to fluconazole in clinical isolates of *C. dubliniensis* belonging to genotype 1 (Moran *et al.*, 1997; Ruhnke, 2000). A study by Moran *et al.* (1997) showed that fluconazole resistance could be generated from susceptible isolates following exposure to fluconazole *in vitro*, demonstrating that this species can rapidly develop resistance. Exposure of *C. dubliniensis* isolates to fluconazole *in vitro* resulted in reduced susceptibility and also an increase in adherence to epithelial cells and increased levels of proteinase secretion (Borg-von Zepelin *et al.*, 2002).

The molecular mechanism of azole resistance in *C. dubliniensis* has been investigated. Homologues of these genes encoding *C. albicans* drug efflux pumps *CDR1* and *MDR1* have been described in *C. dubliniensis* and have been implicated in azole resistance. Moran *et al.* (1997) showed that fluconazole –specific resistance in azole resistant *C. dubliniensis* genotype 1 isolates and *in vitro* generated derivatives is primarily associated with overexpression of the major facilitator CdMdr1p. Although up-regulation of *CdCDR1* was observed in fluconazole resistant clinical isolates it has been shown that CdCdr1p is not essential for fluconazole resistance (Moran *et al.*, 1998). However the majority of *C. dubliniensis* isolates are susceptible to a wide range of antifungal agents and thus the theory that the emergence of *C. dubliniensis* in HIV-infected patients has been due to selection by antifungal therapy is unlikely.

1.3.3 Molecular analysis of *Candida dubliniensis*

DNA fingerprinting with repetitive elements was for many years the method of choice for the molecular epidemiological analysis of pathogenic fungi. Joly *et al.* (1999) screened for and cloned a *C. dubliniensis*-specific repetitive element Cd25. DNA fingerprint studies with this element found that *C. dubliniensis* isolates could be divided into two groups, Cd25 group I and Cd25 group II. Isolates in group I were very closely related (S_{AB} = 0.80), whereas isolates in group 2 were less closely related (S_{AB} = 0.47). A later study by Gee *et al.* (2002) further investigated the genetic diversity of *C.*

Table 1.2 *Candida* species identified in bloodstream infections from a study carried out in England and Wales.

Candida isolate	All study patients	Haematology patients	Intensive care patients	Surgical Patients
C. albicans	88 (64.7 %)	6 (37.5 %)	50 (79.4 %)	38 (69.1 %)
C. dubliniensis	3 (2.2 %)	1 (6.2 %)	2 (3.2 %)	0
C. famata	1 (0.7 %)	0	0	0
C. glabrata	22 (16.2 %)	1 (6.2 %)	4 (6.3 %)	12 (21.8 %)
C. krusei	4 (2.9 %)	4 (25 %)	0	0
C. lusitaniae	1 (0.7 %)	0	1 (1.6 %)	1 (1.8 %)
C. parapsilosis	10 (7.4 %)	2 (12.5 %)	4 (6.3 %)	4 (7.3 %)
C. tropicalis	6 (4.4 %)	2 (12.5 %)	2 (3.2 %)	0
Trichopsoriella sp.	1 (0.7 %)	0	0	0

This table is taken from **Kibbler, C. C., Seaton, S., Barnes, R. A., Gransden, W. R., Holliman, R. E., Johnson, E. M., Perry, J. D., Sullivan, D. J. & Wilson, J. A. (2003).** Management and outcome of bloodstream infections due to *Candida* species in England and Wales. *J Hosp Infect* **54**, 18-24.

dubliniensis using the Cd25 finger print probe. This study found that group I isolates are very closely related, however, they come from very different geographical locations. Also Cd25 group I isolates were largely recovered from HIV-infected patients while Cd25 group II isolates had greater diversity within the group, and the isolates were largely recovered from HIV-negative patients (Gee *et al.*, 2002).

C. dubliniensis isolates have distinct karyotype patterns consisting of nine or ten chromosome sized DNA bands detected by pulsed field gel electrophoresis (PFGE), usually with one or more bands less than 1 Mb in size, this is a feature shared by isolates of type I *C. stellatoidea* . This is in contrast to *C. albicans* isolates which yielded seven chromosome sized DNA bands (Sullivan *et al.*, 1995). In the study by Gee *et al.* (2002) karyotype analysis was also used to analyse *C. dubliniensis* isolates. This study found two major kayotype patterns based on the presence or absence of chromosome-sized bands at approximately 1.7 and 1.75 Mb (Gee *et al.*, 2002).

Gee *et al.* (2002) also carried out a study on the nucleotide sequence analysis of the internal transcribed spacer region (ITS) of the rRNA cluster and identified four distinct genotypes. Seven of the group I isolates sequenced in the study were genotype 1, six of the Cd25 group II isolates were genotype 2, five Cd25 group II were genotype 3 and one Cd25 group II isolate was genotype 4. Sequence variations belonging to the four different genotypes were distributed between the ITS1 and ITS 2 regions. This data strongly suggested that *C. dubliniensis* consists of four distinct genotypes.

A study by McManus *et al.* (2008) used multi locus sequence typing (MLST), a technique based on the nucleotide sequence analysis of a set of housekeeping genes, to investigate the epidemiology and population structure of *C. dubliniensis*. Combinations of 10 loci previously tested for MLST analysis of *C. albicans* were assessed for their discriminatory ability with 50 epidemiologically unrelated *C. dubliniensis* isolates from diverse geographical locations, including isolates from the previously identified Cd25-defined major clades and the four ITS genotypes. Dendograms created in this study revealed a population structure which supports results already identified by DNA fingerprinting and ITS genotyping. This study also revealed significantly less divergence within the *C. dubliniensis* population examined than within the *C. albicans* population (McManus *et al.*, 2008).

1.3.4 Virulence of *C. dubliniensis*

The epidemiological data available to date on the prevalence of *C. dubliniensis* demonstrates that this species is rarely found in the oral flora of healthy individuals. It has also been demonstrated that *C. dubliniensis* is capable of causing systemic infections however it does so less frequently than *C. albicans* (Gilfillan *et al.*, 1998; Kibbler *et al.*, 2003; Vilela *et al.*, 2002). One of the most important virulence factors in *C. albicans* is its ability to adhere to certain human tissues. *C. albicans* is the most adherent and pathogenic species of *Candida*. Several studies have compared the adherence of *C. albicans* and *C. dubliniensis*. A study by de Repentigny *et al.* (2000) investigated the adherence of *Candida* species to small intestinal mucin, and the results of the study showed that *C. dubliniensis* adhered as strongly as *C. albicans* with *C. parapsolosis* and *C. lusitaniae* adhering moderately to the mucin. Gilfillan *et al.* (1998) compared the adherence of four *C. dubliniensis* isolates to that of two control *C. albicans* strains to human buccal epithelial cells (BECs). Adherence of *C. dubliniensis* CD57 was similar to that of *C. albicans*. *C. dubliniensis* CD36 showed no significant difference in adherence compared to *C. albicans* when grown in galactose medium but was significantly more adherent when grown in glucose medium. Adherence of *C. dubliniensis* CD41 and CD43 showed increased adherence when grown in glucose as observed in CD36 (Gilfillan *et al.*, 1998). This increased adherence of *C. dubliniensis* isolates compared to *C. albicans* when grown in the presence of glucose could be linked to the fact that *C. dubliniensis* is found almost exclusively in the oral cavity (Coleman *et al.*, 1997). Gilfillan *et al.* (1998) also compared the virulence of *C. albicans* and *C. dubliniensis* in the mouse model of systemic candidosis and found that mice inoculated with *C. dubliniensis* had a 1.5-2 times greater survival rate compared to *C. albicans*. A study by Vilela *et al.* (2002) injected *C. dubliniensis* and *C. albicans* intravenously into separate infant mice and their survival rate was compared and showed that the mice injected with *C. dublinienisis* had a significantly higher survival rate then those infected with *C. albicans*. Further analysis of the histopathological samples revealed that the kidneys of mice infected with *C. albicans* were filled with abundant hyphae and pseudohyphae, at later periods fungal masses were also seen in the renal pelvis, these masses were surrounded by macrophages and polymorphonuclear leukocytes (PMNs). Also several days after inoculation with *C. albicans* mycelia invaded the brain without causing an inflammatory response. In the mice infected with *C. dubliniensis*, the fungal

cells were found predominantly in the yeast form. The kidneys of mice that died several days after inoculation contained both yeast and elongated cells (Vilela *et al.*, 2002). Thus as observed by both Gilfillan *et al.* and Vilela *et al.* the virulence of *C. dubliniensis* is lower than that of *C. albicans* in systemic models of infection. Stokes *et al.*, (2007) compared *C. albicans* and *C. dublinienisis* on the RHE model of infection and found that *C. dubliniensis* was poorly adherent to the epithelial tissue and grew exclusively in the yeast phase. This is in contrast to *C. albicans* which adhered strongly to the epithelium and produced abundant hyphae, which penetrated and invaded deep into the tissue (Stokes *et al.*, 2007). Stokes *et al.* also compared the gastrointestinal colonisation and dissemination of both *C. albicans* and *C. dubliniensis* in the oral-intragastric infant mouse infection model. When mice were infected with either *C. dubliniensis* or *C. albicans* or both species together the colonisation levels of each species was similar up to and including day 6. However at days 8 and 10 *C. dubliniensis* was no longer detectable, whereas the levels of *C. albicans* remained high. Also Stokes *et al.*, found that when mice were rendered immunocompromised to facilitate dissemination, *C. dubliniensis* was found to be far less able to disseminate into the kidney or liver compared to *C. albicans*. Histological analysis revealed that *C. albicans* was found in both the yeast and hyphal phase in the stomach, liver and kidney. In contrast, in the small number of mice where *C. dubliniensis* was detected, *C. dubliniensis* was only present in the yeast phase in the stomach and kidney. Although hyphal cells of *C. dubliniensis* were observed in the liver (Stokes *et al.*, 2007). The findings of all three of these studies correlate with the findings of Kibbler *et al.* (2003) in that *C. dubliniensis* is less able to penetrate into the bloodstream and cause systemic infection compared to *C. albicans*.

1.4 Comparative genomics of *Candida albicans* and *Candida dubliniensis*

Candida albicans was one of the first eukaryotic pathogens selected for genome sequencing. In October 1996 the Stanford Genome Technology centre began sequencing the SC5314 strain of *C. albicans*. This project was completed in May 2004 (www.sanger.ac.uk/projects/C_albicans). The completion of the *C. albicans* genome and its annotation has provided researchers with important tools for the analysis of this human pathogen. *C. dubliniensis* was first identified and described in 1995, and now

more then 10 years on sequencing of the genome has been completed (www.sanger.ac.uk/sequencing/Candida/dubliniensis/). The completion of this project will allow complete comparison of the two species, which will hopefully provide answers as to why *C. albicans* is more virulent than its very close relative *C. dubliniensis*.

One of the scientific programs of the Broad Instituite is the Fungal Genome Initiative (FGI) which is a partnership between the Broad Instituite and the wider fungal research community. This project produces and analyses sequence data from fungal organisms that are important to medicine, agriculture and industry. Many of the fungi sequenced comprise clusters of closely related organisms. By comparing the sequences from one genome to another, it is hoped that genomic differences can be correlated with phenotypic differences, such as pathogenicity or production of drugs. This will allow scientists to search for genes and regions that underline important behaviours. One such undertaking by the fungal genome initiative is the sequencing and annotation of five *Candida* species (*C. albicans* (Wo-1), *C. tropicalis, L. elongisporus*, *C. guilliermondii* and *C. lusitaniae*), these genomes as well as *C. albicans* SC5314 and three related species being sequenced at the Sanger Institute (*C. dubliniensis*, *C. parapsilosis* and *S. cerevisiae*) provide an excellent opportunity for the comparative analysis of genes and genomes across the *Candida* clade, as well as to the sister *Saccharomyces* clade (Fig 1.1).

The completion of the *C. albicans* genome project has allowed the gene content of different strains to be compared using DNA microarrays. Moran *et al.*, (2004) took total genomic DNA from *C. albicans* and *C. dubliniensis* and co-hybridized these DNAs to *C. albicans* DNA from both species microarray and compared the relative hybridisation efficiency of DNA to each gene specific spot. This led to the identification of thousands of *C. albicans* homologous genes in *C. dubliniensis* without the need for sequence analysis and has resulted in the identification of genes which are highly divergent or even absent in *C. dubliniensis*. Also a group of sequences were examined which were predicted to possess low nucleotide sequence homology as these genes were most likely to be functionally more different or possibly even absent in *C. dubliniensis* (Moran *et al.*, 2004). From this study a group of 19 genes were identified that possessed homology to putative transcriptional regulators (Table 1.3). One of these genes was initially termed *EDT1* (*EFG1*-dependent transcript). Chen *et al.* (2004) referred to

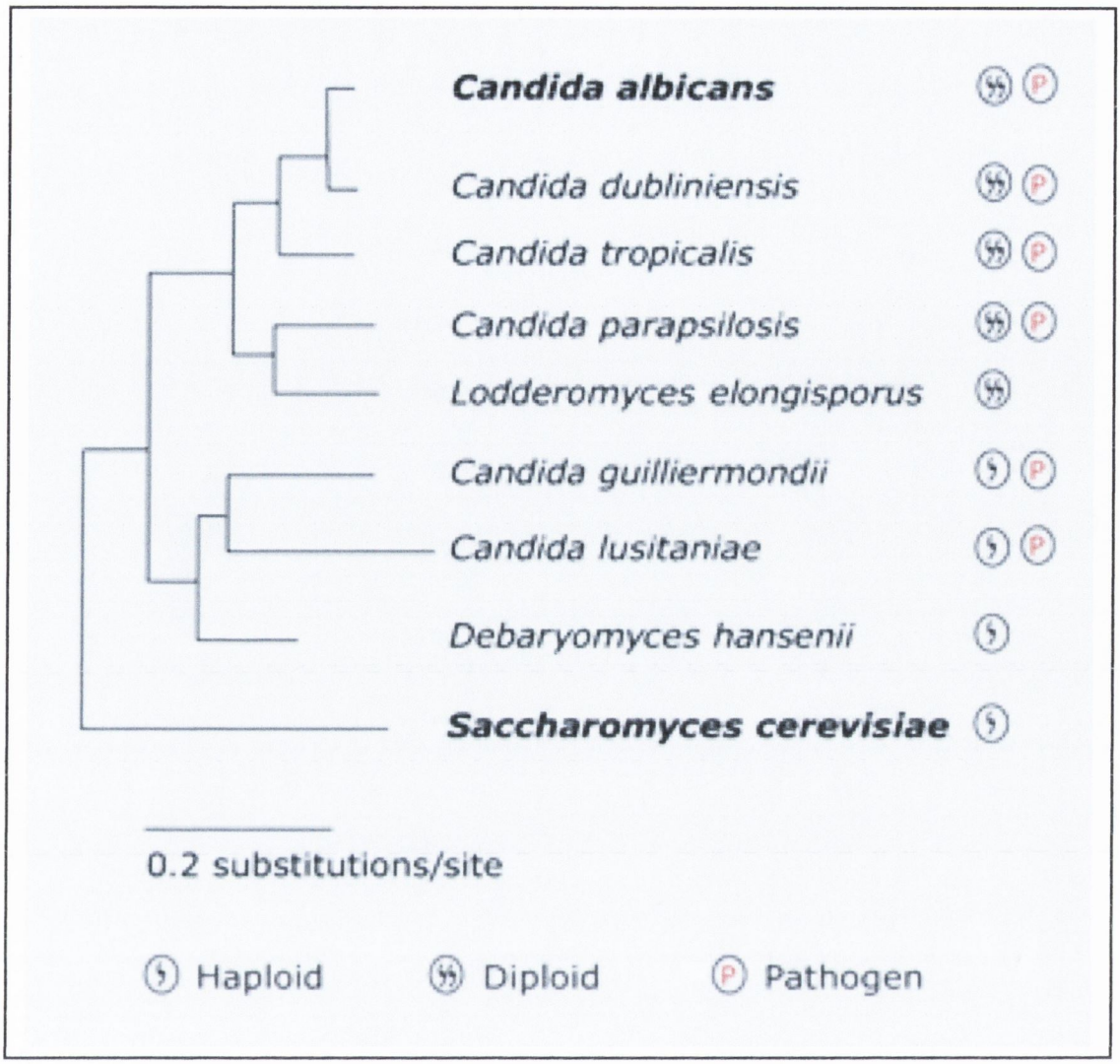

Figure 1.1. Phylogenetic tree of the *Candida* species. This diagram is taken directly from the Fungal Genome Initiative of the Broad Institute. (www.broad.mit.edu/annotation/genome/candida_albicans/MultiHome.html).

Table 1.3. Table of putative transcriptional regulators identified by comparative genomic hybridisation and *in silico* analysis. (Moran *et al.*, 2004).

Gene identifier	Assembly 19 orf	Putative or known function
Putative Transcriptional Regulators		
CTA21/ TLO3	orf19.6112	Homology to *C. albicans* CTA2
CTA22/ TLO10	orf19.3074	Homology to *C. albicans* CTA2
CTA241/ TLO11	orf19.5700	Homology to *C. albicans* CTA2
CTA24.3/ TLO12	orf19.4054	Homology to *C. albicans* CTA2
CTA25/ TLO9	orf19.362	Homology to *C. albicans* CTA2
CTA26/ TLO2	orf19.7680	Homology to *C. albicans* CTA2
CTA27/TLO34	orf19.631	Homology to *C. albicans* CTA2
CTA29/TLO16	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 ScHal9p
IPF9191.3f	orf19.3188	Zinc-finger protein, homology to ScHal9p
IPF8627	orf19.3969	Zinc-finger protein, homology to ScMGA1
IPF19540	orf19.723	Zinc-finger protein
IPF13021	orf19.2647	Zinc-finger protein, homology to ScHAP1
IPF946	**orf19.7561**	**EFG1-dependent transcript EDT1**

unpublished data which found that *CaEDT1* was identified as a key regulator of a filamentation in *C. albicans*.

Recently Zakikhany *et al.* (2007) showed using DNA microarray analysis that transcription of the *C. albicans EDT1* gene increased during infection of RHE tissues. In this study the gene was renamed *EED1*, for epithelial escape and dissemination (Section 1.6.4)

1.5 Factors contributing to morphogenesis in *Candida*

1.5.1 Morphogenesis

Several properties are known to contribute to virulence in *C. albicans*. These include adhesion to host cells, secretion of degradative enzymes and the ability to undergo a reversible morphological transition from the yeast to filamentous growth forms (Calderone & Fonzi, 2001; Lo *et al.*, 1997; Stoldt *et al.*, 1997). The filamentous forms of *C. albicans* include two distinct morphologies, pseudohyphae and hyphae. In pseudohyphae cells are attached end to end but each cell has an elliptical shape with constrictions at the septa (Fig 1.2). In true hyphae, these constrictions are absent and the row of cells show a relatively uniform width. Pseudohyphae and hyphae also differ in other ways including the precise way mitosis and cell division are carried out (Fig. 1.2) (Berman & Sudbery, 2002; Odds, 1988). The production of germ tubes results in a switch to a filamentous growth phase or hypha. The formation of pseudohyphae occurs by polarized cell division when yeast cells growing by budding have elongated without detaching from adjacent cells. Under certain unfavourable growth conditions *C. albicans* can undergo the formation of chlamydospores which are round retractile spore-like structures with a thick cell wall. These different morphological forms often represent a response of the fungus to changing environmental conditions and may permit adaptation to a different environmental niche. The *C. albicans* hyphal form is often found at sites of tissue invasion and cells which are locked in to either the yeast morphology or the filamentous form exhibit attenuated virulence (Braun *et al.*, 2000; Lo *et al.*, 1997; Sudbery, 2001). As mentioned previously the ability to form hyphae has only been observed in *C. albicans* and *C. dubliniensis*, other *Candida* species do not readily form true hyphae. Interestingly, on Pal's and Staib agar *C. dubliniensis* grows in both the pseudohypha and hypha forms whereas *C. albicans* remains exclusively in the

yeast phase (Al Mosaid *et al.*, 2001; Al Mosaid *et al.*, 2003; Staib & Morschhauser, 1999). However, in the presence of *N*-acetylglucosamine *C. albicans* is found in the hypha form whereas *C. dubliniensis* remains in the yeast phase. However, in most environments induction of hyphae is more efficient in *C. albicans* (Al Mosaid *et al.*, 2001; Gilfillan *et al.*, 1998). It is considered that hyphal cells express cell-wall proteins that facilitate adhesion to human tissues, which is important for invasion but also for escape from phagocytosis mediated by neutrophils or macrophages. In contrast, the yeast form is thought to be important for dissemination through the bloodstream (Gow *et al.*, 2002).

1.5.2 MAP kinase pathway

The ability of *C. albicans* to switch from the yeast to hypha growth phase in response to various environmental signals is an excellent example in understanding how signalling pathways coordinate growth and development. The functions of these pathways and regulators on morphogenesis have been reviewed frequently (Berman & Sudbery, 2002; Calderone & Fonzi, 2001; Ernst, 2000; Kadosh & Johnson, 2005; Stoldt *et al.*, 1997). Hypha development in *C. albicans* is controlled by a wide variety of signals. In *Saccharomyces cerevisiae*, the mitogen activated protein kinase (MAPK) pathway is involved in pseudohypha and hypha growth. *C. albicans* contains a MAP kinase pathway capable of activating the yeast to hypha transition. *C. albicans* possess a homolog of *S. cerevisiae* Ste12 (*Cph1*) and a MAPK kinase cascade that includes *Cst*20, *Hst*7, and *Cek1* which are involved in filamentation. Strains with null mutations of any of the genes in the MAP kinase pathway (*CST20*, *HST7*, or *CEK1*) have hypha defects on solid media in response to many hypha inducing conditions (Leberer *et al.*, 1996; Leberer *et al.*, 2001). The *CPH1* mutant strains showed delayed initiation of hypha growth on spider plates, compared to wild-type cells (Lo *et al.*, 1997). Thus mutations in these genes appear to inhibit the yeast to hypha transition under a specific set of growth conditions. They block hypha growth on response to nitrogen limitation on solid medium but not in response to serum, proline or *N*-acetylglucosamine (Ernst, 2000; Leberer *et al.*, 1996; Liu *et al.*, 1994). Parallel signalling pathways must be responsible for the transduction of other morphogenetic signals (Fig.1.3).

The MAPK pathway contains the MAP kinase Cek1, which was first identified by Whiteway *et al.* (1992) in a screen for *C. albicans* sequences that infer dominant

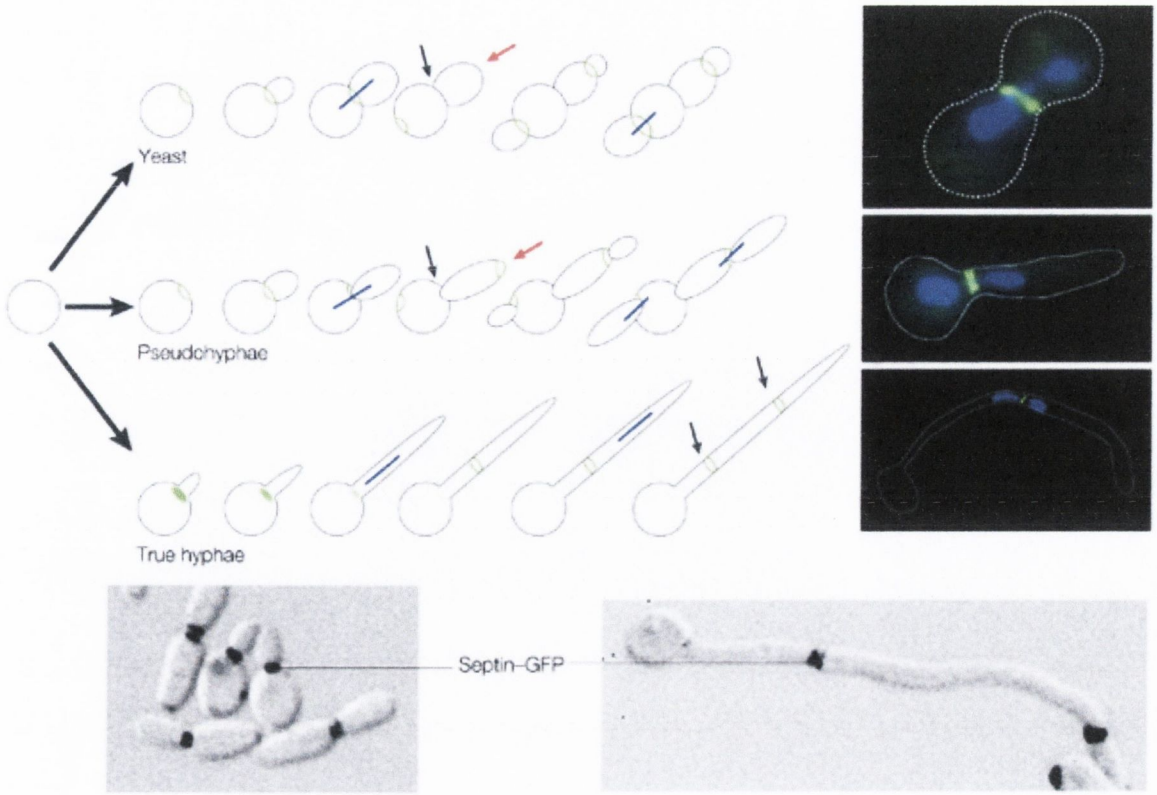

Figure 1.2. The difference between yeast, pseudohyphae, and true hyphae. *Candida albicans* can exist in three forms yeast cells (also known as blastospores), pseudohyphal cells and true hyphal cells. Yeast cells are round and separate readily from each other. Pseudohyphae are elongated yeast cells that remain attached to one another at the constriction septation site and usually grow in a branching pattern. True hyphal cells are long and highly polarised sides and no obvious constriction between cells. A basal septin (green) forms transiently at the junction between the mother and the evaginating germ tube. The sub-apical cells become highly vacuolated and do not branch or bud until the ratio of cytoplasm to vacuolar material increases significantly. All three cell types have a single nucleus per cell before mitosis. Important differences between yeast, pseudohyphal and true hyphal cells include the degree of polarized growth, the positioning of the septin ring (green in diagram and micrographs, and black in light microscope images) and of the true septum relative to the mother cell, The movement of the nucleus (blue line in diagram; stained with DAPI, blue in micrographs) relative to the mother cell and the degree to which daughter cells are able to separate into individuals. GFP, green fluorescent protein.
This diagram is taken directly from a paper written by Judith Berman and Peter E. Sudbery, titled *Candida* albicans: a molecular revolution built on lessons from budding yeast, (2002), *Nat Rev Genet* **3**, 918-930.

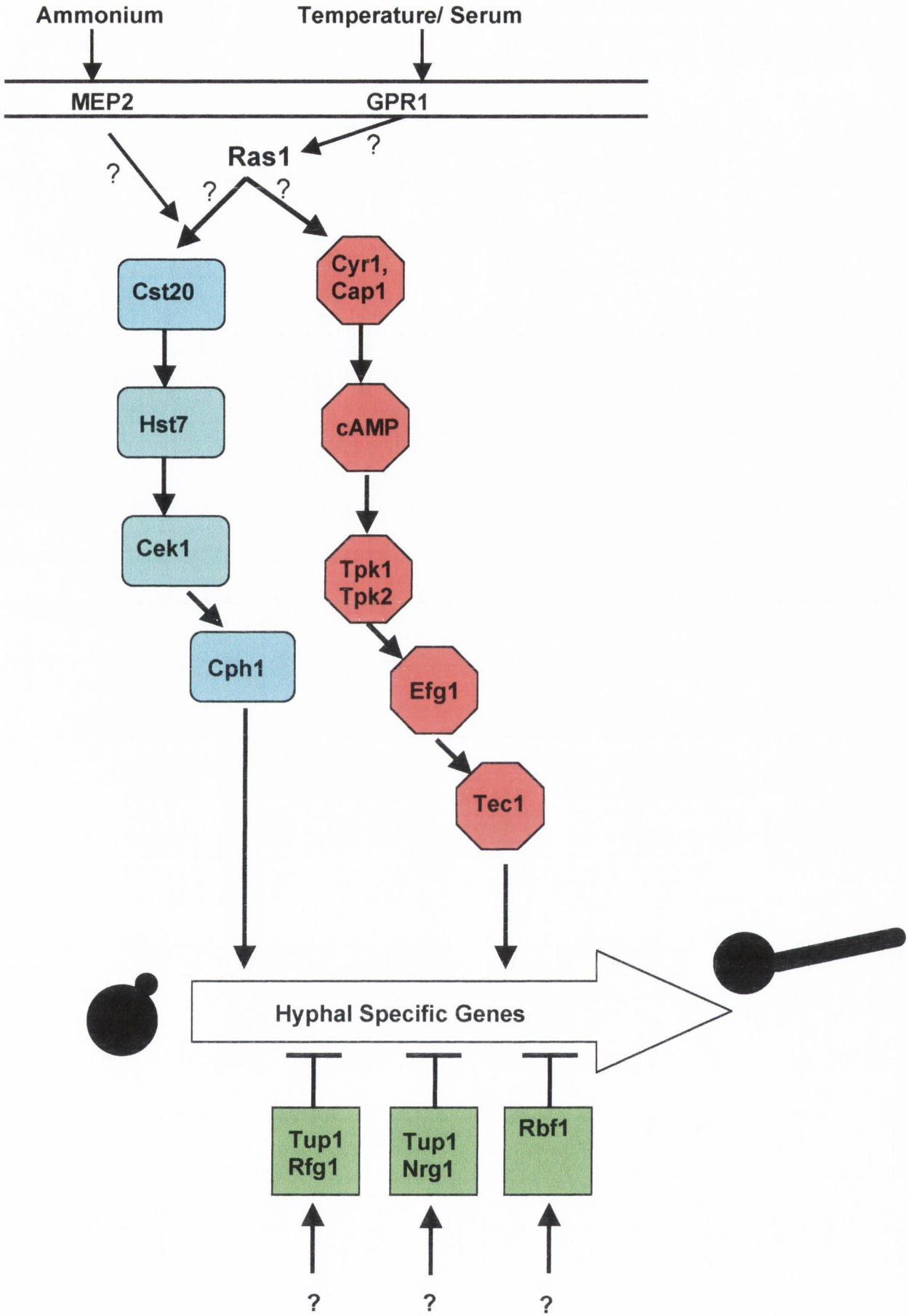

Figure 1.3. Signal transduction pathways that regulate morphogenesis. The Cph1-mediated MAPK pathway (blue), the Efg1-mediated cAMP pathway (red) are two pathways which are know to promote the yeast to hyphal or pseudohyphal growth. Ras1 may act upstream of both these pathways. Negative regulators of these pathways (green) are Tup1, recruited by Rfg1 and Nrg1and also Rbf1 which inhibit the switch from the yeast to the hyphal form.

⊤ = Inhibits hypha formation ? = Pathway not yet defined

negative effects upon mating pheromone-induced cell cycle arrest in *S. cerevisiae.* Previous studies have placed Cek1 below the MAP kinase kinase, Hst7, which is a functional homolog of Ste7. Thus, Cek1 is probably activated directly by Hst7 (Csank *et al.*, 1998; Kadosh & Johnson, 2005; Kohler & Fink, 1996). Cph1 lies downstream of the MAPK module (Fig 1.3). Cph1 is a functional homolog of the *S. cerevisiae* transcription factor Ste12 (Liu *et al.*, 1994). Previous experiments have placed Cph1 below Cst20, Hst7 and Cek1 in this *C. albicans* signalling pathway (Fig. 1.3). Recently Dabas & Morschhäuser identified two genes *MEP1* and *MEP2* which encode ammonium transporters that enable growth of *C. albicans* when ammonium is the only available nitrogen source. Deletion of either of these genes does not affect the ability of *C. albicans* to grow at low ammonium concentrations however Mep2p is involved in the control of morphogenesis as *Δmep2/Δmep2* mutants do not switch to filamentous growth under limiting nitrogen conditions. The control of *MEP2* expression is central to the regulation of nitrogen starvation induced filamentous growth in *C. albicans* (Biswas *et al.*, 2007). This evidence suggests that *MEP2* may act as a sensor of low environmental nitrogen concentration and may act to transduce these signals by an unknown mechanism to the MAPK pathway.

1.5.3 PKA pathway

The cAMP-dependent protein kinase A (PKA) pathway plays a vital role in filamentation in both *S. cerevisiae* and *C. albicans*. In *C. albicans* an increase in cAMP levels results in the yeast to hypha transition and inhibition of the cAMP phosphodiesterase induces this transition (Doedt *et al.*, 2004; Lengeler *et al.*, 2000; Lo *et al.*, 1997; Titz *et al.*, 2006). *C. albicans* has a single gene homologous to the *S. cerevisiae* adenylate cyclase gene. The cyclase is not essential for growth in *C. albicans* but is required for hypha development even when induced by serum. The adenylate cyclase associated protein cap1p, has also been identified and the gene has been disrupted in *C. albicans* (Bain *et al.*, 2001). An increase in cytoplasmic cAMP is seen to occur before the emergence of germ tubes in wild-type strains but not in *Δcap1/Δcap1* mutants (Fig. 1.3). Ras and a receptor-coupled G protein (G protein Gpa2 coupled to receptor Gpr1) act upstream of the adenylate cyclase in *S. cerevisiae*. These stimulate cAMP synthesis in response to intracellular acidification and extracellular glucose (Liu, 2001). In *C. albicans*, Ras1p is an important regulator of hypha development and is

thought to function upstream of the cAMP pathway (Feng *et al.*, 1999). Genes similar to *GRP1* and *GRP2* of *S. cerevisiae* have been identified following sequencing of the *C. albicans* genome, however their role in morphogenesis is unclear. There are two PKA subunits that are activated by cAMP in *C. albicans* known as Tpk1 and Tpk2. Tpk1 and Tpk2 have differential effects on hypha morphogenesis under different hypha inducing conditions. The *tpk2/tpk2* mutant displays strong hypha defects on solid medium containing serum or spider media. However the *tpk2/tpk2* mutant can still form hypha in response to serum stimulation in liquid culture although this process is delayed (Sonneborn *et al.*, 2000). Both of these subunits are positive regulators of hypha morphogenesis and are thought to activate filamentation via phosphorylation of the transcriptional regulator Efg1p (enhanced filamentation growth) (Bockmuhl & Ernst, 2001) (Fig. 1.3).

Efg1p is a basic helix-loop-helix protein which is a member of the APSES family, and plays a major role in hyphal morphogenesis. It influences the yeast to hypha interconversions and also regulates phenotypic switching and chlamydospore formation (Lo *et al.*, 1997; Sonneborn *et al.*, 1999; Sonneborn *et al.*, 2000; Stoldt *et al.*, 1997). Evidence would suggest that Efg1 functions downstream of PKA. Overexpression of *TPK2* is unable to suppress the mutant phenotype of *efg1/efg1* whereas overexpression of *EFG1* can bypass the filamentation defect in the *tpk2/tpk2* mutant (Sonneborn *et al.*, 1999). Induction of hyphae after the addition of serum or GlcNac to liquid or solid media, results in a complete block in hypha formation in strains lacking Efg1p (Lo *et al.*, 1997; Stoldt *et al.*, 1997). On the other hand in microaerophillic/embedded conditions hypha formation is not defective in *efg1/efg1* mutants but appears to be stimulated (Brown *et al.*, 1999; Sonneborn *et al.*, 1999). The APSES family of transcription factors are involved in many developmental processes, members of this family include morphogenetic regulators from other fungi such as Sok2 and Phd1 from *S. cerevisiae* and StuA from *A. nidulans*. Like other bHLH transcription factors, Efg1 can bind directly to E boxes (5'-CANNTG-3') (Leng *et al.*, 2001), and E boxes have been identified in promoters of the hypha specific genes *HYR1*, *HWP1*, *ALS3, ALS8* and *ECE1* (Braun *et al.*, 2000; Leng *et al.*, 2001; Sharkey *et al.*, 1999). Thus, *EFG1* appears to be a transcription factor that might act directly on hypha specific genes regulating their expression (Fig. 1.3).

1.5.4 The pH signalling pathway

C. albicans has the ability to grow in a broad range of host niches, which exhibit significant differences in pH. For example, the pH of mouse blood is pH 7.3, whereas the pH of the rat vagina is pH 4.5. The ability of *Candida* to react to different pH environments is crucial to its pathogenicity. A variety of environmental signals have been implicated in *C. albicans* morphogenesis, each favouring either the yeast or hypha form. The yeast phase is favoured at 25°C and acidic pH, whereas hyphal growth is favoured at 37 °C and a pH near neutral. The pH response in *C. albicans* involves the differential expression of at least three genes *PHR1*, *PHR2* and *RIM101* (Fig 1.4). A screen for genes expressed in a pH-dependent fashion led to the identification of pH β-1,6-glucan crosslinking activity (Muhlschlegel & Fonzi, 1997; Saporito-Irwin *et al.*, 1995; Sentandreu *et al.*, 1998). *PHR1* is an alkaline expressed gene, it is highly expressed at a pH above 5.5 and therefore is required for normal cell morphology at this pH and is also required for systemic infections. *PHR2* is an acid expressed gene and is required for normal cell morphology at acidic pH and also plays a role in virulence in more acidic environments, such as the acidic vaginal environment (De Bernardis *et al.*, 1998; Muhlschlegel & Fonzi, 1997; Saporito-Irwin *et al.*, 1995).

In *C. albicans,* Ramon *et al.* (1999) identified the full length homolog of the *A. nidulans* pH regulator *PacC* which they named *PRR2* (this has since been renamed as *RIM101*). *C. albicans RIM101* is a highly conserved zinc finger domain protein and also has an 84 amino acid C-terminal region which is conserved among other members of fungi (*S. cerevisiae, A. nidulans,* and *Y, lipolytica*). Rim101 activity is regulated by proteolytic processing. Under acidic conditions, Rim101 exists in its full length form (Li & Mitchell, 1997). Under alkaline conditions, the carboxyl terminal is cleaved to yield the active short form. Proteolysis is controlled by pH through a number of gene products, for example, Rim20p, Rim8p, Rim13p and Rim9p. Rim101p acts in conjunction with two other genes Rim8p and Rim20p to induce expression of Pra1p, Phr1p and Rim101p under alkaline conditions (Fig. 1.4) (Ramon & Fonzi, 2003). Recent studies indicate that ESCRT (endosomal sorting complexes required for transport), a group of three protein complexes that sort endoycytic cargo into multivesicular bodies, have been implicated in the Rim101p signalling pathway following a two hybrid assay based in interactions between Rim20p and Snf7p (Barwell *et al.*, 2005). Dfg16 and Rim21 are seven transmembrane domain containing proteins

and may compromise the sensors for external pH (Barwell *et al.*, 2005; Penalva & Arst, 2002). Evidence from this work comes from the Rim8 homolog in *Aspergillus* PalF, which behaves as a pH responsive arrestin which under alkaline conditions results in its phosphorylation and ubiquination (Herranz *et al.*, 2005).

Davis *et al.* (2000) demonstrated that the *RIM101* pathway is necessary *in vivo* for pathogenesis. This was demonstrated using the mouse model of hematogenously disseminated systemic candidiasis, and this showed that both *rim101/rim101* and *rim8/rim8* mutants had significant reductions in virulence. Environmental pH plays a crucial role in *C. albicans*, for nutrient availability, the function of plasma membrane proteins and secreted proteins. It is also a potent regulator of the yeast to hypha transition. The ability to respond to neutral pH is controlled by the *RIM101* pathway which results in the expression of alkaline genes and repression of acidic genes.

1.5.5 *NRG1* and its role in the repression of hypha specific genes.

Hypha development is subject to positive and negative regulations and is mediated by multiple signalling pathways including the MAPK, cAMP and the pH signalling pathways (Brown *et al.*, 1999; Ernst, 2000). Negative regulation is dependent on *CaTUP1* (Braun & Johnson, 1997). Inactivation of *CaTUP1* leads to constitutive filamentous growth and derepression of filamentous genes. *CaTUP1* appears to act independently of the MAPK and cAMP pathways in order to regulate morphogenesis (Fig. 1.3) (Braun *et al.*, 2000; Braun & Johnson, 2000). It is thought that in *C. albicans* Tup1p regulates hypha specific genes in a similar way to Tup1p in *S. cerevisiae*, which represses functional related genes by interacting with their promoter through various DNA binding proteins (Keleher *et al.*, 1992). Both Murad *et al.* (2001), and Braun *et al.* (2001) identified Nrg1p which also represses hypha formation and hypha specific genes in *C. albicans*. Murad *et al.* (2001) identified Nrg1p as a sequence specific DNA-binding protein that targets the Tup1p repressor complex to a subclass of genes that are transcriptionally activated during hypha formation. This Tup1-mediated transcriptional repression is in part mediated by the regulation of the expression of *NRG1* transcription (Murad *et al.*, 2001). Nrg1p is highly expressed in yeast cells but is rapidly downregulated following induction of a stimulus to facilitate hypha development. Saville *et al.* (2003) placed *NRG1* under the control of a tetracycline-regulatable promoter. This study showed that forced overexpression of *NRG1* during murine

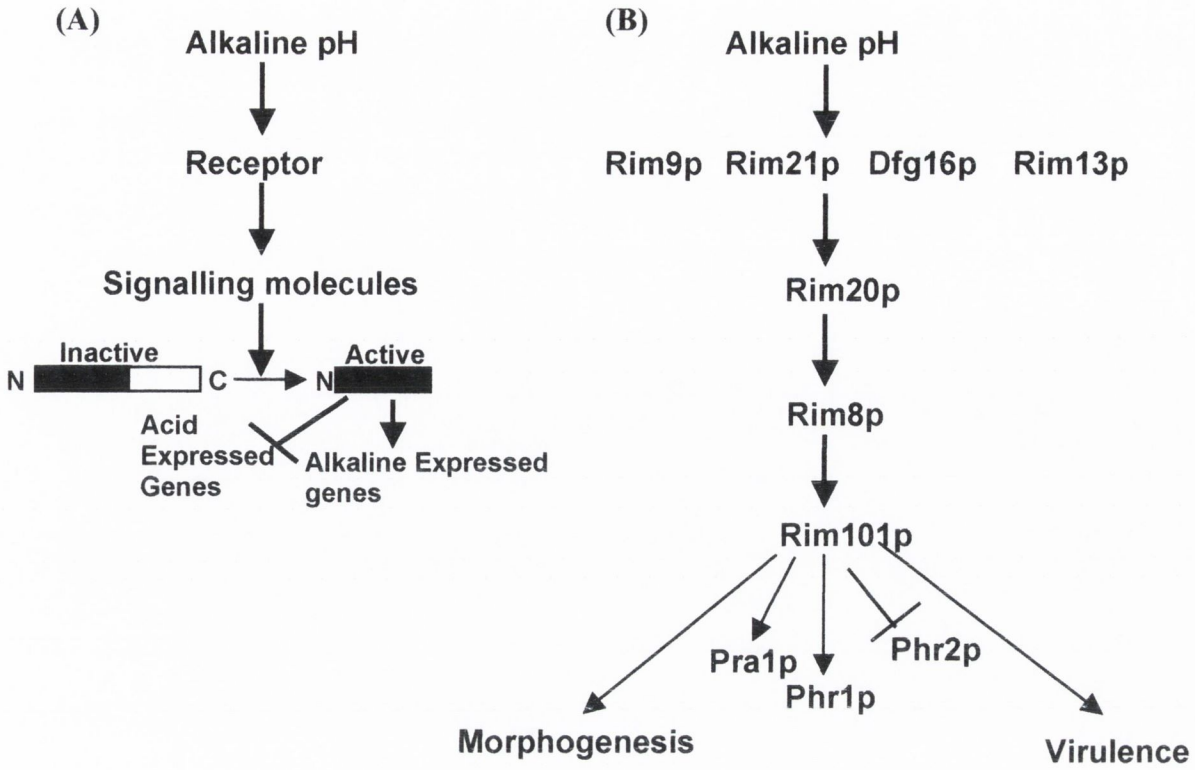

Figure 1.4. (A) This describes the pH signalling pathway, Rim101 activity is regulated by proteolytic processing. Under acidic conditions Rim101 exists in its full length inactive form and under alkaline conditions, a carboxyl terminal is cleaved to yield the active short form. **(B)** Alkaline pH induces the activation of Rim101, the active form of Rim101 regulates the induction/ repression of pH responsive genes and controls morphogenesis in an *EFG1*-dependent manner.

⊤ = Inhibits expression of acidic genes

This diagram is adapted from a paper by **Brown, A. J. & Gow, N. A. (1999).** Regulatory networks controlling *Candida albicans* morphogenesis. *Trends Microbiol* 7, 333-338.

infection prevented filament formation *in vivo* and hence rendered *C. albicans* avirulent (Saville *et al.*, 2003).

A study by Staib & Morschhauser (2005) provided evidence that different signalling pathways maybe involved in the induction of hypha of *C. albicans* and *C. dubliniensis*. This study found that on Staib medium the *C. albicans NRG1* gene is constitutively expressed and thus cells remained in the yeast phase. Interestingly *C. dubliniensis* specifically down regulated Nrg1p expression on Staib medium to allow chlamydospore development. Artificial overexpression of *CdNRG1* suppressed hyphal growth and production of chlamydospores in *C. dubliniensis*. The deletion of *NRG1* in *C. albicans* resulted in chlamydospores formation on Staib agar confirming the major role of *NRG1* in the regulation of morphogenetic processes (Staib & Morschhauser, 2005). Recently, Moran *et al.* (2007) further demonstrated the role of *NRG1* in *C. dubliniensis*. This was carried out by disrupting both copies of *CdNRG1*. Firstly, deletion of both copies of the gene resulted in increased formation of true hyphae in response to serum, exogenous cAMP and CO_2. In co-culture with murine macrophages the wild-type failed to produce filaments following phagocytosis whereas the *Δnrg1/Δnrg1* mutant produced abundant hyphae and had a significantly increased survival and growth rate when compared to the wild-type. In the reconstituted human oral epithelium (RHE) model of infection, the *C. dubliniensis* wild-type failed to filament during infection and adhered poorly to the epithelial surface and after 24 h the epithelium remained largely intact. Following inoculation of the RHE with the *Δnrg1/Δnrg1* mutant, the mutants had produced filaments, which were mainly pseudohypha and also the mutant had caused greater damage to the epithelial tissue when compared to the wild-type strain. This data suggests that differential regulation of *NRG1* between *C. albicans* and *C. dubliniensis* in response to stimuli that promote hyphal growth could in part explain the difference in virulence between these two species (Moran *et al.*, 2007).

1.6 Factors contributing to virulence in *Candida*

1.6.1 Hypha Specific Genes-*HWP1*

The ability of *C. albicans* to cause disease is mediated by the penetration of host tissues. Filamentous forms, in particular true hyphae, embed themselves within the superficial keratinised layer of the epithelium and grow by apical extension. The best described hypha-specific cell wall protein is Hwp1p, first isolated by Staab *et al.* (1999). This protein is encoded by a gene that was initially isolated as cDNA that was found to encode a hypha specific antigen. The structure of the protein indicated it was linked via a GPI-anchor to cell wall ß-glucan (Staab *et al.*, 2004). *HWP1* encodes a cell wall protein that serves as a target for mammalian transglutaminases. Transglutaminases are a family of related enzymes that catalyze Ca_2-dependent post-translational modification of proteins by introducing protein-protein crosslinks between glutamine and lysine residues. These enzymes use Hwp1 as a substrate to form covalent attachments between *C. albicans* and host epithelial cells. *HWP1* is conditionally required for hypha formation. The ability to form hypha is severely reduced in a heterozygous *HWP1* mutant and completely absent in a homozygous mutant. However, in the presence of serum the homozygous mutant produces peripheral hyphae but at greatly reduced levels compared to the wild-type. Transcription of *HWP1* is regulated by Efg1.

Interestingly, comparative genomic hybridisation studies carried out by Moran *et al.* (2004) identified a *C. dubliniensis HWP1* homologue. This *C. dubliniensis HWP1* gene hybridised poorly to *C. albicans HWP1* sequences due to the presence of large deletions in the *C. dubliniensis* ORF. The 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. However the effects of these deletions on transglutaminase substrate activity have not yet been investigated. Stokes *et al* (2007) found that the expression of *HWP1* in both *C. albicans* and *C. dubliniensis* was directly associated with the proportion of germinated cells, indicating that expression of this gene is tightly associated with hypha formation in both species.

1.6.2 The ALS family and their role in adhesion

The *ALS* (agglutinin-like sequence) gene family of *C. albicans* encodes a large family of cell surface glycoproteins which have a three domain structure which is implicated in adhesion to host surfaces (Hoyer, 2001; Hoyer *et al.*, 2008). The *ALS* family include eight genes; *ALS1* to *ALS7* and *ALS9* (recently investigators showed that *ALS3* and *ALS8* are in fact alleles of the same gene, thus *ALS8* was excluded from the nomenclature) (Zhao *et al.*, 2003).

Other species of *Candida* have been found to harbour genes with similarity to the *C. albicans ALS* genes which are characterised by an internal repeated sequence, a Ser/Thr-rich C-terminal domain and a consensus sequence for GPI anchor addition (Eisenhaber *et al.*, 2004). Hoyer *et al.*, (2001) identified three distinct *ALS*-like sequences in *C. dubliniensis* termed *ALSD1, ALSD2* and *ALSD3*. These genes exhibit significance divergence particularly at the 3' ends. This divergence was also observed by Moran *et al.*, (2004) following comparative genomic hybridisation studies. A family of seven *ALS* genes have subsequently been identified in the *C. dubliniensis* genome (Dr. Gary Moran, personal communication).

Deletion of *ALS1* in *C. albicans* and overexpression of the gene in *S. cerevisiae* found that *ALS1* functions in adhesion to vascular endothelial cell monolayers. However deletion of *ALS1* in *C. albicans* did not affect adhesion to buccal epithelial cells, FaDu monolayers or to a reconstituted human epithelial cell model (Zhao *et al.*, 2004). Deletion of many of the *C. albicans ALS* genes has been carried out, however, the deletion of *ALS3* had the greatest effect on adhesion. The mutant strain showed decreased adhesion to endothelial cells, buccal epithelial cells and FaDu monolayers. Overexpression of this gene in *S. cerevisiae* resulted in adhesion to endothelial and epithelial cells (Zhao *et al.*, 2004). A recent study has show that *ALS3* is a fungal invasin that mimics host cell cadherins and induces *C. albicans* endocytosis on oral epithelial cells (Phan *et al.*, 2007).

There are some reports that also suggest that the *ALS* genes contribute to virulence in *C. albicans.* Survival of mice infected with *ALS1* wild-type cells was compared with the *Δals1/Δals1* mutant following injection into the mouse tail vein. The study found that mice infected with the *C. albicans als1/als1* mutant had an increased survival time (Alberti-Segui *et al.*, 2004; Fu *et al.*, 2002). Loss of wild-type *ALS1* function has also been shown to slow hypha formation when compared to the wild-type strain.

1.6.3 The secreted aspartyl proteinases (Saps)

One factor thought to contribute to virulence in *C. albicans* is the production of hydrolytic enzymes which also plays a role in the pathogenicity of bacteria and protozoa (Finlay & Falkow, 1989). Proteinases catalyse the hydrolysis of peptide bonds in proteins but can differ greatly in their specificity and their mechanism of action (Barrett & Rawlings, 1991). Three types of hydrolytic enzymes are produced by *C. albicans* including the secreted aspartyl proteinases (Sap), phospholipases and lipases (Monod *et al.*, 1994; Monod *et al.*, 1998). To date, ten genes encoding Saps have been described in *C. albicans.* All 10 *SAP* genes of *C. albicans* encode preproenzymes 60 amino acids longer than the mature enzymes. These enzymes are processed when transported via the secretory pathway in the endoplasmic reticulum and by a Kex2-like proteinase in the late Golgi department. These mature enzymes contain sequence motifs typical for all aspartyl proteinases. These include two conserved aspartate residues at the active site and conserved cysteine residues which are thought to play a role in the maintenance of the three dimensional structure. Once processed, the enzymes are packaged into secretory vesicles, transported to the plasma membrane, and either incorporated into the cell wall via a GPI anchor (Sap9 and Sap 10) or secreted into the surrounding medium (Naglik *et al.*, 2003).

The presence of 10 *SAP* genes in *C. albicans* indicates that different proteinases may target a variety of host cells and tissues during *C. albicans* infections. *SAP1-3* are mainly associated with an optimum pH activity at pH 3-5 and these proteinases are mainly associated with the yeast phase*SAP4-6* have an optimum pH at pH 5-7 (neutral pH). Hube *et al.* (1994) observed that expression of *SAP4-6* occurred during serum induced germ tube formation. Sap2p, expressed by yeast cells was shown to have a broad spectrum of specificity. Sap1p and Sap3p were discovered to be differentially expressed during phenotypic switching, where a fungus switches its phenotype (Morrow *et al.*, 1992; White *et al.*, 1993). Expression of Sap8p is temperature regulated *in vitro* and is more strongly induced at 30°C in comparison to 37°C, suggesting that this gene may play a role during surface infections.

Experiments carried out *in vitro* demonstrate that *SAP* expression is a highly regulated process. Schaller *et al.,* (1998) used the RHE model of oral candidiasis and reverse transcription PCR and demonstrated that the *SAP* genes had a distinct order of expression (*SAP1* and *SAP3* > *SAP6* > *SAP2* and *SAP8*) during the course of infection. In this model, Schaller *et al.*, found that Sap1p to Sap3p but not Sap4p to Sap6p were

associated with tissue damage. A different expression pattern was observed using the RHE model of vaginal candidiasis; *SAP2, SAP9* and *SAP10* were observed initially followed by *SAP1*, *SAP4* and *SAP5* with *SAP6* and *SAP7* being expressed during the later stages of infection (Schaller *et al.*, 2003). Although the expression patterns were different between the two models of infection, studies with homozygous mutants suggested that Sap1 and Sap2 were the proteases primarily associated with tissue damage (Schaller *et al.*, 1998). These different studies using the RHE model suggest that Sap1 to Sap3 are the main *C. albicans* proteinases contributing to early stages of mucocutaenous infection.

Naglik *et al* (1999), analysed expression of *SAP1-8* from over 130 subjects with oral, vaginal, and asymptomatic carriage of *C. albicans* infections. *SAP2* and *SAP5* were the most commonly expressed and expression of *SAP1* and *SAP3* was found in both oral and vaginal disease. The study also found that all members of the Sap family were expressed during colonisation and pathogenicity. Gene disruption studies of *SAP1-3* found that growth rates of the corresponding mutants in complex and defined media were similar to the wild-type (SC5314). In medium with protein as the sole source of nitrogen *Δsap1/Δsap1* and *Δsap3/Δsap3* mutants had a reduced growth rate. However the *Δsap2/Δsap2* mutants grew poorly in this medium and produced the lowest proteolytic activity. All three mutants were injected intravenously in guinea pigs and mice and the animals had increased survival rates compared to the wild-type. The mutants exhibited attenuated virulence, however the extent of attenuation was not the same in the two models. The results from this study would suggest that *SAP1, SAP2* and *SAP3* all contribute to the virulence of *C. albicans (*Hube *et al., 1997)* (Fig. 1.5). A further study by Sanglard *et al.* (1997) carried out gene disruption studies of *SAP4, SAP5* and *SAP6*. Growth of the triple homozygous mutant (*Δsap4,5,6/Δsap4,5,6*) in complex medium was not affected. However, growth in a medium containing protein as the sole nitrogen source was severely impaired compared to the growth of the wild-type. Hube *et al.* (1997) demonstrated that Sap2p is required for optimal growth in such medium suggesting that Sap4p, Sap5p and Sap6p play an important role in the process of induction of *SAP2*. Guinea pigs and mice were also injected intravenously with the triple homozygous mutant (*Δsap4,5,6/Δsap4,5,6*). Both the guineas pigs and mice had an increased survival time when compared to the wild-type and as before the mutant exhibited attenuated virulence in both models of infection. This study suggested that

Sap4, Sap5, and Sap6 are important for the normal progression of systemic infection by *C. albicans* in animals (Fig 1.5).

Many other *Candida* species are known to possess *SAP* genes including *C. dubliniensis*, *C. tropicalis*, and *C. parapsilosis*. *C. dubliniensis* has a family of Sap encoding genes, however comparative genomic hybridisation studies carried out by Moran *et al.* (2004) revealed that *C. dubliniensis* has only eight members of this family compared to ten in *C. albicans*. In *C. albicans SAP4-6* are associated with hypha formation, however Moran *et al.* (2004) identified only one of these genes in *C. dubliniensis* (*CdSAP4*). In *C. dubliniensis CdSAP4* is expressed during hypha formation, however the absence of *SAP5* and *SAP6* could partly explain the reduced ability of *C. dubliniensis* hyphae to invade tissues (Moran *et al.*, 2004; Schaller *et al.*, 1999; Schaller *et al.*, 2001)

1.6.4 *EED1* is required for epithelial escape and dissemination in *C. albicans*

Orf19.7561 was first described as *EDT1* (*EFG1* dependent transcript) by Chen *et al.* (2004). Chen *et al.* (2004) found that the level of *CaEDT1* mRNA increased with increasing cell densities. The study also referred to unpublished data that identified *CaEDT1* as a key regulator of filamentation (Feng, Q., Chen, H., Guo, B., and Fink G.R., unpublished data). More recently Zakikhany *et al.* (2007) identified new functions for orf19.7561. Zakikhany *et al.*, (2007) carried out an experiment using an oral candidiasis model based on reconstituted human epithelium (RHE) was used to dissect the different stages of fungus-cell interactions from attachment via invasion to tissue destruction. In order to identify genes associated with oral candidosis, Zakikhany *et al.* (2007) took samples from HIV^+ patients who suffered from pseudomembranous oropharyngeal candidosis to compare the genome wide transcriptional profile obtained from each of these stages with the *in vivo* situation and to identify genes associated with oral candidiasis. One of the genes identified as being associated with oral candidosis both *in vitro* and *in vivo* was orf 19.7561 (previously identified as *EDT1*).

Mutants lacking orf19.7561 showed a reduced ability to cause tissue damage during infection of RHE. Also, microarray analysis revealed that orf19.7561 was highly expressed in the early phase of RHE infection. The strain lacking orf19.7561 was the most attenuated of the eight mutants tested during this study. Zakikhany *et al.* (2007) renamed orf19.7561, *EED1* (Epithelial Escape and Dissemination). Further analysis of

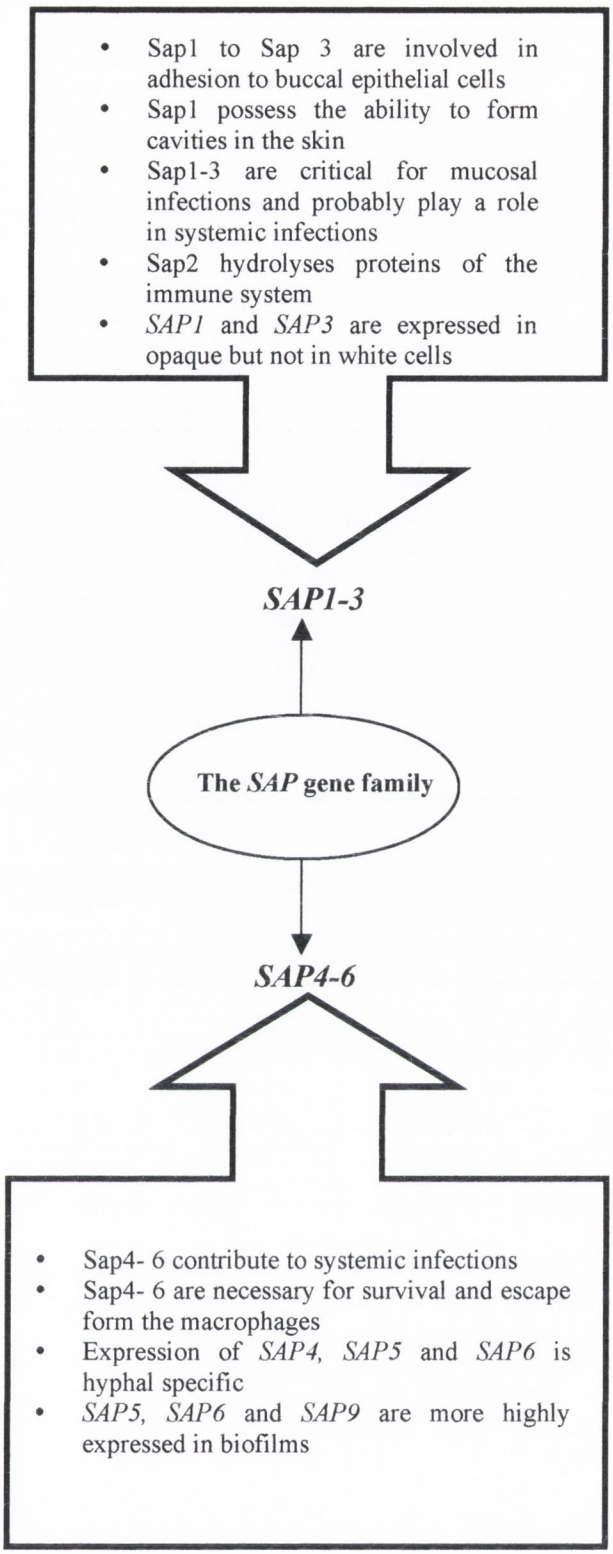

Figure 1.5. A summary of the function of *SAP* gene family identified to date. This diagram clearly demonstrates the broad range of functions of the secreted aspartyl proteinase family in *C. albicans*.

This figure is adapted from a paper by **Naglik, J. R., Challacombe, S. J. & Hube, B. (2003).** *Candida albicans* secreted aspartyl proteinases in virulence and pathogenesis. *Microbiol Mol Biol Rev* **67**, 400-428

the *Δeed1/Δeed1* mutant found that none of the conditions tested for hypha formation (serum, neutral pH, spider medium, and embedded conditions) could induce hyphal growth in the mutant. Similarly, no filaments were observed in liquid hyphal inducing media. In addition dilution of a saturated culture into 10 % serum did not induce true hypha formation, however, the cells did form short pseudohypha chains or remained in the yeast phase (Zakikhany *et al.*, 2007). As the mutant was unable to cause tissue damage on RHE, investigators decided to analyse the *Δeed1/Δeed1* mutant on a monolayer of epithelial cells. *Candida* cells produced short filaments after 1 h and produced elongated pseudohypha cells at 3-7 h which resulted in induced endocytosis. However, after 24 h, no elongated cells were visible, thus indicating that contact with the epithelium is a potent inducer of filamentation and that the *Δeed1/Δeed1* mutants only produced filaments transiently. Zakikhany *et al.* further examined the *Δeed1/Δeed1* mutant on RHE by analysing histological sections after 1 h and 24 h. The investigators found that after 1 h contact with the epithelium resulted in induced membrane ruffling of the *Δeed1/Δeed1* mutant. After 24 h intraepthelial inclusions in the upper part of the tissue, which contained yeast cells and short pseudohypha cells, were visible. Thus, it appears that contact with the epithelium is able to trigger formation of pseudohyphae which permit invasion into the epithelium, however, once inside the epithelium, cells proliferate in the yeast or pseudohyphae phase but remain trapped intracellularly (Zakikhany *et al.*, 2007).

Zakikhany *et al.* demonstrated that *EED1* is not required for adhesion or induced endocytosis but rather for escape and dissemination following invasion into the tissue. It is important to note that previously *EED1* was associated as being a transcript of *EFG1*, and although the function of the *Δeed1/Δeed1* is distinct from the *Δefg1/Δefg1* mutant (Lorenz *et al.*, 2004), it would appear that *EED1* is linked to the Efg1 pathway because *EED1* is downregulated in *Δefg1/Δefg1* mutants, suggesting that Efg1 may be an initiator of *EED1* (Zakikhany *et al.*, 2007).

1.7 Aims of this present study

Comparative analysis of the *C. albicans* and *C. dubliniensis* genomes identified many ORFs whose nucleotide sequence has significantly diverged. One such *C. dubliniensis* ORF is syntenic with *C. albicans* IPF946/orf19.7561. Since this study began, the *C. albicans* orthologue has been described as *EED1*, a regulator of filamentation, which is essential for virulence during infection of Reconstituted Human Oral Epithelium (RHE) (Zakikhany *et al.*, 2007). Therefore the main aims of this study were;

- To identify a *C. dubliniensis* homologue of *EED1*.
- To determine the function of this gene by creating homozygous mutants.
- To directly compare the role of *EED1* in *C. albicans* and the *C. dubliniensis* homologue in virulence.

Chapter 2

Materials and Methods

2.1 General Microbiological Methods

2.1.1 *Candida* strains and isolates.

The *Candida* strains and isolates used in this study are listed in Table 2.1. All yeast isolates were from the culture collection of the Microbiology Research Unit, Division of Oral Biosciences, Dublin Dental school and Hospital, University of Dublin, Trinity College, Dublin 2, Ireland.

2.1.2 Culture media and growth conditions

All *Candida* clinical isolates, strains and derivatives were routinely cultured on Potato Dextrose Agar (PDA) medium (Oxoid, Basingstoke, Hampshire, UK) at pH 5.6, at 37°C. 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 Laboratories, Detroit, Michigan, 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. Strains were also routinely cultured on yeast extract peptone dextrose (YPD) agar at 30°C and 37°C.

The *Escherichia coli* strain DH5α (*supE* Δ*lac*U169 [∅80 *lacZ*ΔM15] *hsd*R17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1; (Sambrook, 1989) was routinely cultured on Luria-Bertani agar (LA), pH 7.4 (Lennox, 1955), at 37°C for 18 h in an orbital incubator (Gallenkamp) at 200 rpm. *Escherichia coli* DH5α was used as the host strain for plasmid pBluescript II KS (-) (Stratagene, La Jolla, California, USA), and plasmid pGEM-T Easy (Promega Corporation (Madison, Wisconsin, USA).

2.1.3 Chemicals, enzymes, radioisotopes, antifungal drugs and oligonucleotides

Analytical-grade or molecular biology-grade chemicals were purchased from Sigma Aldrich Ireland Ltd. (Tallaght, Dublin, Ireland) or Roche Diagnostics Ltd. (Lewes, East Sussex, UK). Enzymes were purchased from Promega and from New England Biolabs (Beverly, Massachusetts, USA) and used according to manufacturer's instructions. [α-^{32}P]dATP (6,000 Ci/mmol; 222 TBq/mmol) was purchased from Amersham International Plc. (Little Chalfont, Buckinghamshire, UK). Custom-

Table 2.1 *Candida* strains and isolates used in this study.

Species	Strain	Coutnry of origin	Body site	Type of patient	Reference
C. albicans	SC5314	USA	Systemic	HIV -ve	(Gillum *et al.*1984)
	132A	Ireland	Oral	HIV +ve	(Gallagher *et al.* 1992)
	Y0109	England	N/A [a]	N/A [a]	(Moran *et al.*, 1997)
	NCPF3153A	England	N/A [a]	HIV -ve	(Hasenclever & Mitchell 1961)
	NCPF8324	England	N/A [a]	N/A [a]	(Moran *et al.*, 1997)
	KJ	Dublin	Oral	HIV +ve	(Moran *et al.*, 1997)
	BWP17	USA	N/A [a]	N/A [a]	(Wilson *et al.*,1999)
C. dubliniensis	CD36	Ireland	Oral	HIV +ve	(Sullivan *et al.*, 1995)
	Wü284	Germany	Oral	HIV +ve	(Staib *et al.*, 2001)
	CD33	Ireland	Oral	HIV +ve	(Sullivan *et al.*, 1995)
	Man 448	England	Oral	AIDS	(Pinjon *et al.*, 1998)
	CD41	Ireland	Oral	HIV -ve	(Moran *et al.*, 1997)
	LP1	Ireland	Oral	HIV -ve	(Gee *et al.*, 2002)
	LP3	Ireland	Oral	HIV -ve	(Gee *et al.*, 2002)
	William II	England	Blood	HIV -ve	(Pinjon *et al.*, 1998)
	96.54	Germany	Oral	HIV +ve	(Pinjon *et al.*, 1998)
	P7718	Israel	wound	HIV -ve	(Polacheck *et al.*, 2000)

[a] N/A Not Available

synthesised oligonucleotides were purchased from Sigma-Genosys Biotechnologies (Europe) Ltd. (Pampisford, Cambridgeshire, UK). DNA molecular weight markers were purchased from Promega. Zymolase 20T (21,600 U/g) was purchased form the Seikagaku Corporation (Tokyo, Japan).

RNase solutions were prepared by dissolving pancreatic RNase (Sigma-Aldrich) at a concentration of 10 mg/ml in 10 mM Tris–HCl (pH 7.5), 15 mM NaCl. This solution was stored in aliquots at –20°C. Proteinase K (Roche Diagnostics Ltd.) solutions were prepared in ultrapurified water from a MilliQ water purification system (Millipore SA- 67120 Molsheim, France) at a concentration of 20 mg/ml and was stored in aliquots at –20°C.

2.1.4 Buffers and solutions

Tris-EDTA (TE) buffer was routinely used in experiments and consisted of 10 mM Tris-HCl, 1 mM EDTA, pH 8.0. Citrate Phosphate buffer (CPB, 0.2M) consisted of, per 100 ml, 58 ml 0.4 M Na_2 HPO_4 and 42 ml 0.2 M citric acid.

TBE buffer was prepared at 10x concentration and consisted of 1 M Trizma base, 1 M boric acid, 0.5 M EDTA. This was diluted in ultrapurified water to a final concentration of 0.5x and was used as the buffer for agarose gel electrophoresis.

Final sample buffer was also prepared at 10x concentration and consisted of 30 % (v/v) glycerol, 0.25% (w/v) bromophenol blue and 0.1 M EDTA, pH 8.0. SSC buffer was prepared at 20x concentration and consisted of 3.0 M NaCl, 0.3 M tri-sodium citrate, pH 7.0.

Phosphate buffer saline (PBS) was prepared from tablets from Sigma Aldrich and was routinely used to wash cells and consisted of 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4.

MOPs buffer used for RNA electrophoresis was prepared at 10x concentration and consisted of 20 mM morpholinepropanesulphonic acid, 5 mM sodium acetate, 1 mM EDTA, pH 7.5. Liquefied phenol washed in Tris-buffer was purchased from Fisher Scientific Ltd (Bishop Meadow Road, Loughborough, UK) and used in the preparation of phenol chloroform (1:1), which was prepared by mixing an equal volume of liquefied phenol and chloroform. This solution was stored at 4°C.

2.1.5 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 YPD plates and incubated for 48 h at 37°C.

2.2 Isolation and analysis of genomic DNA

2.2.1 Extraction of genomic DNA from *Candida* isolates

High molecular weight Genomic DNA was prepared from cells grown in 50 ml of YPD broth in a 250 ml flask at 37°C in an orbital incubator at 200 rpm for 18 h. Cultures were then decanted into 50 ml centrifuge tubes (Sarstedt, Sarstedt Ltd., Sinnottstown Lane, Drinagh, Wexford, Ireland) and centrifuged in a bench top (Sepatech Megafuge 1.0) centrifuge (Heraeus, Germany) at 2,500 x g for 5 min. The supernatant was decanted and the pellet was resuspended in 5 ml 20 mM Citrate phosphate buffer, 40 mM EDTA, 1.2 M Sorbitol, pH 5.6. Cell walls were digested by the addition of 15 mg Zymolase 20T and incubated at 37°C for 30-90 min. The resulting protoplasts were harvested by centrifugation at 2,500 rpm for 5 min in a bench top centrifuge (Sepatech Megafuge 1.0) and the pellet was resuspended in 7.5 ml of 10x TE and 0.75 ml 10% (w/v) sodium dodecyl sulphate (SDS). Added 2.5 ml 5 M Potassium Acetate and incubated on ice for 30 min. The lysed cells are decanted into a 50 ml centrifuge tube and subjected to a clearing spin at 4°C in a Sorvall SS-34 rotor for 10 min at 8,500 rpm. The supernatant was placed into a fresh centrifuge tube and mixed gently with 10 ml ice cold iso-propanol (2-propanol). This was incubated on ice for 5 min and spun in a bench top centrifuge at 2,500 rpm for 5 min. The resulting pellet was dried and reuspended in 1 ml TE buffer. A 100 µl volume of RNAse A (10 mg/ml) was added and this was incubated for 1-2 h at 37 °C. Proteinase K was added to a final concentration of 10 mg/ml and the cell lysates were then incubated for 1-2 hrs at 37°C. The cleared supernatant was extracted four times using an equal volume of a mixture of phenol:chloroform (1:1) and precipitated with the addition of two volumes of ice-cold iso-propanol. The resulting DNA precipitate was then transferred to a fresh tube and washed in ice-cold 70% (v/v) ethanol, dried and resuspended in 150 µl of TE buffer.

2.2.2 Rapid DNA preparation for PCR from *Candida species*

Genomic DNA was prepared from cells grown overnight in YPD at 30°C. Cells were harvested in a 1.5 ml culture in a microfuge tube. The supernatant was removed and the pellet was resuspended in 200 µl breaking buffer (2% Triton X-100, 1% SDS, 10 mM Tris HCL (pH 8.0), 1 mM EDTA, 100 mM NaCl). The cell suspension was added to a 2 ml tube (Sarstedt) containg 0.3 g of glass beads (Sigma), followed by the addition of 200 µl of phenol : chloroform : isoamyl alcohol (24:24:1). Cells were then disrupted in a Fastprep FP120 bead beater (Savant Instruments, Inc. Holbrook, NY) for 1 min at speed 6. Cells were then centrifuged for 10 min at max speed. The aqueous phase was removed to a 1.5 ml microfuge tube and extracted with equal volumes of chloroform: isoamyl alcohol (24:1). This was then centrifuged for 2 min and again the aqueous phase was removed to a fresh 1.5 ml microfuge tube. DNA was precipitated with 400 µl of ethanol at -20 °C. This was then spun for 10 min at max speed and the resulting pellet was washed in 70% (v/v) ethanol and reuspended in 50 µl TE.

2.2.3 General PCR amplification

All primers used in this study are listed in Table 2.2. Amplification reactions were carried out using GoTaq® from Promega in 50 µl volumes containing 1.25 U of GoTaq polymerase (Promega), 10 µl of GoTaq Green FlexiBuffer (Promega), 3 mM $MgCl_2$, 200 µM (each) dATP, dTTP, dGTP, dCTP (Promega), 100 pmol of both a forward and reverse primer, and 10 ng of the DNA template. General PCR reaction conditions consisted of 5 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 50 -60°C, 1 min per 1000 bp at 72°C, followed by 72°C for 10 min.

Amplification products were separated by electrophoresis through 0.8% (w/v) agarose gels containing 0.5 µg/ml ethidium bromide. DNA molecular weight marker was loaded on each gel in an adjacent lane. The products were visualised on a UV transilluminator (Ultra-Violet products Ltd (UVP), Upland, CA, USA) following electrophoresis at 80 V with constant current for 1 hour.

2.2.4 Restriction endonuclease digestion of genomic DNA and agarose gel electrophoresis

Large scale restriction endonuclease digestions of genomic DNA were carried out with 10 µg of genomic DNA in a 40 µl volume containing 12 U of restriction enzyme and the appropriate restriction enzyme buffer according to the manufacturer's instructions. Samples were digested at 37°C for 4-5 h Electrophoresis was performed in 0.8 - 1 % (w/v) agarose gels made with 1x TBE buffer containing 0.5 µg ethidium bromide/ml. Restriction endonuclease-generated DNA fragments in a 1x final sample buffer were applied to the gel wells. Electrophoresis was performed at 60 V. Following electrophoresis, gels were visualised on a UV transilluminator (wavelength 345_{nm}) and photographed using an Imagestore 7500 Version 7.2.2 Gel documentation system.

2.2.5 Southern transfer of DNA from agarose gels

Following the separation of restriction endonuclease digested DNA fragments by agarose gel electrophoresis, DNA fragments were transferred by capillary action using the method of Southern (1975) to nylon membranes. Briefly, gels were soaked in 0.02 M HCl with gentle agitation for 10 min to depurinate the DNA. Following depurination, the DNA was denatured by soaking the gel in denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 45 min with gentle agitation. Following denaturation, the gels were neutralised by soaking in a solution containing 1 M Tris-HCl, pH 7.5, 1.5 M NaCl for a further 45 minutes.

DNA was transferred to Biobond™ Plus nylon membranes (Sigma-Aldrich) using 20x SSC as the transfer buffer. Following transfer, the gel was rinsed in 2x SSC and baked at 80°C for 1 h to fix the DNA or the DNA was fixed using a UV crosslinker (CL-508, UVI tec, Cambridge, Cambridgeshire, UK) set at 0.120 J /cm^2.

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

DNA fragments were labelled with [α-^{32}P] dATP by random primer labelling using the Prime-a-gene kit purchased from Promega. DNA to be labelled was denatured by boiling for 2 min and then added to a reaction mixture containing 1 x labelling buffer, dNTPs (dTTP, dCTP and dGTP), bovine serum albumin, 3 µl of [α-^{32}P] dATP (6,000 Ci/mmol; 220 TBq/mmol) and 5U of Klenow DNA polymerase, and

Table 2.2 Primers used to amplify *C. dubliniensis* and *C. albicans* sequences

Primer	Nucleotide Sequence (5'-3')[a]	Restriction Site	Nucleotide coordinates[b]
EDTP F	ATATAGTGTAGTGATAGTTTCC	*Hind*III	-843 to -822
FLP R	TTCCGTTATGTGTAATCATCC	*Hind*III	+1086 to +1107
3F	ATCGCCGCGGTCGCTTTTCAGAACTTTGCC	*Sac*II	+3323 to +3342
3 R	GGCCGAGCTCTCGAAGAAAAGAAGTCACTC	*Sac*I	+3669 to +3690
5F	GGCCGGTACCAGGGGTGTTGTTGAAGGTTG	*Kpn*I	-304 to -284
5R	AATGCTCGAGGAATTTGATTGTGATGATCC	*Xho*I	+157 to 178
M13F[c]	GTAAAACGACGGCCAGT		
M13R[c]	GGAAACAGCTATGACCATG		
CdEDTF	CCAACCAGTCCTGCTTCTAC		+26 to +44
CdEDTR	CTACTTTTGCCATTTTCATC		+418 to +437
CaEDT F	AAATCCTGAACCAGAAGAGGGTAG		+43 to +62
CaEDT R	AACAGCCTCTTTCCGCATTTC		+573 to +593
CaCEEDF	TTTGAAGCCCAGATGCA		-1868 to -1884
CaCEEDR	GCAGATACCAAACTTCTTCAAG		+3321 to +3343
CdCEDTF	AGTATAACTTTGATACAGGTCCATG		-2033 to -2005
CdCEDTR	AATTGGACCCTGCGTTTA		+3381 to +3398
CdMDPF	ATGCCACCGCGGATAACTTTGATACAGGTCCA TG	*Sac*II	-2009 to -1989
CdMDPR	ATGCCAGCGGCCGCAATTGGACCCTGCGTTTA	*Not*1	+3403 to +3420
CdCompF	GGCCGGTACCAGGGGTGTTGTTGAAGGTTG	*Kpn*I	-298 to -280
CdCompR	GGCCATCGATTGACCTACGTGATGTGTATATC	*Cla*I	+3044 to +3066
CaCompF	GCCGGTACCCAGGGTTTCTCCTTTTGTCC	*Kpn*I	-381 to -362
CaCompR	GGCCATCGATGTGTCACTATTCTACTTTAC	*Cla*I	+2782 to +2801
Ca3F	ATCGCCGCGGAATAGGGGATTGTGAATTAG	*Sac*II	+2921 to +2940
Ca3R	GGCCGAGCTCGATCACCAGATTTGTTGGAA	*Sac*I	+3232 to +3251
CdTetF	GGCCGTCGACATCGAGCAATGAAAACACC	*Sal*I	-8 to +10
CdTetR	ATCGAGATCTTTGGTGGTAGTTTCTGGAG	*Bgl*II	+855 to + 873
[d]CdRtF	GCAGCTCAGCCGCATAAAA		+728 to +737
[d]CdRtR	GGCCTTCTCATGTTTCACTGG		+810 to +830
[d]CaRtF	ACAGCCTCATGTTCAGACACCA		+1939 to +1958
[d]CaRtR	CCTCGAACATTGTACGTGTTGC		+2025 to + 2047
[d]RtActF	AGCTCCAGAAGCTTTGTTCAGACC		+768 to +791[e]
[d]RtActR	TGCATACGTTCAGCAATACCTGGG		+918 to +941[e]

Primer	Nucleotide Sequence (5'-3')[a]	Restriction Site	Nucleotide coordinates[b]
[d]RtTefF	CCACTGAAGTCAAGTCCGTTGA		+851 to +872[f]
[d]RtTefR	CACCTTCAGCCAATTGTTCGT		+901 to +922[f]
[d]RtHWPF	CAGCTGGTATTAAAACTAACCAAGC		+1001 to +1025[g]
[d]Rt HWPR	ATTCTAATGTGGTTGGAATAGCACC		+1108 to +1132[g]
[d]RtNRGF	GTTTGCAAAGTGTGTTCGAG		+712 to +731[h]
[d]RtNRGR	GACGAGCAAAACGGGCTTCA		+816 to +835[h]
[I]TETF2	TCCAGTTACCACTCCTATC		+23 to +43
CdMDPR	GTTCCTGTACACATGATAGA		+1655 to +1674[b]

[a] Restriction endonuclease recognition sites included 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] These are standard primers used for the identification of transformantss

[d] The nucleotide sequences of the PCR primers used to assay gene expression by real-time PCR.

[e] Nucleotide coordinates of *ACT1 from C. albicans* orf19.5007

[f] Nucleotide coordinates of *TEF1 from C. albicans* orf19.5119

[g] Nucleotide coordinates of *HWP1 from C. dubliniensis*, accession no., Cd36_43360

[h] Nucleotide coordinates of *NRG1 from C. dubliniensis,* accession no., Cd36_ 73980, Sequence data for *C. albicans* was obtained from the *Candida* genome database (CGD; http://www.candidagenome.org/) and the CandidaDB database web server (http://genolist.pasteur.fr/CandidaDB/). Searches of the *C. dubliniensis* genome were carried out using the *Candida dubliniensis* GeneDB server (http://www.genedb.org/genedb/cdubliniensis)

[I] Nucleotide coordinates of TETF2 from PNIM1 plasmid, accession no., DQ090840

was incubated at room temperature for 2 h. Unincorporated nucleotides were removed prior to hybridisation by passing the reaction through a size exclusion matrix (SigmaSpin post reaction columns, Sigma-Aldrich) following the manufacturer's instructions.

2.2.7 Southern hybridisation

Hybridisation reactions were carried out in 25 x 3.5 cm bottles (Hybaid, Teddington, Middlesex, UK) in a rotary hybridisation oven (Hybaid) by the method of Sambrook *et al.* (1989). Nylon membranes were rinsed in 6x SSC prior to hybridisation. Membranes were then prehybridised in 15 ml of a solution containing 1x Denhardt's solution (1% [w/v] Ficoll, 1% [w/v] polyvinylpyrrolidone, 1% [w/v] BSA), 6x SSC, 100 µg/ml denatured salmon sperm DNA and 0.5% (w/v) SDS in the oven at 65°C for 2 h.

Radiolabelled probe (~ 2 x 10^6 dpm) was denatured by boiling for 5 min, followed by incubation on ice. The denatured probe was then added to the prehybridisation solution and incubated with the membrane at 65°C for 18 h. Unbound probe was removed from the membranes following hybridisation by washing the membrane in the bottle with a solution of 2x SSC, 0.1% (w/v) SDS at room temperature for 5 min, followed by a wash at room temperature in 0.1x SSC, 0.5% (w/v) SDS for 15 min and a high stringency wash at 65°C in 0.1x SSC, 0.5% (w/v) SDS for 30 min. After washing, the membranes were wrapped in Saran wrap (Dow Chemical Co., Stade, Germany) and placed in an autoradiograghy cassette with a Kodak BioMax MS-1 intensifying screen (Eastman Kodak Company, Rochester, New York, USA) and exposed to Kodak Biomax MS-1 X-ray film at –70°C. Autoradiograms were developed with Kodak GBX developer and fixed in Kodak GBX fixer.

Bound probe was removed from membrane filters by immersing the membrane in a boiling solution of sterile ultra-purified H_2O, followed by a brief rinse in 2x SSC.

2.3 Recombinant DNA techniques

2.3.1 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, 1989). 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 1.5 ml aliquot of this culture was pelleted at 12,000 x *g* for 1 min in a microfuge tube and the plasmid DNA was isolated using the GenElute Plasmid Miniprep kit (Sigma). The culture was completely resuspended using 200 μl of the resuspension solution supplemented with RNase A to degrade total RNA. Cells were then lysed by the addition of 200 μl of an alkaline SDS lysis buffer and cells debris was precipitated by the addition of 350 μl of a neutralisation solution. Cell debris was pelleted by centrifuging at 12,000 x g for 10 min. The cleared lysate was then added to a GenElute binding column and spun at 12,00 x g for 1 min. The bound DNA was then washed with ethanol to remove residual salt and other contaminants. DNA was eluted in 100 μl sterile ultrapurified H_2O .

2.3.2 Polymerase chain reaction (PCR) and purification of PCR amplimers for cloning

Specific *C. dubliniensis* and *C. albicans* genomic DNA sequences were amplified by PCR and cloned into pBluescript or pGEM-T Easy. Oligonucleotide primers were designed containing specific restriction endonuclease cleavage sites (Table 2.2) to amplify the required region. PCR amplification was performed using the Expand Long Template PCR System (Roche Diagnostics Ltd.), or if the expected PCR product was below 3 Kb the Expand High Fidelity PCR system (Roche Diagnostics Ltd.) was used. Amplifications were carried out in 50 μl volumes containing 2.6 U Expand High Fidelity PCR System enzyme mix (Roche Diagnostics Ltd.), 1x Expand Long Template reaction buffer, 2.75 mM $MgCl_2$, 250 μM (each) dATP, dTTP, dCTP, dGTP (Promega), 10 pm of both a forward and reverse primer and 10 ng of genomic DNA template. PCR reactions were carried out in a Thermo Hybaid Multiblock System (MBS) thermal cycler (Thermo Hybaid, Ashford, Middlesex, England). Amplification products were separated by electrophoresis through 0.8 (w/v) agarose gels containing

0.5 μg/ml ethidium bromide. 100 bp and 1 Kb DNA molecular weight markers (Promega) were loaded on each gel. Following electrophoresis at 80V with constant current for 1 h the amplification products were visualised on a UV transilluminator (Ultra Violet Products Ltd) at a wavelength of 345_{nm} and photographed using Image Store 7500 version 7.22 Gel Documentation System. Amplification conditions and specific primers will be described in the relevant sections. All PCR products were either purified using the GenElute™ PCR DNA purification kit (Sigma-Aldrich) according to the manufacturer's instructions or purified from agarose gels using the Qiaex II Gel Extraction Kit (Qiagen Ltd., Fleming Way Crawley, West Sussex, England).

2.3.3 Ligation of DNA fragments

Purified DNA fragments were ligated to pBluescript II KS (-) phagemid digested with the appropriate restriction enzyme(s). Ligation of PCR products to pBluescript was carried out using restriction sites that had been designed within the oligonucleotide primers used in the amplification reactions. Purified DNA fragments were ligated into the appropriate restriction enzyme generated site in the cloning vector. Ligation reactions were carried out in a 10 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 overnight at 4°C. Purified DNA fragments were also ligated using the pGEM-T Easy vector. PCR product amplified by proofreading polymerases was blunt-ended because proofreading polymerases possess 3′→5′ exonuclease activity that removes the 3′-A overhangs necessary for TA cloning. Therefore, 3′-A overhangs can be added to blunt-end fragments using a Taq polymerase after PCR amplification. A-tailing was preformed by incubating the purified PCR product with 1μl of GoTaq Flexibuffer, dATP to a final concentration of 0.2 mM, 5 units of GoTaq polymerase and deionized water to a final reaction volume of 10 μl, this reaction is then incubated at 70°C for 30 min. Ligation reactions were carried out using 2x Rapid Ligation buffer, 50 ng of pGem-T Easy, a 3:1 molar ratio of insert to vector DNA, and 1 U of T4 DNA ligase. For best transformation efficiency reactions were carried out overnight at 4°C.

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

Transformation of *E. coli* strain DH5α with $CaCl_2$ was carried out by the method of (Sambrook, 1989). *Escherchia 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 until the OD_{600} reached ~0.5. The culture was then decanted into ice-cold 50 ml centrifuge tubes (Sarstedt) and chilled on ice for 10 min. Cells were then pelleted by centrifugation at 5,000 x *g* in a Sorvall RC 5B centrifuge (Dupont Co., Denver, Colarado, USA) at 4°C for 10 min. Each pellet was resuspended in 10 ml of ice-cold 0.1 M $CaCl_2$, and re-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 200 µl aliquot of this cell-suspension was transferred to a sterile 1.5 ml microfuge tube on ice for each transformation experiment. Plasmid DNA (up to 50 ng) was added to each tube and incubated for 30 min on ice. A known amount of a standard plasmid preparation was added to a separate tube as a positive control, and a tube which contained no plasmid DNA was also included as a negative control. The tubes were then heat shocked at 42°C for exactly 90 s and rapidly transferred to an ice bath. The cells were then incubated at 37°C in a water bath in the presence of 800 µl LB medium to allow the cells to recover and express the antibiotic resistance marker (ampicillin resistance in the case of pBluescript II KS [-]. The same procedure was carried out when using the pGem-T Easy vector. A 100 µl aliquot of this suspension was then spread on to LA plates containing antibiotic (100 µg ampicillin/ml in the case of pBluescript II KS [-]), 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Boerhringer Mannheim) and 100 µg (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal, Roche Diagnostics Ltd.) and incubated at 37°C for 20 h. Recombinants were identified using blue-white selection as described by(Sambrook, 1989).

2.3.5 Transformation of ABLE®K electroporation competent *E. coli* cells

ABLE® K strains of *E. coli* are designed to enhance the probability of retrieving clones that are toxic to *E. coli* through the reduction of plasmid copy number by ~ 10 fold. This copy number reduction, which is exerted in any ColE1-derived vector, decreases the levels of cloned gene products and enhances the probability that a toxic clone will be propagated. ABLE® K (*E. coli* C lac(LacZω) [Kan McrA$^-$ McrCB$^-$ McrF$^-$

Mrr$^-$ HsdR(r_K^- m_K^-)] [F′ proAB lacI^q ZΔM15 Tn10 (Tet^r)] is a restriction minus derivative of *E. coli* C. The ABLE® K strain contains the lacI^q ZΔM15 gene on the F′ episome, allowing blue-white screening for recombinant plasmids. However, since blue white screening includes gene induction by IPTG, which increases the levels of toxic proteins, blue-white colour screening is not recommended for cloning using the ABLE® K Strain.

Prior to electroporation the cuvettes and microcentrifuge tubes were chilled on ice and the SOC medium (20 g Tryptone, 5 g of yeast extract, 0.5 g NaCl, dH_2O to a final volume of 1 L, autoclaved, supplemented with 10 ml of sterilised 1 M $MgCl_2$ and 10 ml of sterilised $MgSO_4$ and 20 ml of 20 % (w/v) glucose) was preheated to 37°C. The Biorad electroporator (Bio-Rad Laboratories, Alpha Technologies Ltd, Wicklow, Ireland) was set to 200 Ω. ABLE® K cells were thawed on ice and after mixing gently 40 μl of cells were aliquoted into each microcentrifuge tube. DNA (1 μl of plasmid DNA) was added to the cells. The cell-DNA mixture was transferred to the electroporation cuvette. The cuvette was placed in the electroporation chamber and the sample was pulsed once and 960 μl of SOC medium was added immediately to resuspend cells. The cells were transferred to sterile 15 ml centrifuge tubes (Sarstedt). The tubes were incubated at 37°C for 1 h with shaking. 100 μl of the transformation mixture were plated on LB plates containing 100 mg/ml ampicillin. Plates were incubated at 37°C for 24 h.

2.3.6. Cloning using the TOPO® PCR Cloning Kit

The TOPO® XL PCR cloning kit provides a highly efficient, 5-minute one step cloning strategy for the cloning of long PCR products. The plasmid vector pCR® -XL-TOPO® is supplied linearised with single 3' thymidine (T) overhangs for TA cloning® and the topoisomerase covalently bound to the plasmid. The TOPO® Cloning reaction can be transformed directly into competent cells by either electroporation or chemical means.

Firstly the primer pair 5F/3R was used to amplify a 4 Kb fragment of *MDP1* using the Expand Long Template PCR System (Roche Diagnostics Ltd.). Amplifications were carried out in 50 μl volumes containing 2.6 U Expand High Fidelity PCR System enzyme mix (Roche Diagnostics Ltd.), 1x Expand Long Template reaction buffer, 2.75 mM $MgCl_2$, 250 μM (each) dATP, dTTP, dCTP, dGTP (Promega),

10 pm of both a forward and reverse primer and 10 ng of genomic DNA template. PCR reactions were carried out in a Thermo Hybaid Multiblock System (MBS) thermal cycler (Thermo Hybaid, Ashford, Middlesex, England). PCR products were gel purified using crystal violet to visualise the desired band to avoid damage to the PCR products. An 8 µl aliquot of 6X crystal violet loading buffer (30 % Glycerol, 20 mM EDTA, 100 µg/ml Crystal Violet) buffer was added to 40 µl of the PCR product and loaded onto a 0.8 % agarose gel prepared with 1X TAE (50 mM Tris-acetate, pH 8.0, 1 mM EDT1) buffer containing 30 µl of 2 mg/ml Crystal violet solution. The gel was run at 80 V with constant current until the crystal violet in the gel had run a quarter of the way up the gel and the stained PCR product was visible. Following this the PCR product was excised form the gel and transferred to a sterile 1.5 ml microcentrifuge tube. Subsequently 250 µl of 6.6M sodium iodide was added to the agarose and this was mixed by vortexing vigorously. This was then incubated at 50°C until the agarose was completely melted. PCR products were purified from the agarose gel using purification columns supplied with the TOPO PCR cloning kit, according to the manufacturers instructions. The column was then transferred to a sterile 1.5 ml microcentrifuge tube and 40 µl of TE buffer was applied to the column and this was incubated at room temperature for 1 min. The column was then centrifuged at max speed for 1 min to elute the DNA into the microcentrifuge tube. DNA recovery was assayed using ethidium bromide agarose gel electrophoresis (Chapter 2, 2.2.4)

The TOPO® Cloning reaction was carried out in a sterile microcentrifuge tube containing 4 µl of the gel-purified PCR product and 1 µl of pCR®-XL-TOPO® vector. This was then incubated at room temperature for 5 min, following this 1 µl of the 6X TOPO® Cloning Stop solution was added. The tubes were then briefly centrifuged and placed on ice. 2 µl of the TOPO® Cloning reaction was added to one vial of One Shot® electrocompetent cells and mixed gently. The cells and DNA were then transferred to a chilled electroporation cuvette. The cuvette was placed in the electroporation chamber and the sample was pulsed once and 450 µl of SOC medium was added immediately. The cells were transferred to a sterile 15 ml centrifuge tubes (Sarstedt) and were incubated at 37°C for 1 h with shaking. 100 µl of the transformation mixture was plated on LB plates containing 50 µg/ml kanamycin and these were incubated overnight at 37°C. Transformants were identified by PCR using the primer pair M13F and M13R (Table 2.2).

2.4 DNA Sequencing

Commercial DNA sequencing was performed by Cogenics, the genomics services company (Hope end, Takeley, Essex, UK). Nucleotide sequence analysis was performed by the dideoxy chain-terminating method of (Sanger *et al.*, 1977) using an automated Applied Biosystems 377 DNA sequencer and dye-labelled terminators (Applied Biosystems, Foster City, California, USA). The sequencing primers used were either the M13 forward and reverse primers or in the case of direct sequencing of PCR amplimers the PCR primers were used as sequencing primers. DNA Strider™ 1.4f17 software (CEA/Saclay, Gif-sur-Yvette, France) was used for sequence analysis. Searches of the EMBL and the GenBank databases for nucleotide and amino acid sequence similarities were performed using the BLAST family of computer programmes. Alignments of nucleotide and amino acid sequences were carried out using the CLUSTAL W sequence alignment program (Higgins & Sharp, 1988).

2.4.1. Computer analysis of DNA sequences

Homology searches were carried out with BLAST (Basic Local Alignment tool) at a number of different web sites. BLAST allows for rapid searching of nucleotide and protein databases. BLAST searches of GenBank were carried out at NCBI (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). BLAST searches of *Candida* species and related yeasts were carried out using the fungal genome initiative website at the Broad Institute (http://www.broad.mit.edu/cgi-bin/annotation/fgi/blast_page.cgi). Searches of the *C. albicans* genome were carried out using the *Candida* genome database (CGD; http://www.candidagenome.org/) and the CandidaDB database web server (http://genolist.pasteur.fr/CandidaDB/). Searches of the *C. dubliniensis* genome were carried out using the *C. dubliniensis* GeneDB server (http://www.genedb.org/genedb/cdubliniensis)

Clustal W (http://www.ebi.ac.uk/Tools/clustalw2/index.html) was used to align both DNA and protein sequences. It calculates the best match for the selected sequences and aligns them so that the identities, similarities and differences can be seen. Sequences were uploaded in the Pearson (FASTA) format. The parameters used were the default parameters on the website. NJ plot was used to draw phylogenetic trees

based on these alignments. EMBOSS pairwise alignment tool was also used (http://www.ebi.ac.uk/emboss/align/).

The Artemis Comparison Tool (ACT) was obtained from the Sanger website (http://www.sanger.ac.uk/Software/ACT/). ACT is a DNA sequence comparison viewer based on the Artemis software tool. ACT allows visualisation of complete EMBL and GenBank entries or sequences in FASTA or raw format. This tool allows for interactive visualisation of comparisons between genome sequences. This program enables the identification of regions of similarity, insertions and rearrangements at any level from the whole genome to base-pair differences (Carver *et al.*, 2005). EMBL sequence files of *C. albicans* and *C. dubliniensis* chromosomes used in this study with ACT were obtained from CGD and the Sanger centre respectively.

Protein domain searches were carried out using Interpro (http://www.ebi.ac.uk/interpro/) and Pfam (http://www.sanger.ac.uk/Software/Pfam/). These databases contain collections of protein domains each represented by multiple sequence alignments. These databases allow protein sequences to be compared and allow for the identification of putative functional domains.

The *Candida* Gene Order Browser (CGOB) is an online tool for visualising the syntenic context of genes from multiple *Candida* genomes (http://actin.ucd.ie/ygob/). This was used in order to compare the *EED1* locus among different yeast species (Byrne & Wolfe, 2005).

2.5 Analysis of RNA

2.5.1 RNase-free conditions

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

(Sigma) used in RNA extractions were 450-600 microns in diameter and were soaked in hydrochloric acid, washed in distilled water, and baked overnight.

2.5.2 Total RNA extraction from *Candida* isolates

Candida cells were harvested at mid-exponential phase (OD_{600}: 0.8-1.0 unless otherwise stated in specific sections) from 50 ml YPD, Lees pH 4.5 or Minimal medium (SD) (2% glucose, 0.67% yeast nitrogen base without amino acids) broth cultures for RNA extractions by centrifugation at 2,500 x *g* for 5 min. Pellets were frozen immediately in liquid nitrogen. For each sample a 2 ml screw capped Sarstedt tube contaning 0.3 g RNase free glass beads was prepared. The cell pellets were resuspended in 1 ml Tri-Reagent (Sigma) and quickly transferred to the tube containing the glass beads. The cells were then homogenised in a Fastprep FP120 Bead Beater (Savant Instruments, Inc. Holbrook, NY) for 30 s at speed 6. The homogenate was transferred to a fresh 1.5 ml Eppendorf and incubated for 5-10 min (not longer than 40 min) at 15-30°C. After the addition of 200 µl of chloroform the sample was vortexed for 15 s and then centrifuged at 12,000 x g for 15 min at 4°C. The upper aqueous phase was carefully removed to a fresh 1.5 ml Eppendorf tube and 500 µl of isopropyl alcohol was added to the sample and left to incubate at room temperature for 10 min. The sample was then centrifuged at 12,000 x g for 10 min at 4°C. The pellet formed was resuspended in DEPC H_2O, to remove DNA and polysaccharides two volumes of 6 M LiCl was added and incubated at –20°C for 2 h. Following a centrifugation at 12,000 x g for 15 min, the resulting pellet was carefully washed in 1 ml of 70 % (v/v) ethanol. The ethanol was then removed and the pellet was air dried briefly. The pellet was resuspended in a final volume of 30 µl of DEPC H_20 and stored at –70°C. The concentration of each RNA sample was assessed by measuring the A_{260} (1 unit of A_{260} = 42 µg RNA) and 15 µg were loaded on each gel. A test 1 % (w/v) agarose gel was prepared initially to assess the accuracy of concentration determinations and to assess the integrity of each sample. It was found that more accurate determinations of RNA loading could be made by comparison of ethidium bromide staining of the RNA on a

test gel. The integrity of each sample could then be assessed and adjustments made to ensure equal loading of the samples if necessary.

2.5.3. cDNA preparation and quantification

First strand cDNA synthesis was preformed using the Superscript II kit (Invitrogen, Bio Sciences Ltd., Dun Laoighre, Dublin, Ireland.). 1 µg of total RNA and 1 µl of oligo dT $_{12\text{-}18}$ (0.025mM) primer was added to each tube to obtain a total volume of 11 µl. RNase was denatured at 70°C for 10 min and cooled to 42°C to allow primer annealing. Subsequently 4 µl 5X first strand buffer, 2 µl 0.1M DTT and 1 µl RNase inhibitor (40U/ µl) (Ambion, Applied Biosystems, 120 Birchwood Boulevard, Warrington, U.K.), were added to each tube. The mixture was incubated at 42°C for 2 min, and then 1 µl of Reverse Transcriptase (200U/ µl), was added to each sample. The reaction was further incubated at 42°C for 60 min, followed by 70°C for 15 min.

2.5.4 Real-time PCR

Real-time measurment of PCR amplification can be achieved by determining the number of PCR cycles ("cycle threshold" Ct) necessary to achieve a given level of fluresence. In this study, the Ct was fixed in the exponential phase of the PCR. Real-time PCR primers were designed for both the *C. albicans EED1* and the *C. dubliniensis MDP1* genes (Table 2.2) using Primer Express from ABI Prism Version 1.5 (Applied Biosystems, Foster City, CA, U.S.A). The amplicons were between 90 and 110 bp in size. The *ACT1* primer pair were used as a control. Analysis of transcripts was carried out in 15 µl reactions, using SYBR Green PCR Master Mix (Applied Biosystems), in an ABI Prism 7700 Sequence Detection System (Applied Biosystems) for 40 cycles (thermal cycling conditions: initial step of 94°C for 10 min, followed by 40 cycles of 94 °C, 15 s and 59 °C, 30s and 72 °C, 30s). The Ct values for each experiment were recorded. Real-time PCR efficiencies was assessed using the $2^{-\Delta\Delta C}{}_{T}$ method according to Livak & Schmittgen (Livak & Schmittgen, 2001). The relative changes in gene expression were quantified using the $2^{-\Delta Ct}$ equation.

2.6 Targeted gene deletion in *Candida dubliniensis*

2.6.1 Construction of a molecular marker cassette.

The *SAT1*-flipper cassette was used to characterise the function of genes in *C. dubliniensis*. The *SAT1* flipping method relies on the use of a cassette that contains a dominant nourseothricin resistance marker (caSAT1) for the selection of integrative transformants and a *C. albicans* optimised FLP gene that allows the subsequent excision of the cassette, which is flanked by FLP target sequences. Two rounds of integration/ excision generate homozygous mutants that differ from the wild type parent strain only by the absence of the target gene (Reuss *et al.*, 2004). Reintegration of an intact gene copy for complementation of mutant phenotypes is performed in the same way, as described (Reuss *et al.*, 2004) (Fig 2.1). Therefore in *C. dubliniensis* the 5'-3' ends of *MDP1* were cloned into the *Kpn*I/*Xho*I and *Sac*II/*Sac*I sites respectively of the pFS2A. In order to achieve this a 315 bp fragment of the 5' end of the *C. dubliniensis MDP1* gene was amplified using the primer pair 5F/5R (Table 2.2). This was digested with *Kpn*I/*Xho*I restriction enzymes and cloned into the vector pBluescript II KS [-] (Norrander *et al.*, 1983). At the 3' end a 478 bp fragment of the *C. dubliniensis MDP1* gene was amplified using primer pair 3F/3R (Table 2.2) and this was digested with *Sac*II/*Sac*I restriction enzymes and cloned in to vector pBluescript II KS [-]. Both of these fragments were subcloned from pBluescript II KS [-] into the *Kpn*I/*Xho*I and *Sac*II/*Sac*I sites flanking *SAT1* gene in pFS2A to generate pLOC1 (Fig 2.1).

2.6.2 *Candida* transformations

Candida dubliniensis strains were transformed by electroporation (Kohler *et al.*, 1997). Cells from YPD medium pre-culture were diluted 1:10,000 in 50 ml of fresh YPD medium and grown overnight at 30°C to an optical density at 600nm (O.D. $_{600}$) of 1.6 to 2.2, which was found empirically to yield the best transformation efficiency. Cells were transformed with *Kpn*I/*Sac*I fragment from pLOC1 containing the *SAT1* cassette flanked with the 5' and 3' *MDP1* sequence. The fragment was gel purified by Qiaex II. 1 µg per transformation was carried out as outlined by (Staib *et al.*, 2001). *SAT1*-resistant transformants were selected on agar plates containing 100 µg/ml (*C. dubliniensis*) or 200 µg/ml (*C. albicans*) nourseothricin (cloNAT, Werner,

BioAgents, Jena, Germany). Single colonies were picked after 3 to 5 days of growth at 30°C (*C. dubliniensis*) or 37°C (*C. albicans*) and subcultured. Positive transformants were identified by PCR using the primer pair EDTPF-FLPR, which identified a 1,950 bp fragment (Table 2.2).

In order to disrupt the second copy of *C. dubliniensis MDP1,* excision of the cassette was induced by expressing the FLP recombinase. In order to do this positive transformants were grown in yeast peptone maltose (YPM) overnight at 30°C so that the *MAL2* promoter would induce FLP expression (Titz *et al.*, 2006). Putative revertants were streaked onto plates with a drug concentration of 4 µg/ml and 2 µg/ml, which allowed for nourseothricin sensitive revertants to be identified as small colonies. These were then screened by PCR using the primer pair EDTPF/FLPR as before.

2.7 Phenotypic analysis of *Candida* mutants

2.7.1 Induction of hyphae

Hypha production in *Candida* isolates and mutant derivatives was monitored by inoculation of cells in the yeast phase (grown overnight in YPD or Lee's pH 4.5 broth) into media that promote the yeast to hyphae transition (Table 2.3). Cells were washed twice in sterile phosphate buffered saline (PBS) and inoculated to a cell density of 2 x 10^6 CFU/ml in 5 ml of the appropriate inducing medium (Table 2.3), in a 6 well tissue culture plate (Greiner Bio-One, CELLSTAR, Cruinn Diagnostics Ltd., Dublin, Ireland) and incubated statically at 37°C. The percentage of hypha cells was determined every hour from t=0 h to t=6 h using a Nikon TMS-F inverted microscope (Nikon, Japan). One hundred cells were counted and the percentage of cells, which were hypha was determined. This method was used as *Candida* cells began to clump rather rapidly in shaking cultures and can be difficult to count. By using tissue culture plates in a static incubator and an inverted microscope the clumping of *Candida* cells was avoided.

The induction of hyphae on solid media was performed 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. Agar plates were monitored for hypha production every day for five days (Liu *et al.*, 1994). Hypha induction was also examined on Synthetic low dextrose (SLD) medium (0.17% (w/v) YNB without amino acids, 0.5% (w/v) $NH_4(SO_4)_2$, 0.1% glucose, 1.35% (w/v) agar),

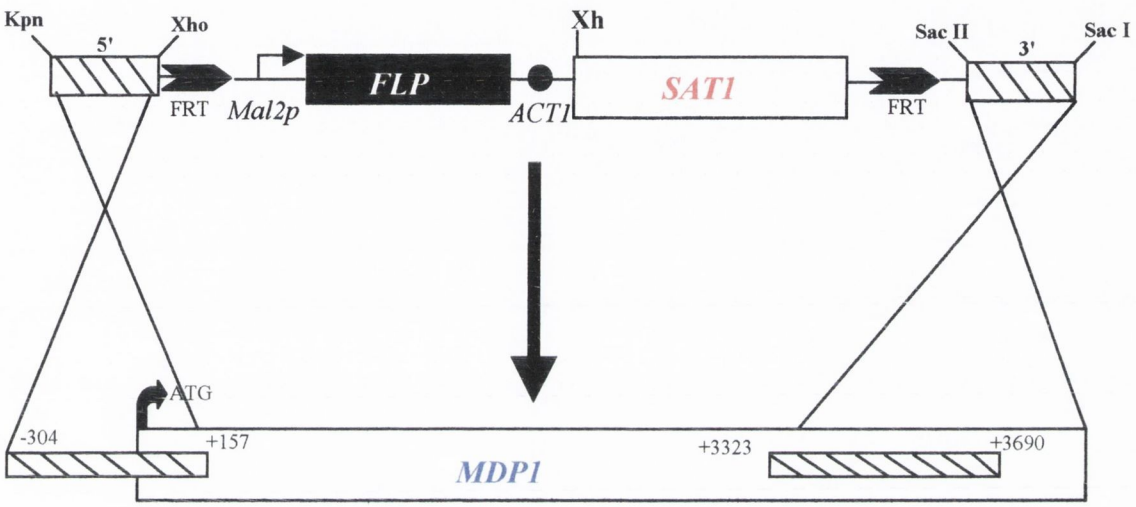

Figure 2.1. Structure of the *SAT1* flipper cassette contained in plasmid pSFS2. The *C. albicans FLP* optimised gene is represented by the black rectangle, (Wirsching *et al.* 2006). The nourseothricin resistance marker (*SAT1*) is represented by the white rectangle. The *MAL2* promoter is represented by the bent arrow and the transcription termination sequence of the *C. albicans ACT1* gene by the filled circle. Unique restriction sites flanking the *SAT1* flipper are marked in bold, *Kpn*I; *Sac*I; *Sac*II; *Xh*, *Xho*I. (Reuss *et al.*, 2004). This *SAT1* flipper cassette was be used to delete both copies of *MDP1*.

Table 2.3 Media that induce hyphal production

Type of Medium	Pre-Culture Media	Experimental media	Supplements	Preculture Temperature	Incubation Temperature
Liquid	Lee's Medium pH 4.5	10 % Serum	Arginine, biotin, and trace metals [a]	30°C	37°C
Liquid	Lee's Medium pH 6	10 % Serum	Arginine, biotin, and trace metals [a]	30°C	37°C
Liquid	Lee's Medium pH 8	10 % Serum	Arginine, biotin, and trace metals [a]	30°C	37°C
Liquid	YPD	10 % Serum	None	30°C /37°C	37°C
Liquid	YPD/ Lees pH 4.5	DMEM	None	30°C	37°C +/- 5% CO_2
Solid	N/A [b]	Spider Medium	None	N/A [b]	37°C
Solid	N/A [b]	YPS	None	N/A [b]	30°C
Solid	N/A [b]	SLD	None	N/A [b]	30°C
Solid	N/A [b]	YNB	Amino acids	N/A [b]	37°C
Solid	N/A [b]	YNB	None	N/A [b]	30°C
Solid	N/A [b]	Lee's Medium pH 6.5	Arginine, biotin, and trace metals [a]	N/A [b]	37°C

[a] 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 $CaCl_2$)

[b] Not Applicable

incubated at 30°C for 5 days. In addition hypha production was examined on Yeast nitrogen base (YNB) medium (0.67% (w/v) YNB with amino acids, 0.5% glucose; 1.35% (w/v) agar), supplemented with 10 % newborn calf serum, which was added to the sterile molten agar and plates were incubated at 37°C and 30°C for 5 days.

Hypha induction was also examined on yeast extract-peptone-sucrose (YPS) medium (1 % yeast extract, 2 % Bacto Peptone, 20 % sterilised sucrose) by plating a suspension of approximately 100 cells from an overnight culture and incubating at 30°C and 37°C for five days. Embedded conditions were also examined using a sandwich of YPS medium by pouring 15 ml of agar into a Petri dish and left to solidify and then plating a suspension of approximately 100 cells on top of this and when this was dry pouring another 15 ml of YPS agar on top of this and incubated at 30°C for five days. Therefore, cells were plated between two solidified layers of agar ('sandwiched') (Brown *et al.*, 1999).

2.7.2 Growth rate determination at elevated temperature and osmotic stress

Overnight broth cultures of *C. albicans* and *C. dubliniensis* strains were grown in YPD broth at 30°C. The cell density of each overnight culture was determined using an improved Neubauer haemocytometer. Cells from these cultures were then added to fresh YPD (with and without NaCl) at 30°C, 37°C and 42°C to yield a final concentration of $2x10^6$ cfu per ml. These cultures were incubated in shaking in a Gallencamp orbital incubator set at 200 rpm. At specific time points, aliquots of each culture was removed and the optical densities at 600 nm ($O.D._{600}$) measured using a Genosys 2 spectrophotometer, (Thermo Spectronic, Dublin, Ireland). These values were then used to plot a growth curve for each isolate grown under each condition. Growth curve analysis was carried out using Prism 4 for Macintosh, version 4.0. The equation to determine exponential growth was Y = Start*exp*(K*X), where Y = start and increases exponentially with rate constant K, the doubling time equals 0.69/K. Experiments were performed on three separate occasions

2.7.3 Spot plate assay

Susceptibility of *C. albicans* and *C. dubliniensis* isolates and mutant derivatives were tested qualitatively by spotting serial dilutions of yeast cultures on to inhibitor

containing agar plates. Overnight broth cultures were firstly diluted to 1 x 10^7 cells/ml. Four 10-fold serial dilutions of this first dilution were made and 10 µl of each dilution was spotted onto each plate and these were incubated at 37°C for 48 hrs (Table 2.4)

2.8 Cell Culture

2.8.1 General cell culture techniques

The murine macrophage like cell line RAW 264.7 was obtained from the American Type Tissue Culture Collection (ATCC). The complete medium used to maintain the cell line consisted of Dulbeccos modified eagles medium (DMEM) supplemented with 10 % (v/v) heat-inactivated fetal bovine serum (Sigma-Aldrich) and 100 U ml^{-1} penicillin and 100 mg ml^{-1} streptomycin (Sigma-Aldirch). Cells were grown at 37°C under an atmosphere with 5 % (v/v) CO_2, in 90 mm diameter tissue culture plates (Sarstedt).

2.8.2 Co-culture of macrophages with *Candida*

Co-culture of murine macrophage the cell line RAW 264.7 with *Candida* was carried out in flat bottomed 96 well dishes (Sarstedt). For each experiment a macrophage suspension of 1.5 x 10^6 cells ml^{-1} was prepared in complete medium. For each *Candida* strain tested 16 wells were dispensed with 100 µl of the macrophage suspension and this was cultured overnight (16 h) to form confluent monolayers. The following day the confluent monolayers are washed with fresh complete medium and a final volume of 150 µl of medium was added to each well. *Candida* strains were cultured overnight in 5 ml YEPD broth at 37°C with shaking. Cells were washed twice in PBS (Sigma-Aldrich) and a suspension of 1.5 x 10^6 cells ml^{-1} was prepared in complete medium. A 50 µl aliquot of this suspension was added to quadruplicate wells containing the confluent washed monolayers and quadruplicate wells containing complete medium alone. This yielded 7.5 x 10^4 *Candida* cells per well, giving a MOI of 1:2 (*Candida*: macrophages). Cells from this well (50 µl) were then serially diluted 1:4 in the three adjacent wells to yield MOIs of 1:8, 1:32, 1:128 respectively. Wells

Table 2.4 Media used for spot plate assays

Condtions Tested	Concentration	Temperature
YPD alone	-	37°C
NaCl	0.6 M	37°C
NaCl	1 M	37°C
NaCl	1.5 M	37°C
NaCl	1.6 M	37°C
Sorbitol	1.5 M	37°C
H_2O_2	10 mM	37°C
Calcoflour white	800 μg/ml	37°C
$CaCl_2$	0.5 M	37°C
SDS	100 μg/ml	37°C
Amphotericin B	0.2 μg/ml	37°C
CongoRed	200 μg/ml	37°C
Menadione	50 μM	37°C
Menadione	100 μM	37°C
YPD	-	42°C

containing complete medium alone acted as macrophage free growth controls. The inoculated plates were incubated at 37°C in 5% (v/v) CO_2 for 16 h.

Following incubation, the survival of the *Candida* cells was assessed by comparing their growth with the macrophage-free control wells using an XTT (Sigma-Aldrich) dye reduction assay. The XTT system is a means of measuring the activity of living cells via mitochondrial dehydrogenases. The key component is the sodium salt of (2,3-bis[2-Methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide inner salt) XTT. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring of XTT yielding orange formazan crystals which are soluble in aqueous solutions. The resulting orange solution is spectrophotometrically measured. An increase or decrease in cell numbers results in a concomitant change in the amount of formazan formed, indicating the degree of cytotoxicity caused by the test material. Briefly, each well was washed three times with sterile water, which resulted in lysis of the macrophages in those wells containing monolayers. Cells were then incubated with 200 μl of a 400 mg ml-1 XTT solution containing coenzyme Q (50 mg ml-1) for 45 min. Control incubations with wells containing lysed macrophages in the absence of *Candida* cells were also performed to demonstrate that lysed macrophages alone had no XTT reduction activity. Following incubation, 100 μl of the reduced dye solution was removed and the absorbance measured at 480 nm in a Tecan Genios microplatereader (Tecan UK, Reading, UK). We have previously demonstrated a linear relationship between absorbance of XTT at 480 nm and the number of viable *Candida* cells between 5×10^4 and 5×10^7 (Fig 2.2). Absorbance values were corrected against a cell-free blank and the percentage growth of each *Candida* strains was determined relative to the positive growth control (100%) for that MOI. Statistical analysis was carried out using the 2way ANOVA test, P values smaller than 0.05 were considered to statistically significant. Each experiment was performed on at least four occasions.

Staining of macrophages infected with *Candida* with acridine orange was carried out with infected macrophage monolayers grown on sterile glass coverslips. The medium was removed and a solution of acridine orange (10 mg ml^{-1}) in PBS was added to duplicate wells and incubated for 10 min. The stain was then removed and the cells washed once in PBS. 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).

2.8.3 Infection of RHE tissues with *Candida*

Reconstituted human oral epithelial tissues were purchased from Skinethic Laboratories (Nice, France) and used as described previously (Schaller *et al.*, 1999; Stokes *et al.*, 2007). Prior to arrival of the tissues *Candida* strains were grown on PDA for 48 h at room temperature, following this several colonies were resuspended in PBS and washed twice. Subsequently 10 ml of YPD broth were inoculated with 2 x 10^5 cells and this inoculum was incubated at 25°C with shaking at 250 rpm overnight. Upon delivery of the RHE tissues, they were placed immediately into 1 ml of maintenance medium (without antibiotics) and these were incubated overnight at 37 °C, 5% CO_2. Following this 10 ml of YPD broth were inoculated with 4 x 10^6 cells from the 25°C culture, and these were incubated overnight at 37°C with shaking at 250 rpm. The following day the overnight *Candida* cultures were washed thrice in PBS and resuspended in 1 ml PBS to a concentration of 4 x 10^7 cells. Reconstituted epithelium samples (0.5 cm^2) were then inoculated with 50 µl of this inoculum, controls were inoculated with 50 µl of PBS. Inoculated cultures were incubated at 37°C, 5% (v/v) CO_2 at 100% humidity for 6 h, 12 h, and 24 h.

Prior to sectioning and staining for light microscopy, RHE tissues were fixed in 4% (v/v) paraformaldehyde in PBS (pH 7.4), dehydrated in ethanol and embedded in paraffin wax. Sections were stained with Periodic Acid Schiff (PAS) reagent for visualization of fungal elements (Luna, 1968). Tissues were examined using a Nikon Eclipse 600 microscope.

The release of lactate dehydrogenase (LDH) from epithelial cells into the cell-culture medium was measured to quantify the extent of epithelial cell damage. The CytoTox 96® nonradioactive cytotoxicity assay (Promega, Madison, WI) was used to measure the amount of LDH in each sample. The CytoTox 96® assay quantitatively measures LDH a stable cytosolioc enzyme that is released upon cell lysis. Released LDH in culture supernatants is measured with a 30 min coupled enzyme assay, which results in the conversion of a tetrazolium salt (INT) into a red formazan product. The amount of colour formed is proportional to the number of lysed cells. The reaction was assayed at 480 nm using a Genios plate reader (Tecan UK). One unit of LDH activity is equivalent to 1 mM formazan formed per reaction. For statistical comparison *t* tests were used and P values smaller than 0.05 were considered to be statistically significant. Infections were carried out on two separate occasions.

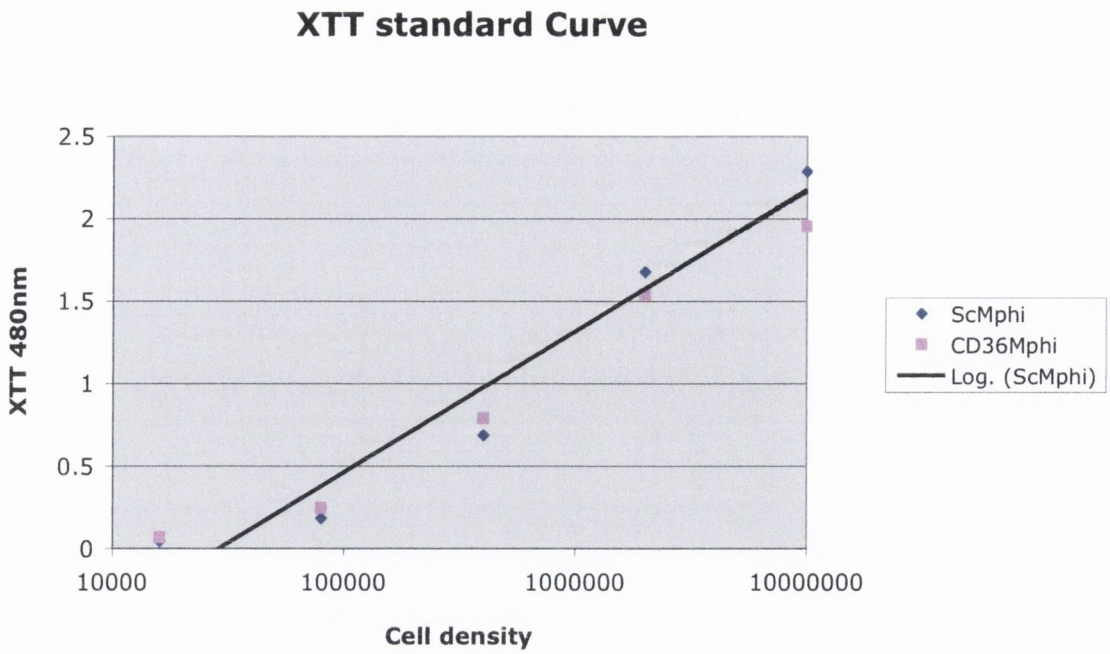

Figure 2.2. Standard curve generated to demonstrate the linear relationship between absorbance of XTT and the number of viable *Candida* cells

Chapter 3

Identification and Expression of a *Candida dubliniensis* *EDT1* Orthologue

3.0 Introduction

Candida albicans and *Candida dubliniensis* are genetically very similar and share many traits associated with virulence in *C. albicans*. *Candida albicans* was one of the first eukaryotic pathogens selected for genome sequencing. In October 1996 the Stanford Genome Technology Centre supported by grants from the NIDR, NIH, and the Burroughs Wellcome Fund, began sequencing of strain SC5314 of *Candida albicans*. This project was completed in May 2004 (www-sequence.stanford.edu/group/candida/). The completion of the *C. albicans* genome and its annotation has provided researchers with an important tool for the analysis of this human pathogen. *Candida dubliniensis* was first identified and described in 1995 and now more than 10 years on after its original identification the nucleotide sequence of the *C. dubliniensis* genome has been completed (see www.sanger.ac.uk/sequencing/Candida/dubliniensis/). It is hoped that the ongoing *C. dubliniensis* project will allow for a complete comparison between the genomes of the two species to be performed in order to determine how virulence has evolved between these two similar species.

The completion of the *C. albicans* genome project and the construction of DNA microarrays has allowed genomes of non-sequenced strains to be compared. *C. albicans* microarrays were used by Moran *et al.* (2004), to attempt a comparison of the entire *C. albicans* and *C. dubliniensis* genomes. In this study total genomic DNA from *C. albicans* and *C. dubliniensis* was co-hybridzed 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 process is known as comparative genomic hybridisation (CGH) and was used at the time because there was no genome sequence available for *C. dubliniensis*. This study led to the identification of thousands of orthologues of *C. albicans* genes in *C. dubliniensis* and provided an initial insight into the numbers of genes which are highly divergent or even absent in *C. dubliniensis*. One such ORF deemed absent or highly divergent in *C. dubliniensis* was IPF946/orf19.7561 (Moran *et al.*, 2004). At the time that this current study began, no function had yet been assigned to the *C. albicans* gene. However, subsequently the *C. albicans* orthologue has been described as *EED1* (epithelial escape and dissemination), a regulator of filamentation, which is crucial for virulence during infection of Reconstituted Human Oral Epithelium (RHE) (Zakikhany *et al.*, 2007). In a separate study, orf19.7561 had been described as *EDT1* (*EFG* dependent transcript). In this study, Chen *et al.* (2004)

described *EDT1* as a cell density regulated gene and cited unpublished findings that *EDT1* was a key regulator of a filamentation in *C. albicans*. It was observed that the level of *EDT1* mRNA increased with cell density relative to rRNA controls. In the same study, Chen *et al.*, (2004) identified tyrosol as a density-sensing molecule which plays an important role in both growth and morphogenesis in *C. albicans*. *EDT1* mRNA levels were shown to be induced by tyrosol. In a previous study by Hornby *et al.* (2001), another quorum sensing molecule, farnesol, was identified to inhibit the formation of germ tubes in cultures of high cell density. Therefore, this suggests that morphogenesis in *C. albicans* is under positive and negative control by the actions of tyrosol and farnesol.

The aims of this chapter were to identify a *C. dubliniensis* orthologue of *EED1*/*EDT1* using genome sequence analysis tools such as BLAST. Attempts were made to infer a function for *EDT1* based on homology and domain strucutre. Following this, gene expression analysis was carried out to identify whether the *C. dubliniensis* *EDT1* gene was regulated by cell density or hypha formation, using realtime PCR.

3.1 Results

3.1.1 Identification of a *C. dubliniensis EED1* homologue

Data from the comparative genomic hybridisation (CGH) studies carried out by Moran *et al.*, (2004) indicated that the *C. albcans* orf19.7561/ IPF946 may have no orthologue in *C. dubliniensis*, or one which is significantly divergent at the nucleotide sequence level. Since the completion of the CGH study, the entire nucleotide sequence of *C. dubliniensis* CD36 has been determined.

In order to identify a *C. dubliniensis EED1* homologue, a number of nucleotide and amino acid sequence searches of the *C. dubliniensis* genome were carried out. BLAST searches of the *C. dubliniensis* genome sequence with the *C. albicans EDT1* sequence were carried out using both the nucleotide and amino acid sequences (blastn and blastp). These BLAST searches using the *C. dubliniensis* GeneDB server did not yield any significant matches. Further BLAST searches were carried out using the Broad Institute website against three other *Candida* species (*C. guilliermondii*, *C. lusitaniae, C. tropicailis*) and other yeast species to determine whether these species had any orthologous genes to *EED1*. BLAST searches were carried out using the Multi-fungi BLAST tool and once again this yielded no significant matches to *EED1*. A nucleotide BLAST search of the *Candida* Genome database with the *C. albicans EED1* nucleotide sequence identified itself and also similarity to *SSN6* and orf19.6309 which is a functional homolog of *S. cerevisiae* Cyc8p/Ssn6p (E value = 1.9 e -16). The protein BLAST of *EED1* in this database yielded no significant matches, including no matches to the deduced sequences of *SSN6* or orf19.6309.

As no significant matches to *EED1* were identified in any *Candida* genome BLAST searches it was decided to compare the *EED1* locus on chromosome R with the corresponding region in *C. dubliniensis*. This was carried out using the Artemis Comparison Tool (ACT). This tool allows interactive visualisation between genome sequences and associated annotations (Carver *et al.*, 2005). Homologous sequences are joined by coloured bands that represent matching regions (Fig 3.1). This analysis demonstrated that the regions flanking the *EED1* gene in *C. albicans* were highly conserved in *C. dubliniensis*. The regions upstream and downstream of *EED1* were found to be highly conserved on *C. dubliniensis* chromosome R. This included orthologues of the upstream genes orf19.7557 and *YTA6* and downstream genes *BET2*

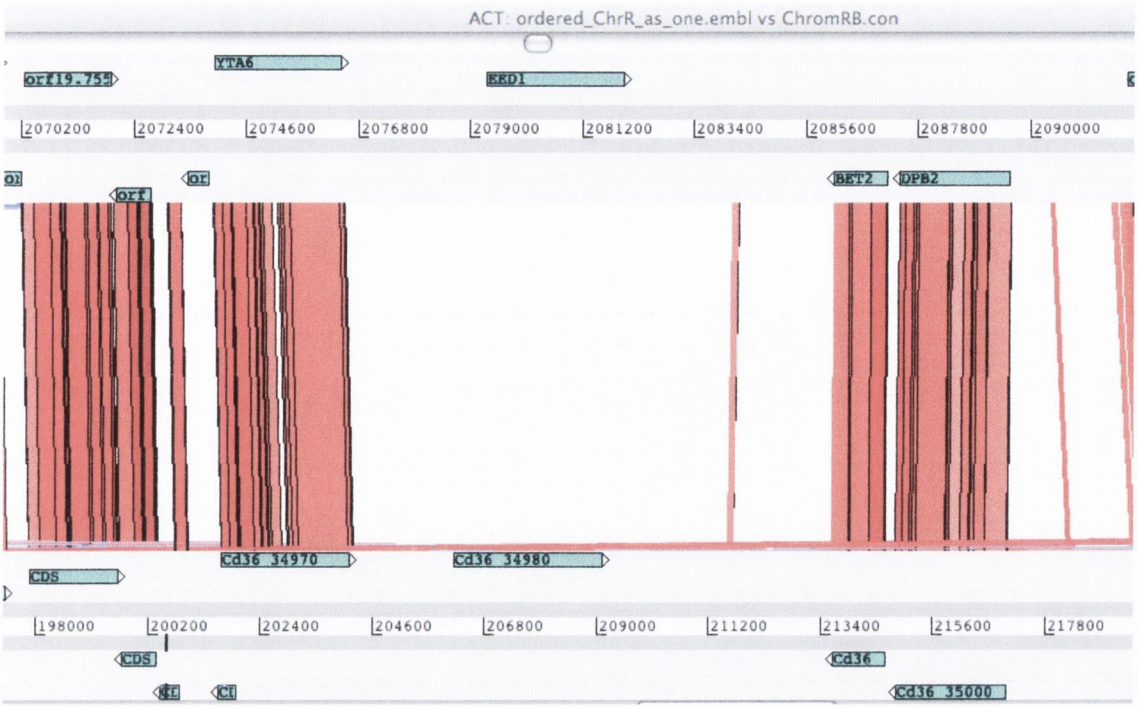

Figure 3.1 An alignment of the region of chromosome R from *C. albicans* (top) containing *EED1* with the same region of *C. dubliniensis* chromosome R (bottom) generated with the Artemis comparison tool (ACT). Red lines connect sequences with high similarity. Absent match lines indicate an area of low homology. Genes are indicated in blue, where a possible gene function has been assigned and genes that do not yet have an assigned function are indicated in black.

and *DPB2* (Fig 3.1). The intervening region containing *EED1* was poorly conserved in *C. dubliniensis*. However, a significant ORF could be identified in this region of *C. dubliniensis* chromosome R, termed Cd36_34980. It was investigated whether Cd36_34980 could be a divergent orthologue of *EED1*

3.1.2 Analysis of a putative *EED1* orthologue in *C. dubliniensis*

A protein alignment of Eed1p and Cd36_34980 (identified using ACT) was carried out using EMBOSS pairwise alignment tool. As shown in figure 3.2, the *C. dubliniensis* protein sequence contained a high percentage of proline (14.3 %) and glutamine (19.8 %) residues. Interestingly the *C. albicans* protein also had a high percentage of proline (10.1 %) and glutamine residues (16.2%) (Table 3.1). The alignment demonstrated that the proteins shared only 22% identity and 32 % similarity. Interpro was utilised to identify putative functional sites in these unknown protein sequences (Mulder *et al.*, 2007). This search identified a Myb-like DNA binding domain in *C. dubliniensis* Cd36_34980 (754 aa to 802 aa) (Fig 3.2). Following identification of this Myb-like DNA binding domain in Cd36_34980, the protein sequences of both Cd36_34980 and *EED1* were used to interrogate the Pfam database. Pfam also identified this Myb-like DNA binding domain in the same region (754 aa to 802 aa) of Cd36_34980. In *EED1*p, Pfam identified a match to itself (Edt1p) but no other significant domains were found (Fig 3.3). Both protein sequences were used to interrogate the Broad Institute Multi-fungi BLAST tool. The BLAST results from this website for Eed1p found that this protein had no significant matches to any other proteins. However Cd36_34980 had similarity to proteins from other yeast species. This protein had similarity to a *Pichia guilliermondii* hypothetical protein thought to play a role in translation (PGUG_00122.1; E value = 1e-06) and to a *C. tropicalis* hypothetical protein similar to a potential DNA binding protein (CTRG_00932.2; E value = 6e-05). Another protein to have similarity to Cd36_34980 was *Saccharomyces cerevisiae* disrupter of telomere silencing protein 6 (SCRG_04565/ *DOT6*; E value = 5e-04). Telomere silencing proteins in yeast are thought to play a role as both transcriptional activators and also as repressors. Telomeric DNA-binding proteins such as Dot6p contain at least one Myb-like DNA binding domain (Giaever *et al.*, 2002). All matches in these proteins identified from this BLAST search were to the 750-800 aa region of Cd36_34980, which has been identified as a Myb-like DNA binding domain (Fig 3.4).

In order to compare the *EED1* locus in *C. albicans* and *C. dubliniensis* with that of other *Candida* species, the *Candida* Gene Order Browser (CGOB) was used. This tool was used to select the *C. albicans EED1* gene and display the neighbouring genes in the genome as well as the syntenic regions of other *Candida* and yeast genomes. Nine genomes currently feature in the *Candida* Gene Order Browser. These are *C. albicans, C. parapsilosis, C. dubliniensis, Lodderomyces elongisporus, C. lusitaniae, Pichia guilliermondii, P. stipitis, C. tropicalis, Debaryomyces hansenii* and *Saccharomyces cerevisiae.* Following searches of the CGOB the *EED1* gene in *C. albicans* was identified (identified in this browser as *DEF1*) and the regions upstream and downstream of *EED1* (Fig.3.5). From the browser window, the genes upstream *YTA6* and downstream *BET2* are conserved among all the yeast species. However the intervening regions containing *EED1* are poorly conserved. However CGOB did identify a syntenic *C. dubliniensis* gene Cd36_34980, which was previously identified at this location using ACT. This program identified a syntenic gene in *D. hanseii* (DEHAOE3597g), *P. stipitis* (Pstip 28806), *C. guilliermondii* (PGUG_00122.1), *L. elongisporus* (LELG_05325) and *C. parapsolosis* (CP_02994). The CGOB also identified a syntenic gene in *C. albicans* WO-1 strain, however on further analysis of this sequence it was found that the sequence was of poor quality and a complete ORF could not be identified. A syntenic gene was also identified in *C. tropicalis* and as before, further analysis revealed that the sequence was of poor quality and a complete ORF could not be identified. Following the identification of these syntenic genes Pfam searches were carried out, using the respective amino acid sequences of these proteins. This search identified a Myb-like DNA binding domain in all of these proteins, with high homology to the Myb-like DNA binding domain in Cd36_34980 (Fig 3.6). Clustal W analysis and phylogenetic analysis were carried out to examine the relationship between these proteins containing this Myb-like DNA binding domain. Firstly a protein alignment was carried out on the entire proteins of these syntenic sequences using Clustal W (Fig 3.7). This alignment shows that, overall, there is little homology between the proteins encoded in each species. Interestingly, the protein sequences of each species are high in both proline and glutamine (Table 3.2). The alignment does show that, regions of homology between all species in the Myb-like DNA binding domain and it is evident that all species have a conserved C- terminal motif (IXXLLN). The phylogenetic tree generated demonstrates how divergent the *C. albicans EED1* gene is compared to the other syntenic genes identified in the same region by CGOB

Table 3.1 Percentage of amino acids present in Eed1p and CD36_34980

Gene Name	Amino Acid	Abbreviated Name	Percentage Present in Sequence
***EED1* (887 aa)**	Arginine	arg R	6.8
	Glutamine	gln Q	**16.2**
	Leucine	leu L	6.9
	Proline	pro P	**10.1**
	Serine	ser S	9.8
CD36_34980 (964 aa)	Arginine	arg R	5.9
	Glutamine	gln Q	**19.8**
	Leucine	leu L	7.7
	Proline	pro P	**14.3**
	Serine	ser S	8.9

```
Length: 1173
Identity:     261/1173 (22.3%)
Similarity:   367/1173 (31.3%)
Gaps:         495/1173 (42.2%)
Score: 522.5

CaEED1              1 MERRQFNTSNIRNGTGRPRKTPRSKLYMVYPPL-----------------     33
                                .|||.||    ||..
CD36_34980          1                 MKTPNSK----YPTSPASTPERNTSVTIAEST     28
CaEED1             34 --SGEDSTNPEPEEGSSQENNPTEPSSSQSNSVQNQDQSEDQSQLPQQEL     81
                      ||::|||.|.|:...|.:.|.|..||||||    .:...:.|||||..
CD36_34980         29 VPSGQNSTNSEQEQNRLQASAPLESGSSQSNS----PRDPVEGQLPQQAP     74
CaEED1             82 NTQQELNTQQELNTPS---------------PRAS--------------     101
                      .|.........::.||              |:.|
CD36_34980         75 ETPPPQEPTTPVSGPSAAPANEVPSNGKEAPPKKSTLSGRSLWEKDEFGF     124
CaEED1            102 ----------------NTSTETPAPLSPIQPGIRNIPLGLLLPQEKVGR     134
                              |..:.||:|::.|.|||      |..|.||
CD36_34980        125 YVAFREAVTKEHRIKDENGKSRTPSPITGIPPGI------LTPPGEK---     165
CaEED1            135 LMGYPFYRDFNFTLNPERYQKLIYVFQILKNA-ARNHRNGASLLRKYFLL     183
                                  .::.::|.. |..|...||::.  |:.
CD36_34980        166 ----------------------SIYPMVKMCKAVPHMMTASMVN--FVD     190
CaEED1            184 ARRSKRTTDMFVTTIEEMRKRLLENSRKRELEEAQERE-ESNKRQHTESS     232
                      .:|..          :||.|.:.::|     :||.|.. |.:..|..::|
CD36_34980        191 GQRDG----------DEMYKMIKQSS-----QEASEHPGEGSGYQRNKNS     225
CaEED1            233 AEPNAELSTESTTESNAESGAEPNAEPSAESTTESNVESGAEPNAE--SG     280
                      .:|:::.|             .|...||        :.:.|:|:.:  .|
CD36_34980        226 RQPSSQTS-------------PPKQAPS--------ILTAAQPHKKHCPG     254
CaEED1            281 AESGAEPTAESNAELKQRIWEILSYRLEQSNNETNNTGESNSTSQQPRQ-     329
                      ..|...|                ||:..|......:...|...|..|.|
CD36_34980        255 GTSKNPP---------------SYQSLQRPAPVKHEKASKYYSFGPLQK     288
CaEED1            330 LPNNELIMNIRVLQKNTHAKPVLGRIKFTPDKSNKTSLTGLQNKVHSTNT     379
                      ||.       |..|......|.|
CD36_34980        289 LPP-------RPWQPAPAPPPPL--------------------------     304
CaEED1            380 QQSQKHPQQILTNSETHKPQQYLAQLQQQMVHQTNSHEPLQKRSPPPQQQ     429
                            ||:....|....|||...|.|||.: |...:.|.|...||.|||
CD36_34980        305 ------PQRRRQPSPPPPPQQPQQQQQQQQL-QPMPNLPQQPCEPPVQQQ     347
CaEED1            430 Q---QKQPSVPTSSVPLQVSQKQNQQQQELPLPPQPQPQQRTAPSAVKQQ     476
                      |   ..|......|||.:  |:|::|.|  ||.|:..|||:.......||
CD36_34980        348 QLPRSPQREQQHGSVPFE--QQQSRQLQ--PLAPEAGPQQQVQLQLQLQQ     393
CaEED1            477 QLMQMQ--PPPQQQQQQQRHQPLQQSPPTMPL----QQQPVPPVQQVQTV     520
                      .|.|.|  ||.||||||.|:..|..|..|..||    |||.:.|..|.|..
CD36_34980        394 VLQQSQQLPPSQQQQQSQQLPPPPQQQPPQPLEKQQQQQHLQPQPQPQPQ     443
CaEED1            521 PPPLLQPQTQLSQQQQQQQQQAQLQMQVPRCYQYQNRPSSQQRQYSQTPQY     570
                      |.|..|||.|..|||||||||.|.::.    .|.|:|...|:.::.:..|.
CD36_34980        444 PQPQQQPQQQPQQQQQQQQQQTELS----SQQQSRQQQQRPRHRRLEQL     489
CaEED1            571 NQPPPQQKVY-----------------------------------AL-P     583
                      .|...::|.|                                     || |
CD36_34980        490 QQQERREKSYMSPALRDFRDLIKELEKQQHQQRNIIQKPKRKRNSNALRP     539
CaEED1            584 PQQVYAPPPRQVYAQP-TIACKQQYPQQLYEQAPQEGLSYQHHYQQVQQR     632
                      |||     .:|:..|| .::|.|:..|.|.|...|..|..|...:|:||.
CD36_34980        540 PQQ-----SKQLQVQPHHLSCVQEQLQDLQEIQQQLNLQQQQQPRQLQQL     584
CaEED1            633 QNQQPYMQSAPTYQQPHVQTPKSTRSNKQEKQRLPKGQEQVPKATRTMFE     682
                      |..:...:.....|..:             :|.|.|.|:|
CD36_34980        585 QQLRQLQEQLDLLLQQLL----------RQELQKQQQQ----------     612
CaEED1            683 AFTGLNIAVEKLRQRTLDNGREPERLRTEYVN----VLSLPERAAEKSTS     728
                          ||:..::|.|         ::.:..||.    .:|..:|..::..:
CD36_34980        613 ----LNLQQQQLNQ---------QQQQQSYVQAPQPTISYQQQQIQQPVA     649
CaEED1            729 RSKQSSNQKPVVKQQSSFPPPIKHQQTQE-------------QQGNILP     764
                      ::.|.|    :|.||...||.::.|.:|:             :|..|||
CD36_34980        650 QASQGS----LVYQQPQIPPQVQAQPSQQYQQPYAQVNLQSYNEQPAILP     695
CaEED1            765 PVSQLL-------------------------------------------     770
                      ||||||
CD36_34980        696 PVSQLLSRPSRLPPSSKLPPPSQLPLQQGLPVYSQQSGPGIFNTFNGNDL     745
CaEED1            771 ------------AIQSSTVTSR--------------------------     780
                             :||...|..|
CD36_34980        746 AVVGVPRRGVGSWSIQEDHVLWRLKKDHNFGWEQIAKYFLDRTASACESR     795
CaEED1            781 -------GSNASGAVMGSGN------TQRVASRSFTNTFVAEAVVNNANN     817
                            ||.....::|:.|      ...:.:.........|.:.||||.
CD36_34980        796 WKKIFESGSRLGKNIVGNSNINGNNGASNIGTGLSAGATTTETMANNANG     845
CaEED1            818 R-------------------------------------------GGPVP     823
                      |                                           ..|.|
CD36_34980        846 RVEPSPVPAPAPAPAPAPTMTLRPAPAPAPAPALALTLVPAPAPAPAPAP     895
CaEED1            824 PTGPETNTRGGRASTRSSGRPR--GNRSTQRAEGNVTGRVARSTDGS---     868
                      |..|:||||..|...|...|.:  |...|::.....|   .|||.||
CD36_34980        896 PPVPKTNTRETRRKRRGGARGKYGGRNKTRKVREETT---TRSTTGSSND     942
CaEED1            869 ----QSQNSGKASKISNIRNLLN    887
                          ||:.||:.||.|.|.:||
CD36_34980        943 SPRLQSRQSGRISKPSIINSLL     964
```

Figure 3.2. Protein alignment of *CaEED1* (top) and Cd36_34980 (bottom) using EMBOSS pairwise alignment tool. The highlighted region (red) in Cd36_34980 corresponds to a Myb-like DNA binding domain identified using ClustalW. Pairwise scores are calculated as the number of identities in the best alignment divided by the number of residues compared (gap positions are excluded). Both of these scores are initially calculated as percent identity scores and are converted to distances by dividing by 100 and subtracting from 1.0 to give number of differences per site.
Alignment score is calculated in two ways - fast and slow (more accurate mode). The scores are calculated from separate pairwise alignments. These can be calculated using 2 methods: dynamic programming (slow but accurate) or by the method of Wilbur and Lipman (extremely fast but approximate) (Myers & Miller, 1988; Wilbur and Lipman, 1983).

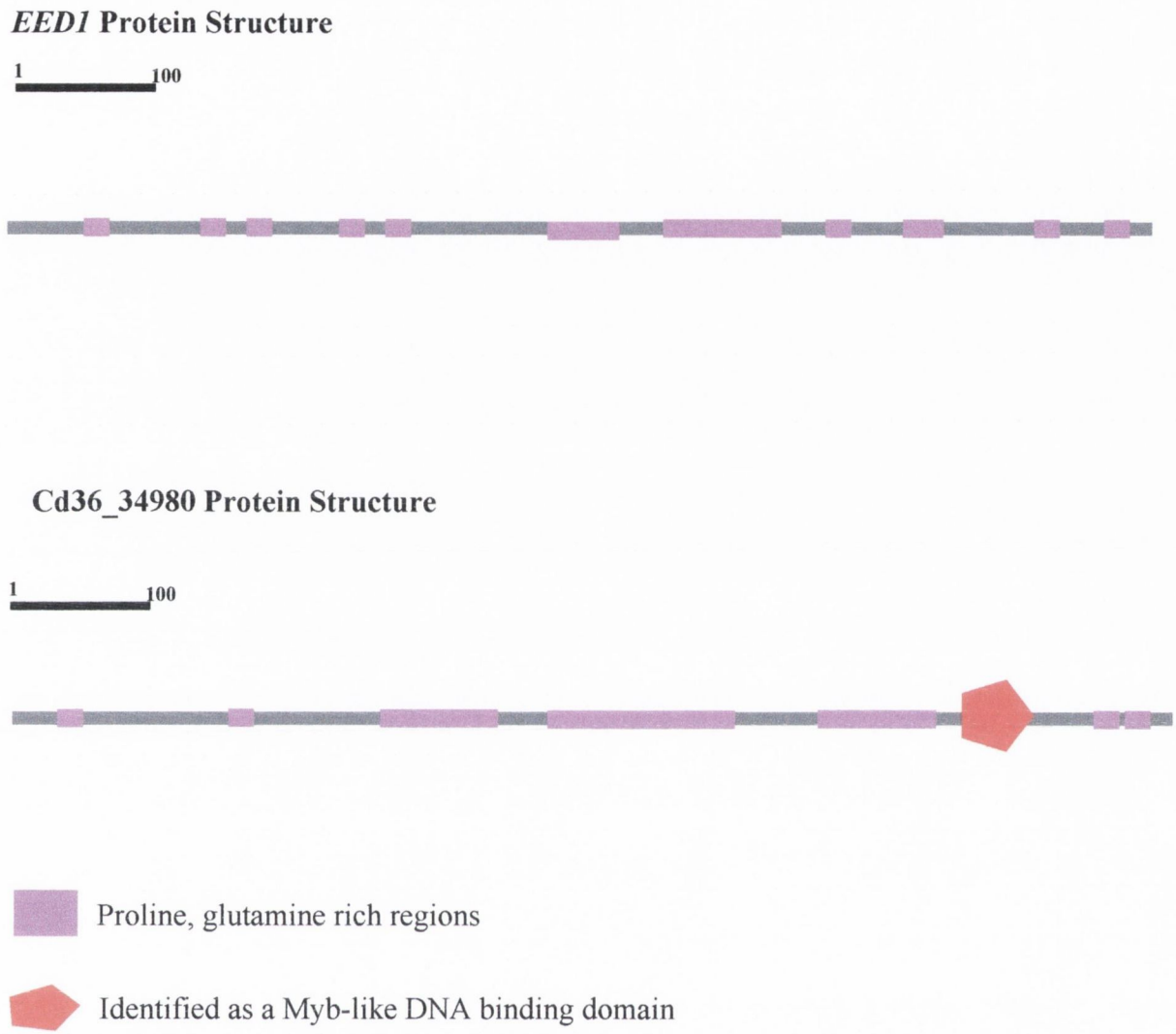

Figure 3.3. Pfam sequence search results of the Eed1p and the Cd36_34980 proteins. No significant domains were found in Eed1p. In Cd36_34980 a Myb-like DNA binding domain was identified from 754-802 aa.

SeqA	Name	Len(aa)	SeqB	Name	Len(aa)	Score
3	CTRG_00932	595	4	CAWG_01508	600	71
1	PGUG_00122	175	2	LELG_05325	561	34
1	PGUG_00122	175	5	Cd36_34980	964	28
1	PGUG_00122	175	6	SCRG_04565	669	23
4	CAWG_01508	600	6	SCRG_04565	669	20
1	PGUG_00122	175	3	CTRG_00932	595	18
2	LELG_05325	561	5	Cd36_34980	964	18
3	CTRG_00932	595	6	SCRG_04565	669	18
1	PGUG_00122	175	4	CAWG_01508	600	17
2	LELG_05325	561	6	SCRG_04565	669	11
2	LELG_05325	561	3	CTRG_00932	595	8
2	LELG_05325	561	4	CAWG_01508	600	8
3	CTRG_00932	595	5	Cd36_34980	964	7
4	CAWG_01508	600	5	Cd36_34980	964	7
5	Cd36_34980	964	6	SCRG_04565	669	6

Species	Gene Name
C. tropicalis	CTRG_00932.2
P. guilliermondii	PGUG_00122.1
S. cerevisiae	SCRG_04565
C. albicans	CAWG_01508
C. dubliniensis	Cd36_34980

```
PGUG_00122      ------------------------------------------------------------
LELG_05325      -MALLMSNRNVFNSPRLESSPSPAAY---LPGSTNQLRTQPQDELQQINPSKRRKLSLVP  56
CTRG_00932      ----------MSSAEVDSNTTSDSK----SGSTAAATTTTTQKNPSRTPTSWDAEDDIL  45
CAWG_01508      ----------MSSPEIEASGSSESK----SGS-------SSQKNPSRTPTSWDAEDDIL  38
CLUG_00389      DVPTPNLGTDRLHSRTFDSISSALKNNMANESSDPTPSASPPSKNFSRTPTSWDASDDVL  67
Cd36_34980      PPPLPQRRRQPSPPPPQQPQQQQQQQQLQPMPNLPQQPCEPPVQQQQLPRSPQREQQHG 360
SCRG_04565      LHSLTRQPHSSSTAMSKNEAQESSPSLPASSSSSTSASASASSKNSSKNPSSWDPQDDLL  81

PGUG_00122      ------------------------------------------------------------
LELG_05325      TTPLNIMNEYSTQTQASGNHTAKSIGLRLPSPSSLLNSFTSGNDSSSAQ-APTIPITTGL 115
CTRG_00932      LMHLKDNQKLGWKEIASHFNNRTPNACQFRWRRLKSGNLKNPPKSAAAL-GAQFKQNPNM 104
CAWG_01508      LMHLKDNQKLGWKEIASNFTNRTPNACQFRWRRLKSGNLKNPPKSAAAL-GAQFKQNPNM  97
CLUG_00389      LMHLKDNQKLGWKEIASHFHNRTPNACQFRWRRLKSGNLKNPPKSAAAI-GSLAATSITE 126
Cd36_34980      SVPFEQQQSRQLQPLAPEAGPQQQVQLQLQLQQVLQQSQQLPPSQQQQQ-SQQLPPPPQQ 419
SCRG_04565      LRHLKEVKKMGWKDISQYFPNRTPNACQFRWRRLKSGNLKSNKTALIDINTYTGPLKITH 141

PGUG_00122      ------------------------------------------------------------
LELG_05325      TGSTGTWVGEGGAQTGVAVAEYGPPH---------MVRQN---------------------- 146
CTRG_00932      NAATKKKKATP-TPTSSSSSSSTTKN---------KENKN-TNSNLNDSIG---NISRED 150
CAWG_01508      NSAPKKKKATTKNNKSTTESTDNTTE---------KEAKSGKVSKTPKSSGPTNNGSKGN 148
CLUG_00389      TPSPSQ------GPGSPAGSAPKKAP---------KRRKS-------------------E 152
Cd36_34980      QPPQPLEKQQQQQHLQPQPQPQPQPQPQQQPQQQPQQQQQQQQQQQQTELSSQQQSRQQQQ 479
SCRG_04565      GDETTNAQQKPSKKVEENVLTEDTAEFTTTSSIPIPSRKTSLPSFHGSMSFSQSPSNVTP 201

PGUG_00122      ------------------------------------------------------------
LELG_05325      -------------------IRPSYIEGYSYPNQYSHEQYRPSVSLIPDASHHHHHHQHQH 187
CTRG_00932      TASQTPTSNSTSN-FTYNPMTTGTFQGFDNSISTALAGLNALSNSPSYISGSSPPTPRGG 209
CAWG_01508      TPSSSSNSTTNSNNFTYNPMTTGTFQGFDNNISTALAGLNALSNSPSYISNFSPATPKG- 207
CLUG_00389      TPTSSLAP-------VYSPLANTTFTGYD-TVSTALAGLSALSST----SGASVGSQQSG 200
Cd36_34980      RPRHRRLEQLQQQERREKSYMSPALRDFRDLIKELEKQQHQQRNIIQKPKRKRNSNALRP 539
SCRG_04565      TTIVSNAASSMPFAPPTLPAALPHHPHQHLHHHPHHKTLKPRSNSHSFTNSLNQDHIVRS 261

PGUG_00122      -------------------------MAQAIRPFPPSMPYG-------------------I  16
LELG_05325      QHQHQPVQLQ------HQLPHQLHDPTKVQLPMSTSQSYSPTIAQPGASGQREGPIGGQV 241
CTRG_00932      MTSPNIRGS---ISG-TSLSHRYEASPGASGIGDDRRRSSVNGPTS----SNGGYYVDVS 261
CAWG_01508      LTSPTIHGSGPHHSGNPNLAHKYEASPASDRSLDQRRRSSATGSVSGHRNSNGGYYVDVS 267
CLUG_00389      LINT------------TSHPQTFDNSPKSDVALDPQTHSAAAAGTS------GGYFTDIS 242
Cd36_34980      PQQSKQLQVQP--HHLSCVQEQLQDLQEIQQQLNLQQQQQPRQLQQLQQLRQLQEQLDLL 597
SCRG_04565      NDEEKYGFIPKVFVRSRRSSFAYPQQVAITTTPSSPNSSHVLLSSKSRRGSLANWSRRSS 321

PGUG_00122      NMGPALPPHPQSYQFQPPAPLSYDAHSRQSSF-------------------VYGANTS---  55
LELG_05325      YAPPMQDPQSQAHQYMPYQPLQMETTNQPMQQ-------------------VLQVSQVPAP 283
CTRG_00932      VDPTMNLPHNKSHPTTPSA-LTPRNSTSGADKASNLATNHRASISGIASISALRNNSIIQ 320
CAWG_01508      VDPTMNLPHNKSHPTTPSS-LTPRNSTSAGDKGSNFT--HRGSISGVANISALRSNSIIQ 324
CLUG_00389      VDPTSNLPHNRPHPVPMSSGLTPRSSNVDQHT-------------------MPIHNNSIIQ 284
Cd36_34980      LQQLLRQELQKQQQQLNLQQQQLNQQQQQQSYVQAPQP-------TISYQQQQIQQPVAQ 650
SCRG_04565      FNISSNNTSRRSSMILAPNSVSNIFNVNNSSSNTASTS----------NTNSRRESVIKK 371
                              :                                        .

PGUG_00122      -MQPMFPQPSHVEHASAAAAASTP-YYYPQHSQ--------PQYIATYYQTDERPIRR-- 103
LELG_05325      PLPPLPPQPQPQPPSQMPNTYQMP-IVQPQTDLYTQTYSQGPQYYYVAPHQAQAPQHQGF 342
CTRG_00932      FTTDERGSISSLGRASVSSLPSKS-MKIPHHESGNTALAHLPVLFGGTGGSVSGPSRNSS 379
CAWG_01508      ITTDERGSISSMGRASVSSLPSKS-MNIPHHQSGNNSLAHLPVLFGGTG-SISGPSRHSS 382
CLUG_00389      IVRDDRTSVSSQSRHSMSSLPSNS-MNIPHHQSNNNALAHLPVLFGGNS--ISGPSRNTS 341
Cd36_34980      ASQGSLVYQQPQIPPQVQAQPSQQ-YQQPYAQVNLQSYNEQPAILPPVSQLLSRPSRLPP 709
SCRG_04565      EFQQRLNNLSNSGGPTSNNGPIFPNSYTFMDLPHSSSVSSSSTLHKSKRGSFSGHSMKSS 431
                          .                                    .         .

PGUG_00122      ------------------------------------------------KSKSKQSLSWTP 115
LELG_05325      APQVQQEMT--QAQQLHYLHGGHMVPGFAGVSSTFSPGMYHSEPMAHLRRRTKQSSTWSP 400
CTRG_00932      VSG-ASGLQ--QSALSSLRNGSTANSTTGYFSRSGSVVIPHNADKKDEEPLKFDKERSEM 436
CAWG_01508      VSGGSVGGQ--QTTLPSLRSGSVVGSNIGYFSRSGSVVIPHTADKKEEEPFKFDKERLEA 440
CLUG_00389      ISG-PTGLPHLASTLSSIRNGSIVGPPQAYLRRS-SMSVVHTERRGSEKDVKNEKSKVKE 399
Cd36_34980      SSKLPPPSQ---------LPLQQGLPVYSQQSGPGIFNTFNGNDLAVVGVPRRGVGSWSI 760
SCRG_04565      CNPTNLWSKDEDALLMENKKRNLSVMELSILLPQRTEVEIQWRLNALSSDADMLSPTHSP 491

PGUG_00122      QEDKLLLELKDVHKLGWREISNHFKDRTANACQFRWRRIVSGIT---------------- 159
LELG_05325      QEDKLLRELKEVQKLGWREISTFFHDRTPNACQFRWRRIISSLASLHQTQSSG--GNGGG 458
CTRG_00932      DNKKFNKIRRNMPPSTVSNRTKRKPGTPQPKLDIPWTMEEDELLINRRNRE----LSFAE 492
CAWG_01508      SNKKLQKIRRNMPPS--RTKTKRKPGTPIPKLDIPWTMEEDELLINRRNRE----LSFAE 494
CLUG_00389      QENEKESSER--------SETPKKSASTQPIFKLPWSMEEDELLINRRRKE----LSFAE 447
Cd36_34980      QEDHVLWRLKKDHNFGWEQIAKYFLDRTASACESRWKKIFESGSRLGKNIVGNSNINGNN 820
```

```
SCRG_04565      QKTLSKKTRPRMFKTGSTTDDDKGSDKEDVMSDGSNDDDEDNVDPLHRAKQSSNKTVFSS 551
                .:                                .       .

PGUG_00122      ---------------------------------PNTSP----------------------- 164
LELG_05325      GSGGLDINATASNYPSGTVGEGSSSNIGSQTMATTSPSIPSRRASVAHVNMIPHTLQPFG 518
CTRG_00932      LSILLPQRTEGEIWARIDHLEKLRNGGHRTANSRDSRRLRQESIGLDDMDDLYDDDDDDD 552
CAWG_01508      LSILLPQRTEGEIWTRIDYLENLRNGGHRSANSRDSRRARQESIGLGDVDGFYDDDDDDD 554
CLUG_00389      LSILLPQRTEGEIWARIDALERLRNG-HRASMSRENRRRRQSSLGLDDVDDFYSEVDSVI 506
Cd36_34980      GASNIGTGLSAGATTTETMANNANGRVEPSPVPAPAPAPAPAPTMTLRPAPAPAPAPALA 880
SCRG_04565      SSSNISSKDVSPDPIFSPDPADDSSNTSDAGSRCTITSDTSSSAATMNRTPNSKNPQDIA 611


PGUG_00122      ---------------------------------AKRHSINYLLN----------------- 175
LELG_05325      EISPRLARSASHTPILETSEEGEEEEEREEKKGKQHKINFLLN----------------- 561
CTRG_00932      --VLRVSDDDDD-VLVDVDDMT---LPKKKKRRMSSAVNPLSVRSTIRK----------- 595
CAWG_01508      DDVLQVSDDDDDEVLVDIDDIP---HPRKKKRRMSSAVNPLSVRGSIRK----------- 600
CLUG_00389      GAIDSDEDDEDEDALLDVDDSIPGQMKRARKRRASSAVNPLSVRDNIRRRL--------- 557
Cd36_34980      LTLVPAPAPAPAPAPPPVPKTNTRETRRKRRGGARGKYGGRNKTRKVREETTTRSTTGSS 940
SCRG_04565      LLNNFRSEAITPRPKPSSTTTSITTENTNNMINHSSSTTTTTNNSPLPSINTIFKDML-- 669


PGUG_00122      ------------------------
LELG_05325      ------------------------
CTRG_00932      ------------------------
CAWG_01508      ------------------------
CLUG_00389      ------------------------
Cd36_34980      NDSPRLQSRQSGRISKPSIINSLL 964
SCRG_04565      ------------------------
```

Fig 3.4. Alignment of Cd36_34980 against sequences that produced significant similarity following BLAST searches on the Broad Institute Web BLAST. The table at the top of the alignment shows the alignment score of each protein identified.

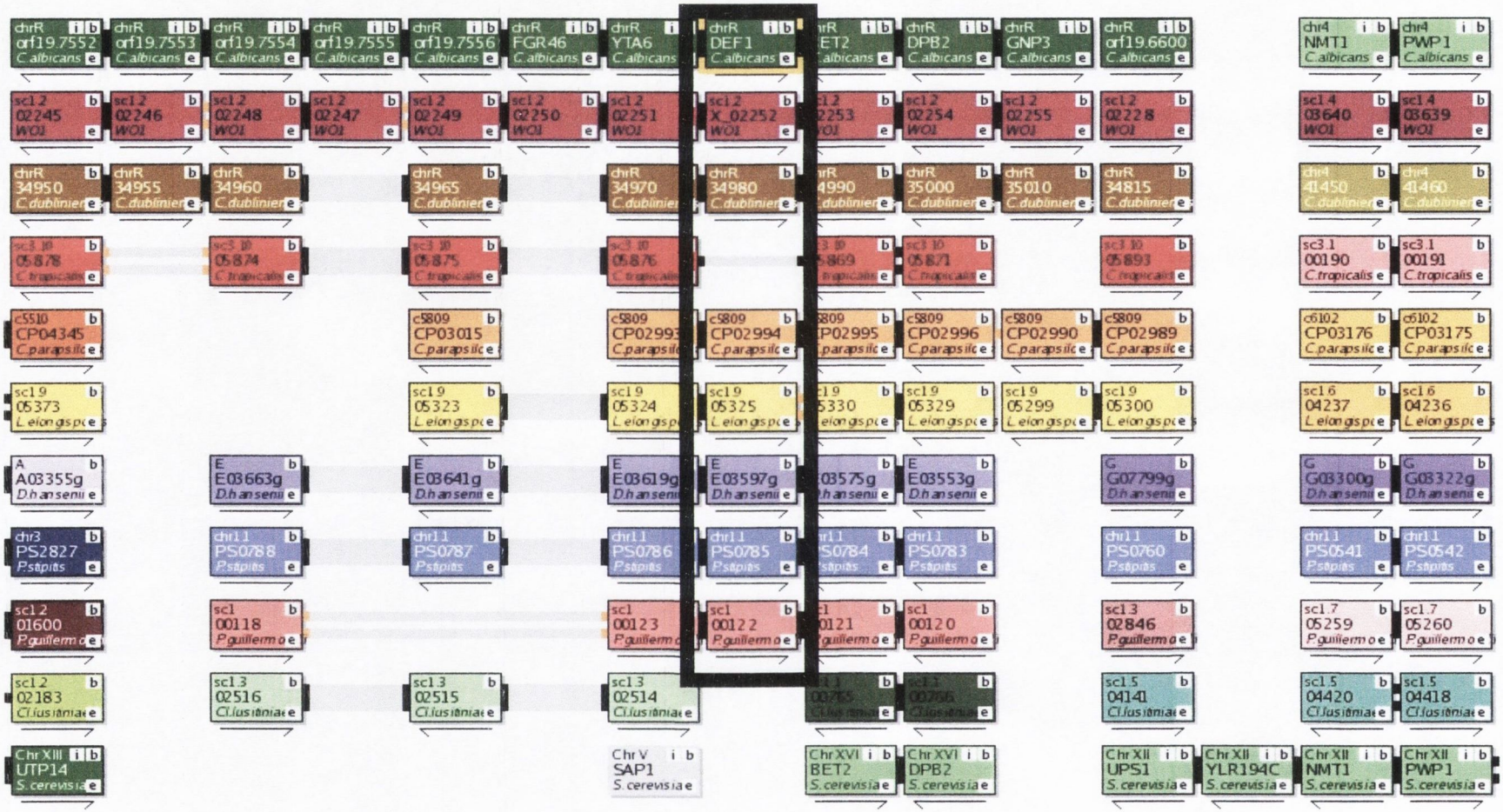

Fig 3.5 The *Candida* Gene Order Browser (CGOB) displaying the *C. albicans EED1* gene (dark green, top) highlighted with a yellow box and the neighboring genes in this genome as well as the syntenic regions of the other selected *Candida* genomes.Each colour represents a different species; *C. albicans* (Dark green top), *C. albicans* WO-1 gene (dark pink), *C. dubliniensis* (brown), *C.tropicalis* (red), *C. parapsolosis* (orange), *L. elongisporus* (yellow), *D. hanseii* (purple), *P. stipitis* (blue) and *P. guilliemondii* (peach). The genes identified as having synteny to *EED1* are outlined in the black box.

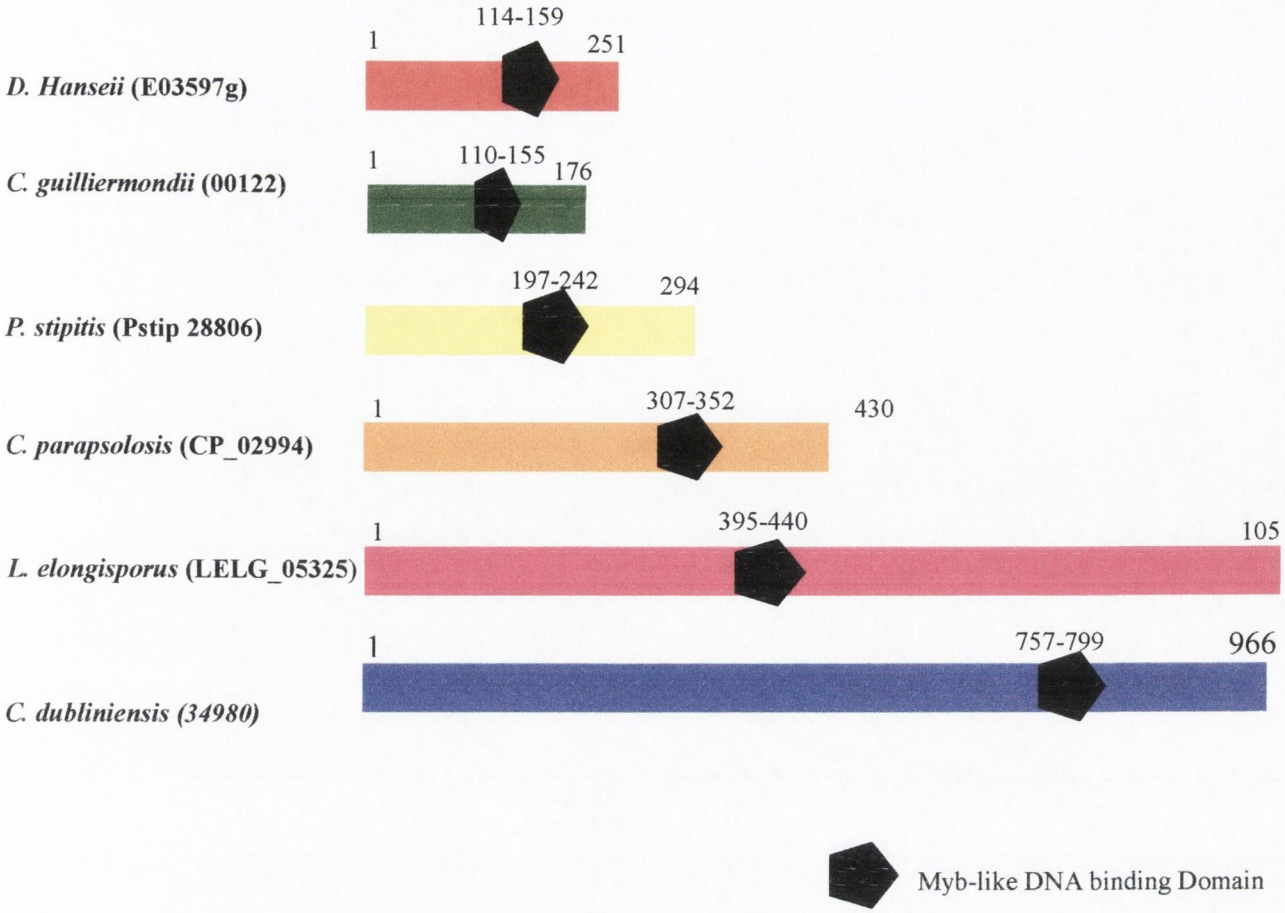

Figure 3.6. Comparison of size and location of Myb-like DNA binding domains (black) in proteins identified as being syntenic with Eed1p using the CGOB.

SeqA	Name	Len(aa)	SeqB	Name	Len(aa)	Score
1	Pstip_28806	293	2	PGUG_0122	175	38
2	PGUG_0122	175	5	CP_02994	429	36
2	PGUG_0122	175	4	LELG_05325	561	34
2	PGUG_0122	175	3	DEHAOE3597g	250	32
3	DEHAOE3597g	250	4	LELG_05325	561	32
3	DEHAOE3597g	250	5	CP_02994	429	30
1	Pstip_28806	293	4	LELG_05325	561	28
1	Pstip_28806	293	5	CP_02994	429	27
4	LELG_05325	561	5	CP_02994	429	27
1	Pstip_28806	293	3	DEHAOE3597g	250	25
2	PGUG_0122	175	6	Cd36_34980	965	25
1	Pstip_28806	293	6	Cd36_34980	965	22
3	DEHAOE3597g	250	6	Cd36_34980	965	22
2	PGUG_0122	175	7	EED1	887	20
5	CP_02994	429	6	Cd36_34980	965	20
6	Cd36_34980	965	7	EED1	887	18
4	LELG_05325	561	6	Cd36_34980	965	17
1	Pstip_28806	293	7	EED1	887	14
4	LELG_05325	561	7	EED1	887	14
5	CP_02994	429	7	EED1	887	14
3	DEHAOE3597g	250	7	EED1	887	9

Species	Gene Name
P. stipitis	Pstip_28806
C. guilliermondii	PGUG_0122
L. elongisporus	LELG_05325
C. dubliniensis	Cd36_34980
D. hanseii	DEHAOE3597g
C. parapsolosis	CP_02994
C. albicans	EED1

```
Pstip28806      ------------------------------------------------------------
PGUG_0122.1     ------------------------------------------------------------
DEHAOE3597g     ------------------------------------------------------------
LELG_05325      ----------------------MALLMSNRNVFNSPRLESSPSPAAYLPGSTNQLRTQP 37
CP_02994        ----------------------MLTLMGNKDIFKPSTFPKRKRKHEQAQVAKPRNLRYK 37
Cd36_34980      SRQPSSQTSPPKQAPSISTAAQPHKKHCPGGTSKNPPSYQSSQRPAPVKHEKASKYYSFG 284
EED1            TESTTESNAESGAEPNAEPSAESTTESNVESGAEPNAESGAESGAEPTAESNAELKQRIW 300

Pstip28806      ------------------------------------------------------------
PGUG_0122.1     ------------------------------------------------------------
DEHAOE3597g     ------------------------------------------------------------
LELG_05325      QDELQQINPSKRRKLSLVPTTPLNIMNEYSTQTQASGNHTAKS------IGLRLPSPSSL 91
CP_02994        PSIEALNILKKPINLGEGDMSEAETSLESVPNPEEGDSRADDS------FSS---QNFQV 88
Cd36_34980      PLQKLPPRPWQPAPAPPPPLPQRRRQPSPPPPPQQPQQQQQQQQSQPMPNSPQQPCEPPV 344
EED1            EILSYRLEQSNNETNNTGESNSTSQQPRQLPNNELIMNIRVLQKNTHAKPVLGRIKFTPD 360

Pstip28806      --------------------MSNYNPNQPPYFGSFGHSRTVRQDSEKFPDIETGKLYEKT 40
PGUG_0122.1     ------------------------------------MAQAIRP----FP----------P 10
DEHAOE3597g     ------------------------------------------------------------
LELG_05325      LNSFTSGNDSSSAQAPTIPITTGLTGSTGTWVGEGGAQTGVAVAEYGPPHMVRQNIRPSY 151
CP_02994        KDKHSSTQGSPSAPLPSSNVLFRSQQNQSPVLPHINIGQNLTLPSR-HVNVSRSQQTESV 147
Cd36_34980      QQQQSPRSPQREQQHGSVPFEQQQSRQLQPSAPEAGPQQQVQSQSQSQQVSQQSQQSPPS 404
EED1            KSNKTSLTGSQNKVHSTNTQQSQKHPQQILTNSETHKPQQYSAQSQQQMVHQTNSHEPSQ 420

Pstip28806      TFSYSQNEQNTSLSPVTETPMYYAARNSNQDKYFLSDSPSHFQSYHPVPPGMPAPTLHHA 100
PGUG_0122.1     SMPYGIN-----MGPA--------------------LPPHPQSYQFQPP---APLSYDA 41
DEHAOE3597g     ------------------------MSYNNHQN-QKYHPYSFRPDQNRHGYYCINQVPILH 35
LELG_05325      IEGYSYPNQYSHEQYRPSVSLIPDASHHHHH-QHQHQHQHQPVQLQHQLPHQLHDPTKV 210
CP_02994        PEAATVVSQQSLPQHMPEQQLPPMHTLQSYPTSQQNLPLQQETRYYYSSSQSQAPAEPYQ 207
Cd36_34980      QQQQQSQQLPPPPQQQPPQPLEKQQQQQHLQPQPQPQPQPQPQQQPQQQPQQQQQQQQQQ 464
EED1            KRSPPPQQQQQQKQPSVPTSSVPLQVSQKQNQQQQELPLPPQPQPQQRTAPSAVKQQQSMQ 480

Pstip28806      ISPIVNENNYPL-----------------------------------QMQAMPHQSQLP 124
PGUG_0122.1     HS-----------------------------------------------RQSSFVYGANTS 55
DEHAOE3597g     LQPPTTQTTIP-------------------------------------PLQIPGIRST 56
LELG_05325      QLPMSTSQSYSPTIAQPGASGQREGPIGGQVYAPPMQDPQSQAHQYMPYQPLQMETTNQP 270
CP_02994        LIPPPQSQTTP-------------------------------------YPPSLIPLQQIP 230
Cd36_34980      QTESSSQQQSRQQQQRPRHRRLEQLQQQERREKSYMSPALRDFRDSIKELEKQQHQQRNI 524
EED1            MQPPPQQQQQQQ-------------------------------------RHQPLQQSPPTMP 505

Pstip28806      LQQ------------------------------------------------------- 127
PGUG_0122.1     MQP------------------------------------------------------- 58
DEHAOE3597g     LQT------------------------------------------------------- 59
LELG_05325      MQQ------------------------------------------------------- 273
CP_02994        TTY------------------------------------------------------- 233
Cd36_34980      IQKPKRKRNSNALRPPQQSKQLQVQPHHLSCVQEQLQDLQEIQQQLNLQQQQQPRQLQQL 584
EED1            LQQQPVPPVQQVQTVPPPSSQ------------------------------------- 526

Pstip28806      ------------------------------QQPYPFSYYPQVTHQAPVQQPYGSHY---- 153
PGUG_0122.1     ------------------------------MFPQP-SHVEHASAAAAASTPY-------- 79
DEHAOE3597g     ------------------------------TIPQAQVQAQPH-LLAPIPQPQ-------- 80
LELG_05325      ------------------------------VLQVSQVPAPPLPPLPPQPQPQPPSQMPNT 303
CP_02994        ------------------------------SHNPEYYPSYQPPQLPPHPQYIPMHHMQQH 263
Cd36_34980      QQLRQSQEQLDLLLQQLLRQELQKQQQQLNLQQQQLNQQQQQQQSYVQAPQPTISYQQQQI 644
EED1            ----------------------------PQTQLSQQQQQQQQAQLQMQVPRCYQYQNRP 557

Pstip28806      -----------VYYQQPPQGYITGAPHSTGQLPVYMLGIIP------------------- 183
PGUG_0122.1     -----------YYPQHSQPQYIATYYQTDERPIRRK----------------------- 104
DEHAOE3597g     -----------VYYQPYPPQQYYTYVQPQGESSLPSN----------------------- 106
LELG_05325      YQMPIVQPQTDLYTQTYSQGPQYYYVAPHQAQAPQHQGFAPQVQQEMTQAQQLHYLHGG- 362
CP_02994        YYQQPMS----SSSSAPAQHQFYYNVVPYTMPDPYAG----------------------- 296
Cd36_34980      QQPVAQASQGSLVYQQPQIPPQVQAQPSQQYQQPYAQVNSQSYNEQPAILPPVSQLLSRP 704
EED1            SSQQRQYSQTPQYNQPPPQQKVYALPPQQVYAPPPRQVYAQPTIACKQQYPQQLYEQAPQ 617

Pstip28806      ------------------------------------GEMMLLARRKSKQSTTYSPKEDR 206
PGUG_0122.1     ---------------------------------------------SKSKQSLSWTPQEDK 119
DEHAOE3597g     ---------------------------------------KPRKTKHFSTWTPAEDK 123
LELG_05325      -----------------HMVPGFAGVSSTFSPGMYHSEPMAHLRRRTKQSSTWSPQEDK 404
CP_02994        ------------------------------------YVGVPRKSKQSSTWSADEDK 316
Cd36_34980      SRLPPSSKLPPPSQLPLQQGLPVYSQQSGPGIFNTFNGNDLAVVGVPRRGVGSWSIQEDH 764
EED1            EGSS------------YQHHYQQVQQRQNQQPYMQSAPTYQQPHVQTPKSTRSNKQEKQ 664
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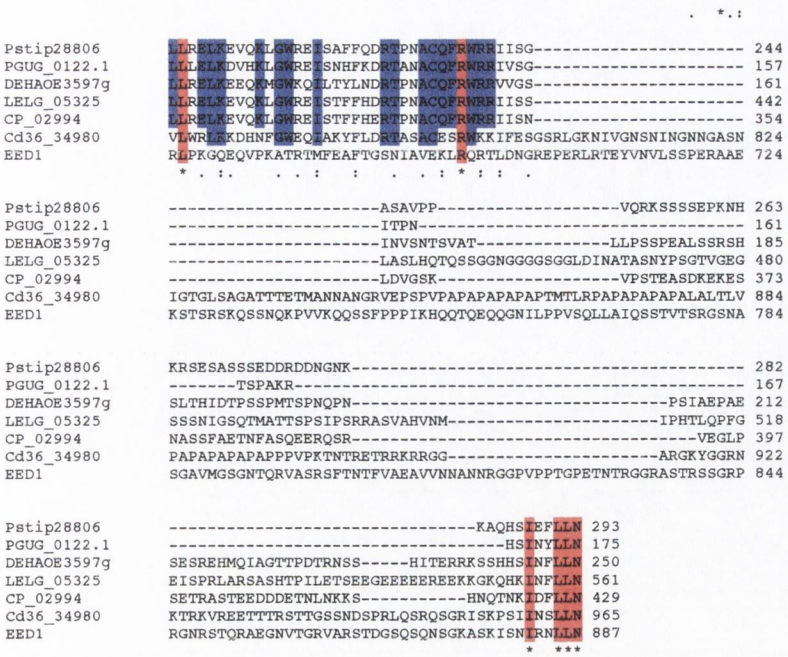


Fig 3.7. Clustal W alignment of syntenic sequences obtained from the CGOB. Regions in red show areas of 100 % homology and the regions marked in blue indicate areas of homology between the species with the exception of *C. albicans EED1*. The table at the top shows the Clustal W alignment score of each protein found to be syntenic to *EED1*, identified using CGOB.

Table 3.2. Percentage Proline and Glutamine residues found in syntenic genes idenified with CGOB

Gene Name	% Proline	% Glutamine
EED1	10.1	16.2
Cd36_34980	14.3	19.8
CP_02994	10.9	10
E03597g	12	9.6
Pstip_28806	10.5	9.5
PGUG_00122	11.9	8
LELG_05325	10.9	11.7

(Fig 3.8). It is also evident that closely related species have more similarity between the encoded proteins, for example it is well known that *C. parapsolosis* and *L. elongisporus* are closely related, as are *P. stipitis* and *P. guilliermondii* and this is reflected in figure 3.8. Due to the high homology between each species at this Myb-like DNA binding domain an alignment of this region was also carried out using Clustal W (Fig 3.9).

Several other proteins were identified at the beginning of section 3.2.2 as having homology to Cd36_34980 using BLAST, including proteins from *C. tropicalis* (CTRG_00932), *S. cerevisiae* (SCRG_04565), *C. lusitaniae* (CLUG_00389.1) and *C. albicans* (CAWG_01508). As these sequences were not syntenic with *EED1* alignments were carried out to determine whether there was any homology between these sequences and the syntenic genes identified with CGOB. The phylogenetic tree generated from this alignment demonstrates that the genes identified at the *EED1* locus with CGOB represent a distinct group of Myb-domain proteins, distinct from those non-syntenic genes identified using BLAST. These include *C. tropicalis* (CTRG_00932), *C. lusitaniae* (CLUG_00389.1) and *C. albicans* (CAWG_01508), which seem to have higher homology to *DOT6* in *S. cerevisiae* (Fig 3.10).

Following the identification of an *EED1* orthologue in *C. dubliniensis* and subsequent analysis of its protein sequence, it was decided to refer to Cd36_34980 as *MDP1* (Myb domain Protein 1) as no role in epithelial escape and dissemination has yet been attributed to this protein.

3.1.3 Intra-strain difference in the *EED1* and *MDP1* sequence

Due to the level of diversity between these two sequences, we amplified a region of *EED1* and *MDP1* from multiple unrelated isolates to determine whether these sequences were conserved within each species. The primer pair CdMDPF/R and (Table 2.2) were used to amplify *C. dubliniensis* DNA from different strains to obtain a product of 497 bp. These sequences were aligned using Clustal W. The alignments show single nucleotide polymorphisms (SNPs) (Fig 3.11). In particular *C. dubliniensis* isolates LP1 and JP3, both genotype 3 strains, showed base changes from T to C at 162 bp compared to the genome sequence of strain CD36 (genotype1). These isolates showed a base change at 167 bp from G to T, at 176 bp a base change from C to T was observed and at 241 bp a G to A substitution was seen. There were other SNP's observed in CD41 and MAN448, both genotype 2 strains, including a G to A

substitution at 242 and 246 bp and a C to T substitution at 266bp. Cd36, Cd33 and JP5 strains were identified previously as genotype 1 strains and had identical sequences. The *C. albicans* alignment of the 527 bp sequence amplified with CaEDTF/R (Table 2.2) of *C. albicans* strains SC5314, 3153A, 132A, Y0109, 8324, KJ, along with *EED1* was carried out and demonstrated that these PCR sequences are 98% identical with the exception of the *C. albicans* clinical isolate 8324, which exhibited differences. At 178 bp, 8324 has lost a GGA codon. However at 235 bp this GGA codon appears to have been reinserted in 8324. Overall this 8324 sequence displayed 86% homology to the other sequences and no additional information is available on this isolate (Moran *et al.*, 1997)(Fig 3.12).

3.1.4. Expression analysis of *EED1* and *MDP1* by quantitative real-time PCR

In order to carry out quantitative analysis of *EED1* and *MDP1* gene expression, an internal control was optimised. Previous studies have found that most housekeeping genes such as *ACT1* (encoding beta-actin), *PMA1* (plasma membrane ATPase pump) and *TEF1* (transcript elongation factor) can suffice as internal controls (Livak & Schmittgen, 2001). In this study, the housekeeping gene *ACT1* (orf19.5007) was used. The analysis was carried out using the $2^{-\Delta\Delta CT}$ method used by (Livak & Schmittgen, 2001). Livak and Schmittgen (Livak & Schmittgen, 2001) found that standard housekeeping genes usually suffice as internal control genes. For the $\Delta\Delta C_T$ calculation to be valid the amplification efficiencies of the target gene (*MDP1* and *EED1*) and reference gene (*ACT1*) must be approximately equal. The method to assess if two amplicons have the same efficiency is to look at how the ΔC_T varies with template dilution. (Fig 3.13). For each dilution sample amplifications were performed using primers for *MDP1/EED1* and *ACT1*. The average C_T for both sets of primers was determined and a plot of the log of the DNA dilution versus C_T was made (Fig 3.13). If the value of the slope is close to zero, the efficiencies of the target and reference genes are similar. The slopes of the investigated transcripts were *MDP1* (0.0529), *EED1* (0.0499) and *ACT1*, (0.0517), therefore the assumption holds that the ΔC_T method may be used to analyse the data (Livak & Schmittgen, 2001).

In a study by Chen *et al.*, (2004) it was found that *C. albicans* shows a significant lag in growth when diluted into fresh minimal medium. From this study it appears that cell density controls the morphological switch from the yeast form to the filamentous

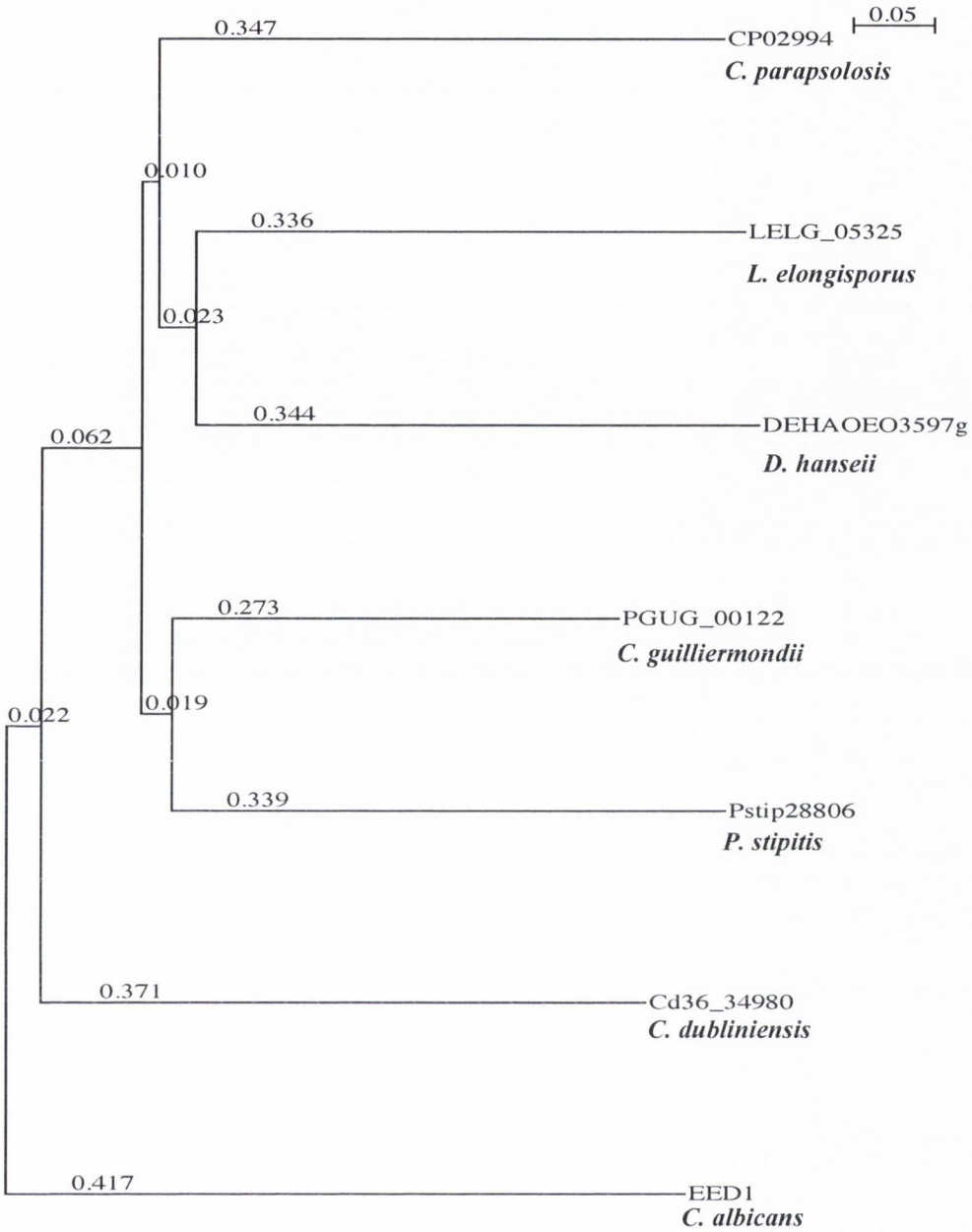
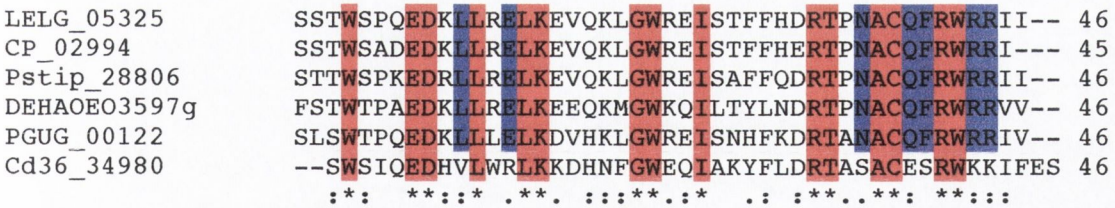


Figure 3.8. Phylogenetic tree generated from an alignment of the protein sequences encoded by syntenic genes of *EED1* identified with CGOB. From this tree the *C. albicans EED1* genes is the least closely to related to the other genes, followed by *C. dubliniensis* Cd36_34980. *C. guilliermondii* (PGUG_00122) and *P. stipitis* (Pstip28806) are more closely related to each other as are *C. parapsolosis* (CP_02994), *L. elongisporus* (LELG_05325) and *D. hanseii* (DEHAOEO5397g).



Species	Gene Name
P. stipitis	Pstip_28806
C. guilliermondii	PGUG_0122
L. elongisporus	LELG_05325
C. dubliniensis	Cd36_34980
D. hanseii	DEHAOE3597g
C. parapsolosis	CP_02994

Figure 3.9. Clustal W alignment generated from sequences corresponding to the Myb-like DNA binding domains found in each of the syntenic genes from each species identified with the CGOB. The amino acids highlighted in red are homologous between all species and those highlighted in blue are homologous between the species identified by CGOB with the exception of Cd36_34980.

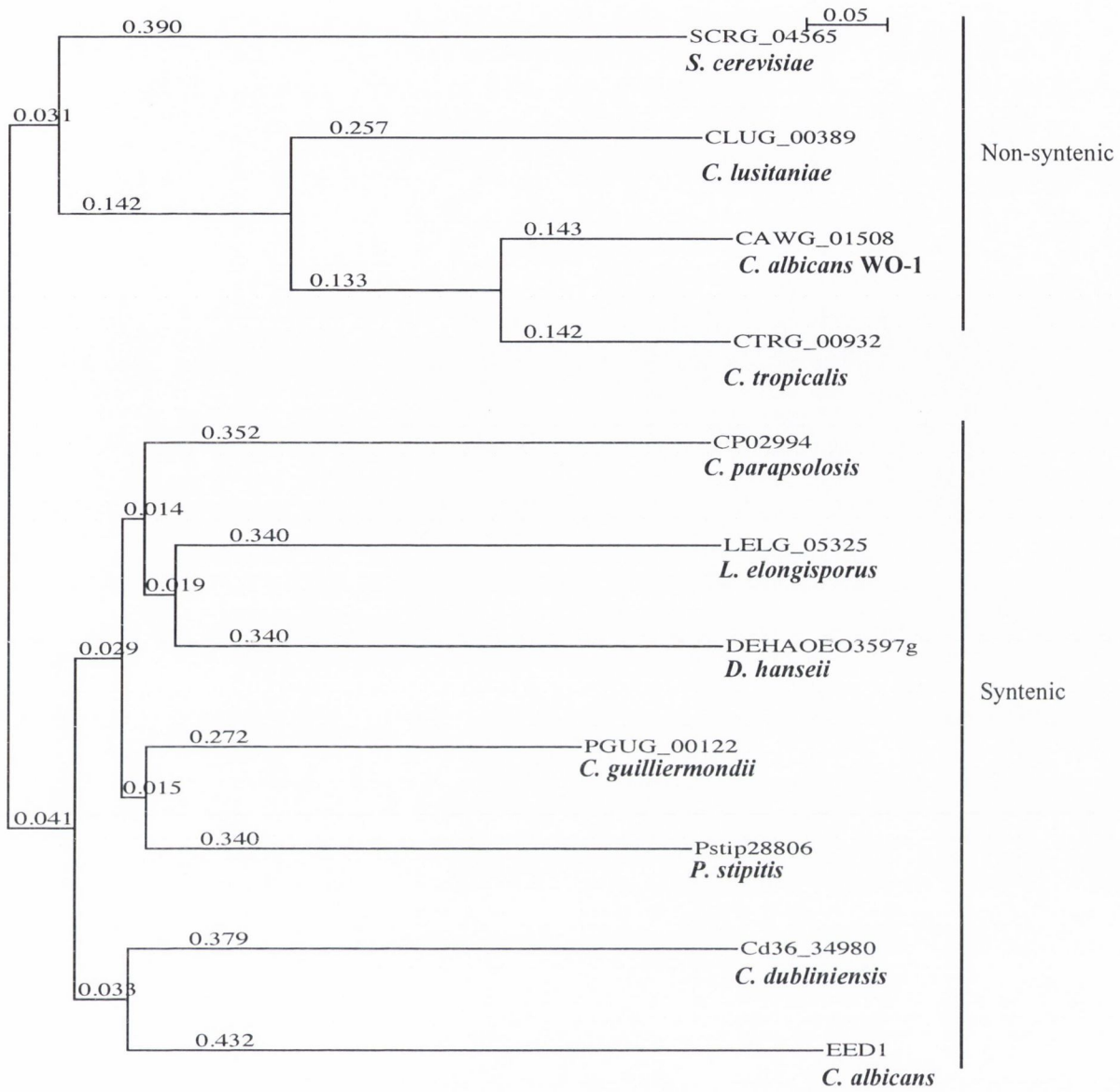


Figure 3.10. Phylogenetic tree of syntenic and non-syntenic sequences identified with CGOB and BLAST searches carried out using the Multi-fungi BLAST tool on the Broad Institute website.

```
CLUSTAL 2.0.5 multiple sequence alignment

MAN448          GCCACAACAGGCTCCAGAAACACCACCACCCCAAGAACCAAC 120
CD41            GCCACAACAGGCTCCAGAAACACCACCACCCCAAGAACCAAC 120
JP5             GCCACAACAGGCTCCAGAAACACCACCACCCCAAGAACCAAC 120
CD36            GCCACAACAGGCTCCAGAAACACCACCACCCCAAGAACCAAC 120
CD33            GCCACAACAGGCTCCAGAAACACCACCACCCCAAGAACCAAC 120
LP1             GCCACAACAGGCTCCAGAAACACCACCACCCCAAGAACCAAC 102
JP3             GCCACAACAGGCTCCAGAAACACCACCACCCCAAGAACCAAC 120
                ******************************************

MAN448          TACGCCCGTCTCAGGTCCTTCTGCCGCTCCTGCTAATGAAGTTCCCAGTAATGGTAAGGA 180
CD41            TACGCCCGTCTCAGGTCCTTCTGCCGCTCCTGCTAATGAAGTTCCCAGTAATGGTAAGGA 180
JP5             TACGCCCGTCTCAGGTCCTTCTGCCGCTCCTGCTAATGAAGTTCCCAGTAATGGTAAGGA 180
CD36            TACGCCCGTCTCAGGTCCTTCTGCCGCTCCTGCTAATGAAGTTCCCAGTAATGGTAAGGA 180
CD33            TACGCCCGTCTCAGGTCCTTCTGCCGCTCCTGCTAATGAAGTTCCCAGTAATGGTAAGGA 180
LP1             CACGCCCGTCTTAGGTCCTTCTGCCGCTCCTGCTAATGAAGTTCCCATTAATGGTAAGGA 162
JP3             CACGCCCGTCTTAGGTCCTTCTGCCGCTCCTGCTAATGAAGTTCCCATTAATGGTAAGGA 180
                ********** *********************************** ************

MAN448          GACTCCTCCTAAGAAATCAACACTGTCTGGTAGATCCCTTTGGGAAAAGGATGAGTTTGG 240
CD41            GACTCCTCCTAAGAAATCAACACTGTCTGGTAGATCCCTTTGGGAAAAGGATGAGTTTGG 240
JP5             GGCTCCTCCTAAGAAATCAACACTGTCTGGTAGATCCCTTTGGGAAAAGGATGAGTTTGG 240
CD36            GGCTCCTCCTAAGAAATCAACACTGTCTGGTAGATCCCTTTGGGAAAAGGATGAGTTTGG 240
CD33            GGCTCCTCCTAAGAAATCAACACTGTCTGGTAGATCCCTTTGGGAAAAGGATGAGTTTGG 240
LP1             GACTCCTCCTAAGAAATCAACACTGTCTGGTAGATCCCTTTGGGAAAAGGATGAGTTTGG 222
JP3             GACTCCTCCTAAGAAATCAACACTGTCTGGTAGATCCCTTTGGGAAAAGGATGAGTTTGG 240
                * ************************************************************

MAN448          TTTTTATGTTGCTTTTCGGGAAGCTATTACCAAGGAGCATAGAATTAAAGATGAAAATG 299
CD41            TTTTTATGTTGCTTTTCGGGAAGCTATTACCAAGGAGCATAGAATTAAAGATGAAAATG 299
JP5             TTTTTATGTTGCTTTCCGGGAAGCTGTTACCAAGGAGCATAGAATTAAAGATGAAAATG 299
CD36            TTTTTATGTTGCTTTCCGGGAAGCTGTTACCAAGGAGCATAGAATTAAAGATGAAAATG 299
CD33            TTTTTATGTTGCTTTCCGGGAAGCTGTTACCAAGGAGCATAGAATTAAAGATGAAAATG 299
LP1             TTTTTATGTTGCTTTCCGGGAAGCTGTTACCAAGGAGCATAGAATTAAAGATGAAAATG 281
JP3             TTTTTATGTTGCTTTCCGGGAAGCTGTTACCAAGGAGCATAGAATTAAAGATGAAAATG 299
                *************** ********* *********************************
```

Figure 3.11. Alignment of the nucleotide sequences of the *MDP1* gene in the *C. dubliniensis* strains CD33, JP5, CD41, Man448, LP1, JP3, generated with the ClustalW sequence alignment program (Higgins & Sharp 1988). Asterisks indicate identical residues, spaces indicate dissimilar residues. The gaps indicate the difference in size of the PCR amplimers.

```
1
KJ              ACAAACATGTCTGTTGTTCTTTTAGACCTTCTCGCTAACAGGAAATACTTTCTAAGTAGA 60
EED1            ACAAACATGTCTGTTGTTCTTTTAGACCTTCTCGCTAACAGGAAATACTTTCTAAGTAGA 60
3153            ACAAACATGTCTGTTGTTCTTTTAGACCTTCTCGCTAACAGGAAATACTTTCTAAGTAGA 60
132A            ACAAACATGTCTGTTGTTCTTTTAGACCTTCTCGCTAACAGGAAATACTTTCTAAGTAGA 60
8324            ACAAACATGTCTGTTGTTCTTTTAGACCTTCTCGCTAACAGGAAATACTTTCTAAGTAGA 60
                ************************************************************

KJ              GAAGCTCCATTTCTGTGATTACGAGCAGCATTTTTAAGTATCTGAAACACATAAATAAGT 120
EED1            GAAGCTCCATTTCTGTGATTACGAGCAGCATTTTTAAGTATCTGAAACACATAAATAAGT 120
3153            GAAGCTCCATTTCTGTGATTACGAGCAGCATTTTTAAGTATCTGAAACACATAAATAAGT 120
132A            GAAGCTCCATTTCTGTGATTACGAGCAGCATTTTTAAGTATCTGAAACACATAAATAAGT 120
8324            GAAGCTCCATTTCTGTGATTACGAGCAGCATTTTTAAGTATCTGAAACACATAAATAAGT 120
                ************************************************************

KJ              TTCTGAT-ATCTCTCTGGATTTAGGGTAAAATTAAAATCGCGGTAAAATGG-ATATCCCA 178
EED1            TTCTGAT-ATCTCTCTGGATTTAGGGTAAAATTAAAATCGCGGTAAAATGG-ATATCCCA 178
3153            TTCTGAT-ATCTCTCTGGATTTAGGGTAAAATTAAAATCGCGGTAAAATGG-ATATCCCA 178
132A            TTCTGAT-ATCTCTCTGGATTTAGGGTAAAATTAAAATCGCGGTAAAATGG-ATATCCCA 178
8324            AGACGGCCAACTTTTT---CTTGTGGTAATAATAATCCCAGAGGAATATTTCGAATTCCT 177
                    *   * ** * *    **  ***** * ***   *   * ** **     ** **

KJ              TAAGACGGCCAACTTTTT---CTTGTGGTAATAATAATCCCAGAGGAATATTTCGAATTC 235
EED1            TAAGACGGCCAACTTTTT---CTTGTGGTAATAATAATCCCAGAGGAATATTTCGAATTC 235
3153            TAAGACGGCCAACTTTTT---CTTGTGGTAATAATAATCCCAGAGGAATATTTCGAATTC 235
132A            TA-GACGGCCAACTTTTT---CTTGTGGTAATAATAATCCCAGAGGAATATTTCGAATTC 234
8324            GGTTTCTGATATCTCTCTGGATTTAGGGTAAAATTAAAATCGCGGTAAAAT--GGATATC 235
                    * *  * ** * *    **  ***** * ***   *   * ** **   **  **

KJ              CTGGTACAAACATGTCTGTTGTTCTTTTAGACCTTCTCGCTAACAGGAAATACTTTCTAA 295
EED1            CTGGTACAAACATGTCTGTTGTTCTTTTAGACCTTCTCGCTAACAGGAAATACTTTCTAA 295
3153            CTGGTACAAACATGTCTGTTGTTCTTTTAGACCTTCTCGCTAACAGGAAATACTTTCTAA 295
132A            CTGGTACAAACATGTCTGTTGTTCTTTTAGACCTTCTCGCTAACAGGAAATACTTTCTAA 294
8324            CCATAACAAACATGTCTGTTGTTCTTTTAGACCTTCTCGCTAACAGGAAATACTTTCTAA 295
                *    ************************************************************

KJ              GTAGAGAAGCTCCATTTCTGTGATTACGAGCAGCATTTTTAAGTATCTGAAACACATAAA 355
EED1            GTAGAGAAGCTCCATTTCTGTGATTACGAGCAGCATTTTTAAGTATCTGAAACACATAAA 355
3153            GTAGAGAAGCTCCATTTCTGTGATTACGAGCAGCATTTTTAAGTATCTGAAACACATAAA 355
132A            GTAGAGAAGCTCCATTTCTGTGATTACGAGCAGCATTTTTAAGTATCTGAAACACATAAA 354
8324            GTAGAGAAGCTCCATTTCTGTGATTACGAGCAGCATTTTTAAGTATCTGAAACACATAAA 355
                ************************************************************

KJ              TAAGTTTCTGATATCTCTCTGGATTTAGGGTAAAATTAAAATCGCGGTAAAATGGATATC 415
EED1            TAAGTTTCTGATATCTCTCTGGATTTAGGGTAAAATTAAAATCGCGGTAAAATGGATATC 415
3153            TAAGTTTCTGATATCTCTCTGGATTTAGGGTAAAATTAAAATCGCGGTAAAATGGATATC 415
132A            TAAGTTTCTGATATCTCTCTGGATTTAGGGTAAAATTAAAATCGCGGTAAAATGGATATC 414
8324            TAAGTTTCTGATATCTCTCTGGATTTAGGGTAAAATTAAAATCGCGGTAAAATGGATATC 415
                ************************************************************

KJ              CCATAAGACGGCCAACTTTTTCTTGTGGTAATAATAATCCCAGAGGAATATTTCGAATTC 475
EED1            CCATAAGACGGCCAACTTTTTCTTGTGGTAATAATAATCCCAGAGGAATATTTCGAATTC 475
3153            CCATAAGACGGCCAACTTTTTCTTGTGGTAATAATAATCCCAGAGGAATATTTCGAATTC 475
132A            CCATAAGACGGCCAACTTTTTCTTGTGGTAATAATAATCCCAGAGGAATATTTCGAATTC 474
8324            CCATAAGACGGCCAACTTTTTCTTGTGGTAATAATAATCCCAGAGGAATATTTCGAATTC 475
                ************************************************************

KJ              CTGGT 480
EED1            CTGGT 480
3153            CTGGT 480
132A            CTGGT 479
8324            CTGGT 480
                *****
```

Figure 3.12. Alignment of the nucleotide sequences of the *EED1* gene in *C. albicans* strains, SC5314, 3153A, 132A, α Y109, 8324, and KJ, generated with the ClustalW sequence alignment program (Higgins & Sharp 1988). Asterisks indicate identical residues, spaces indicate dissimilar residues.

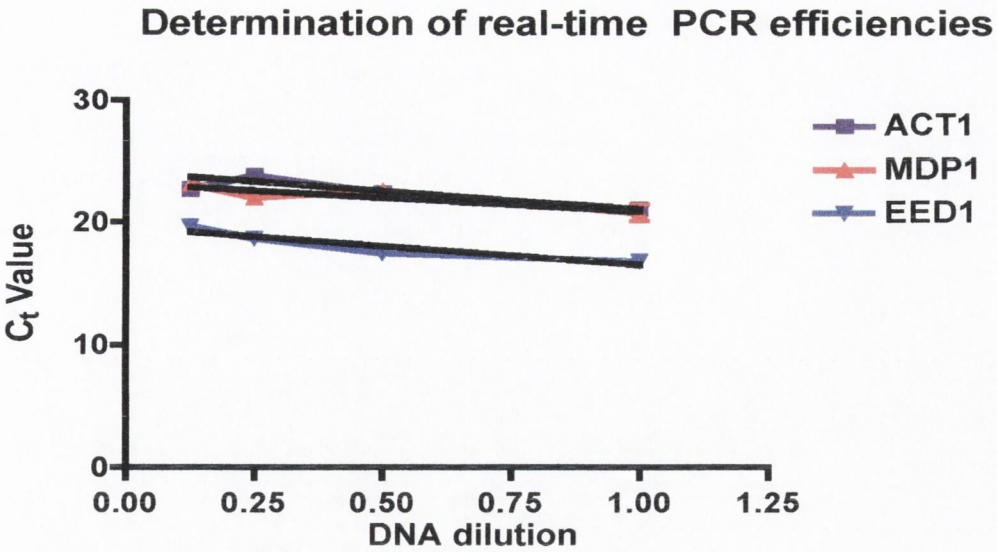


Figure 3.13. A plot of of log the DNA dilutions versus C_T values. The efficiency amplification of the target genes (*MDP1* and *EED1*) and internal control (*ACT1*) examined using real-time PCR and SYBR green detection. Serial dilutions of DNA were amplified by real-time PCR using gene-specific primers.

form. This study also found that *EED1* expression increases with increasing cell densities (Chen *et al.*, 2004). In this current study both *EED1* and *MDP1* relative expression with increasing cell densities was investigated. This was carried out by growing both strains in synthetic minimal media (SD) at 30°C for 24 h. Following this, cells were collected by centrifugation and inoculated into fresh SD at the following cell densities; 10^8, 10^7, 10^6, and 10^5 cells /ml. The cells were incubated for a further 60 min at 30°C. Subsequently, RNA isolation and cDNA synthesis was carried out for real-time PCR analysis. The real-time results showed that *EED1* expression relative to expression of the *ACT1* control agreed with the findings of Chen *et al.*, (2004) that *EED1* expression increased with increasing cell densities (Fig 3.14). Interestingly, the expression of *MDP1* relative to the expression of the *ACT1*, decreased with increasing cell densities (Fig 3.14). These initial results suggest that these two genes may be functionally divergent. It must be noted here that *MDP1* expression was 100 times lower than that of *EED1* when grown in SD.

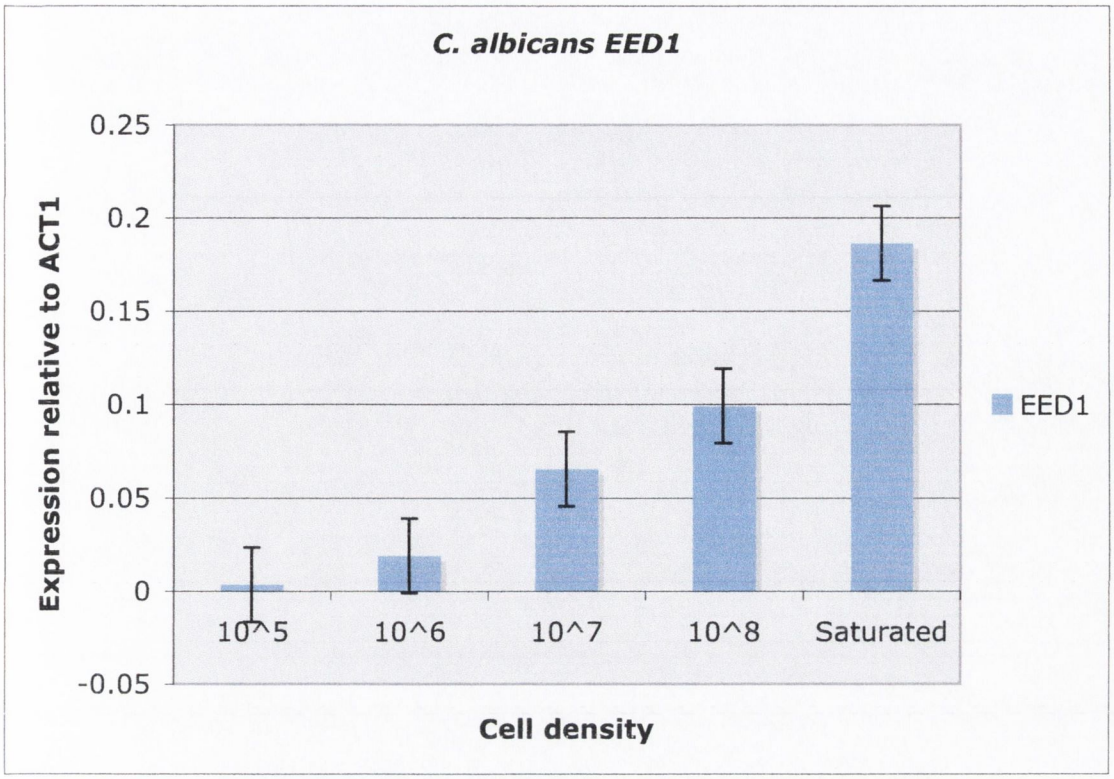
Expression analysis of both *EED1* and *MDP1* relative to *ACT1* was investigated under different conditions and also in different strains of *C. albicans* and *C. dubliniensis* (Table 3.3). The expression of *EED1* and *MDP1* in 10% newborn calf serum was investigated. Cultures were grown overnight in YPD or Lee's medium pH 4.5 at 30°C, cells were washed twice in PBS and inoculated to a cell density of 2 x 10^6 CFU/ml in 10 % newborn calf serum. Cells were collected by centrifugation at 1h, 3h and 5h after inoculation into 10 % serum and subsequently RNA isolation and cDNA synthesis was carried out for real-time PCR analysis. The results indicated a difference in transcriptional regulation of *EED1* when *C. albicans* is pre-cultured in YPD or Lee's pH 4.5. Following pre-culture in YPD significant expression of *EED1* relative to *ACT* was detected at T_0. However expression of *EED1* dropped by 6-fold at 1h and the level of expression of *EED1* did not increase relative to *ACT1* by 5h (Fig 3.15). Expression levels of *EED1* following pre-culture in Lee's pH 4.5, drops initially when the strain is diluted into fresh medium. However expression of *EED1*, when *C. albicans* is shifted from a low pH medium, increased 7-fold over the course of the experiment (Fig 3.15).

Expression of *MDP1* following pre-culture in YPD and subsequent inoculation into 10% newborn calf serum, found that under these conditions *MDP1* was expressed at extremely low levels relative to *ACT1* (Fig 3.16). However, by pre-culturing in Lee's pH 4.5 followed by inoculation into 10% newborn calf serum, *MDP1* expression increased 2-fold relative to *ACT1* within the first hour, however expression levels

dropped at 3h but increased again by the end of the experiment (5h) (Fig 3.16). Expression of *MDP1* was 30-fold higher in cells pre-cultured in YPD compared to cells grown in synthetic minimal medium or Lee's medium. However, overall expression of *MDP1* relative to *ACT* under both pre-culture conditions was very low compared to *C. albicans*.

The expression level of *HWP1* (hypha wall protein) was investigated under the same conditions as *MDP1*. This was performed in order to demonstrate that these experimental conditions induced hypha-specific gene expression This experiment was performed using *C. dubliniensis* strain Wü284 and from the real-time results it is apparent that *HWP1* expression relative to *ACT1* increased dramatically at 3h and decreased again at 5h (Fig 3.17).

The expression of *EED1* was also examined in the following *C. albicans* strains, CAF2-1 (wild type *EFG1*), CAN6 (Δ*cph1*/Δ*cph1*), CAN33 (Δ*efg1*/Δ*efg1*) and MMC3 (Δ*nrg1*/Δ*nrg1*) following pre-culture in YPD. *EED1* is expressed in CAF2-1 at almost similar levels to *EED1* relative to *ACT1*. However there is a 25-fold drop in expression of *EED1* relative to *ACT1* in MMC3 and CAN6 compared to CAF21. There is an 8-fold drop in expression of *EED1* relative to *ACT1* in CAN33 compared to CAF2-1 (Fig 3.18). From these results it would suggest that these transcriptional regulators play a role in the regulation of *EED1*. Interestingly, *EED1* is not expressed in MMC3 (Δ*nrg1*/Δ*nrg1*). This was unexpected as *NRG1* is a transcriptional repressor and is down regulated under conditions of filamentous growth (Saville *et al.*, 2003).



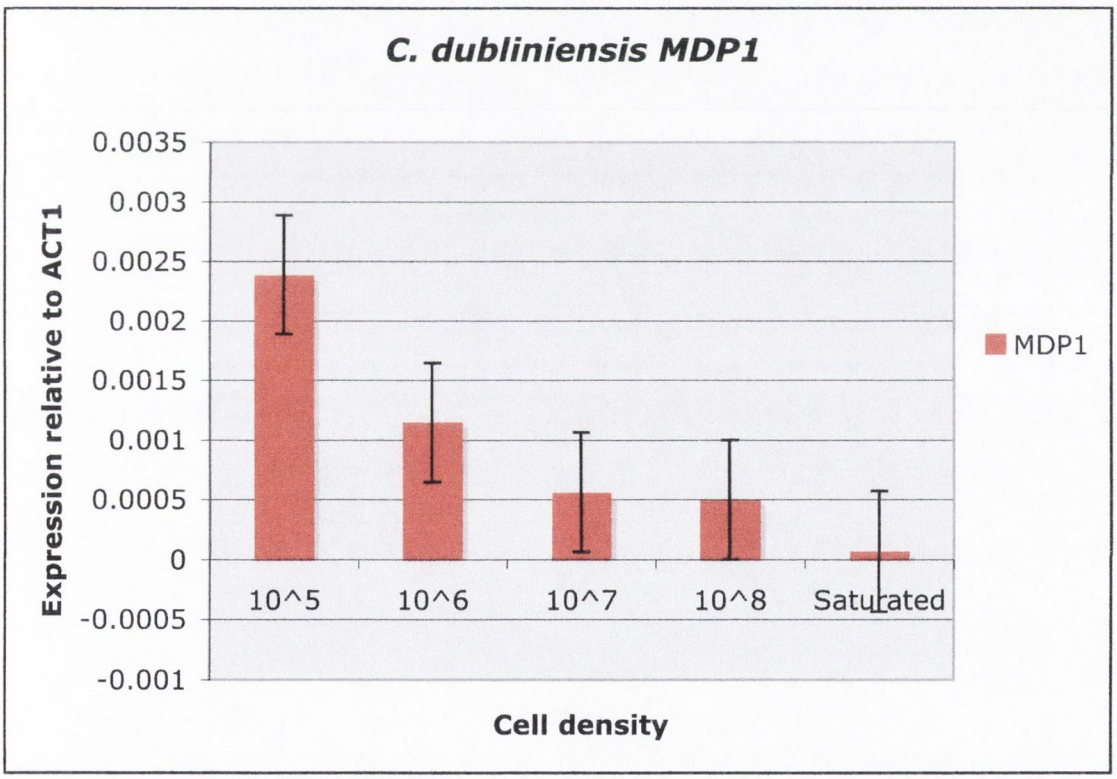


Figure 3.14. Relative Expression of *EED1* (top blue) and *MDP1* (bottom red) following pre-culture in SD and subsequent inoculation into fresh SD at different cell densities. In these graphs the scale used is different as the expression of *MDP1* is so low when compared to *EED1*. Experiments were repeated on at least three separate occasions.

Table 3.3 Experimental conditions for real-time PCR

Strain	Gene	Preculture conditions	Experimental Conditions
Wü284	***MDP1***	YPD	10 % newborn calf serum
		Lees pH 4.5	10 % newborn calf serum
		Saturated SD	SD (10^8-10^5 cells /ml)
BWP17	***EED1***	YPD	10 % newborn calf serum
		Lees pH 4.5	10 % newborn calf serum
		Saturated SD	SD (10^8-10^5 cells /ml)
Wü284	***CdHWP1***	YPD	10 % newborn calf serum
		Lees pH 4.5	10 % newborn calf serum
Wü284	***CdNRG1***	YPD	10 % newborn calf serum
		Lees pH 4.5	10 % newborn calf serum
CAF 21	***EED1***	YPD	YPD
MMC3 (Δ*NRG1*)	***EED1***	YPD	YPD
CAN 33 (Δ*EFG1*)	***EED1***	YPD	YPD
CAN 6 (Δ*CPH1*)	***EED1***	YPD	YPD

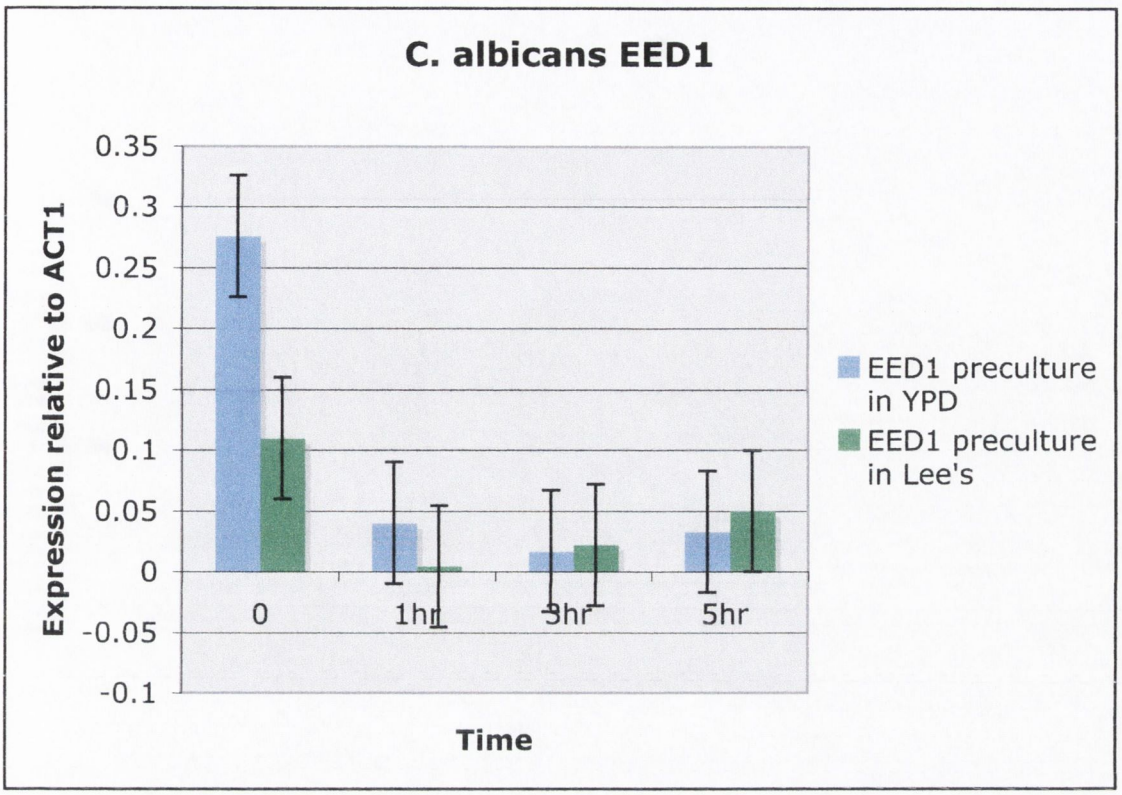


Figure 3.15. Relative Expression of *EED1* following pre-culture in YPD (blue) and relative expression of *EED1* following pre-culture in Lee's pH 4.5 (green) and subsequent inoculation into 10% serum. Experiments were repeated on three separate occasions.

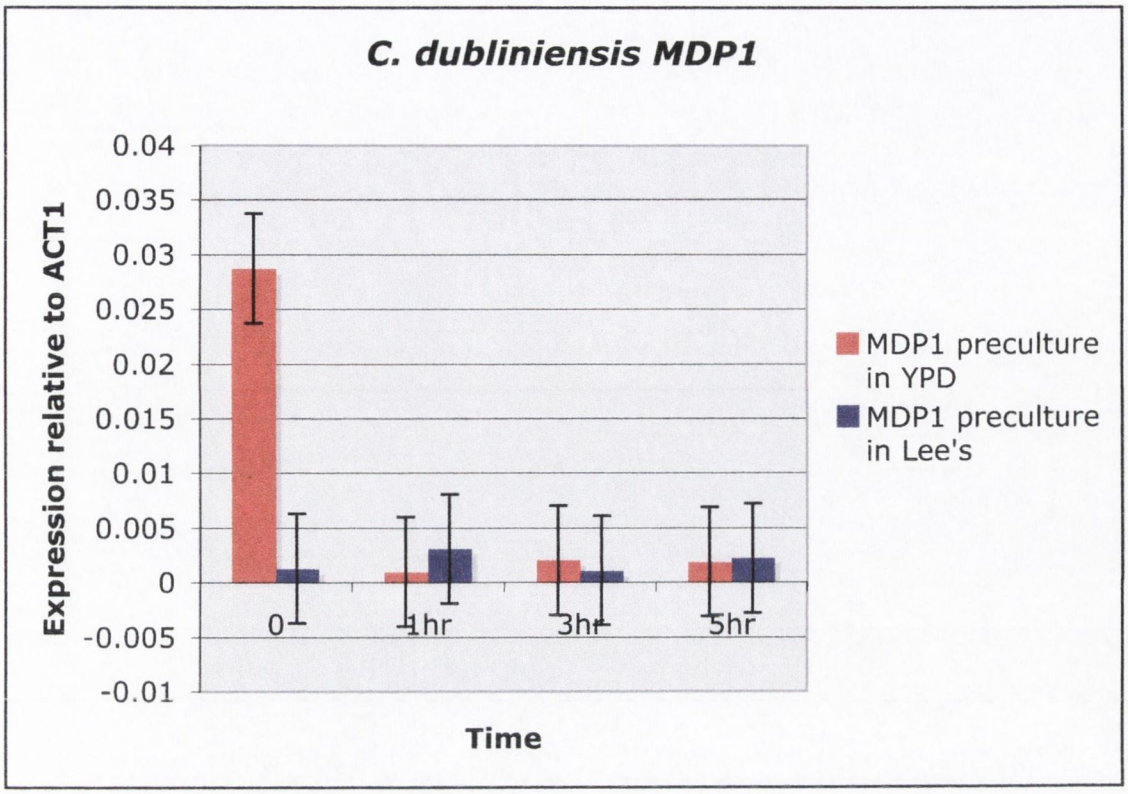
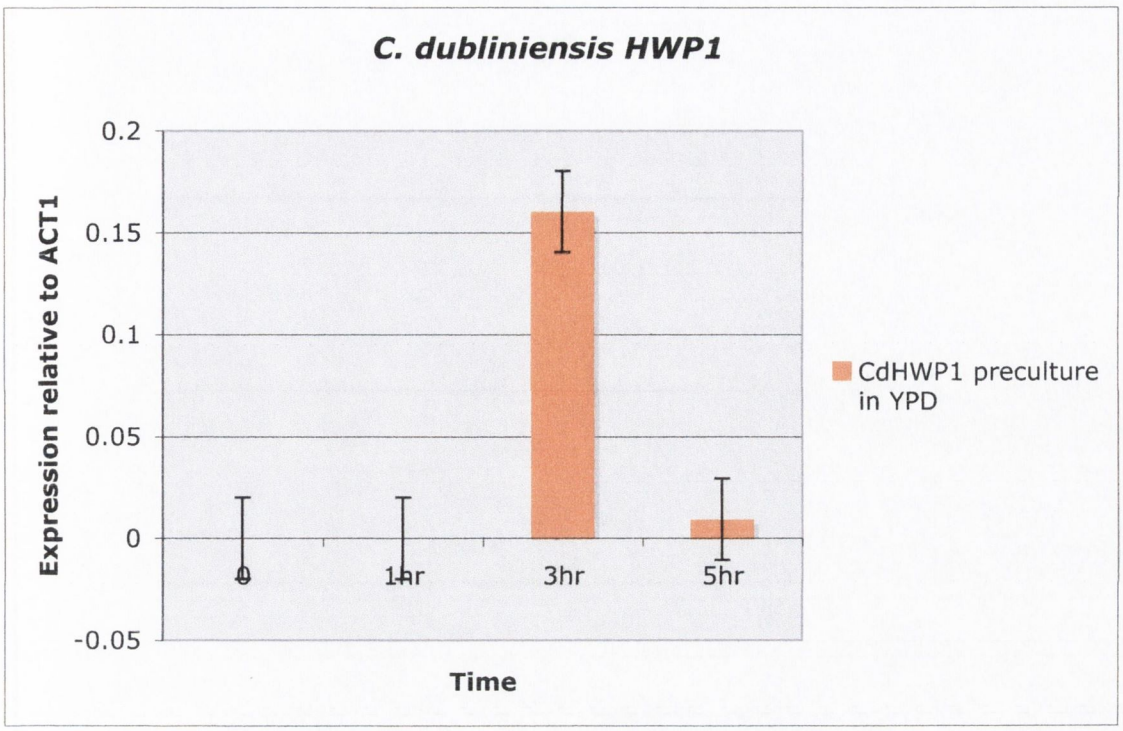


Figure 3.16. Relative Expression of *MDP1* following pre-culture in YPD (red) and relative expression of *MDP1* following pre-culture in Lee's pH 4.5 (purple) and subsequent inoculation into 10% serum. Experiments were repeated on three separate occasions.



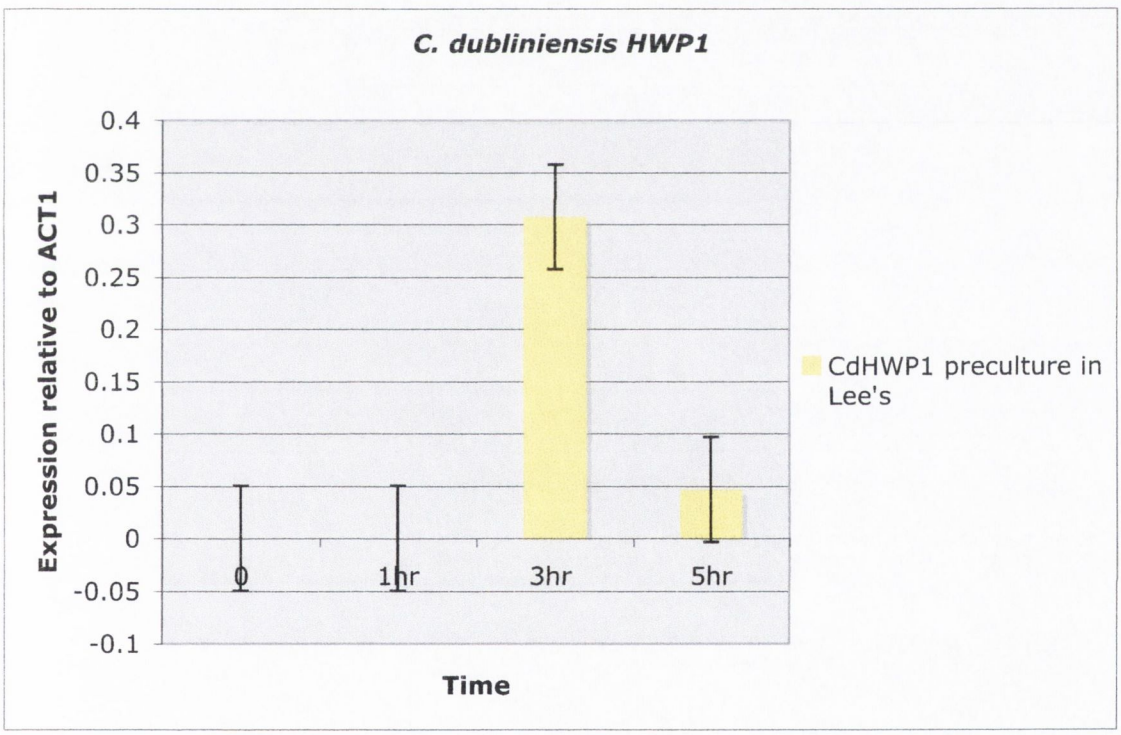


Figure 3.17. Relative Expression of *HWP1* following pre-culture in YPD (orange) and relative expression *HWP1* following pre-culture in Lee's pH 4.5 (yellow) and subsequent inoculation into 10% serum. Experiments were repeated on at least three separate occasions.

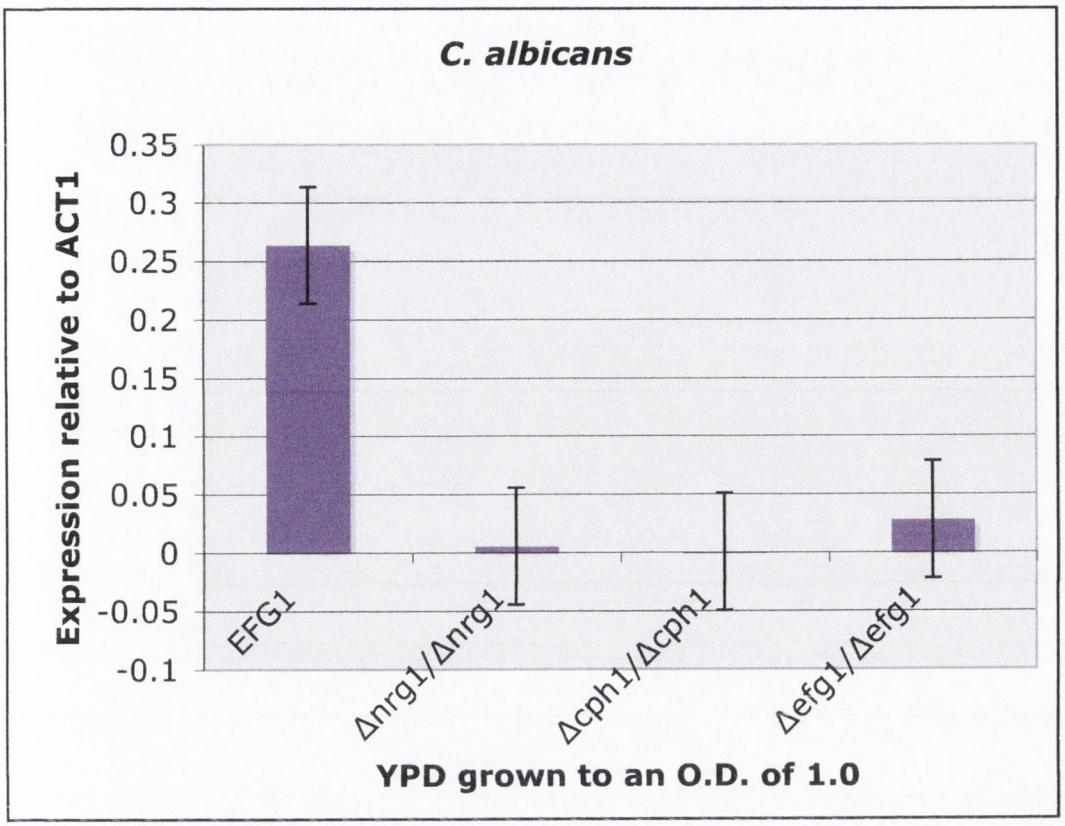


Figure 3.18. Expression of *EED1* relative to *ACT1* in the following *C. albicans* genotypes *EFG1*, *Δnrg1/Δnrg1*, *Δcph1/Δcph1*, *Δefg1/Δefg1*following pre-culture in YPD overnight, overnight cultures were diluted into fresh YPD and grown to an O.D. of 1.0. Experiments were repeated on three separate occasions.

3.2 Discussion

3.2.1 Identification of a *C. dubliniensis* homologue and its Myb-like DNA binding domain

Candida species are the most important opportunistic fungal pathogens of humans responsible for both superficial and systemic infections. *Candida albicans* is responsible for the majority of infections, but other species are becoming increasingly common. *Candida dubliniensis* was first identified in 1995 (Sullivan *et al.*, 1995) and was identified as a germ tube and chlamydospore positive yeast with high similarity to *C. albicans*. The completion of the sequencing of the *C. albicans* and the *C. dubliniensis* genomes and comparison of these genomes will provide an insight as to why *C. albicans* is more virulent than *C. dubliniensis*. These projects should also provide answers to the evolution of *Candida* species and the mechanism required for it to adhere to human cell surfaces. *C. albicans* microarrays have also permitted a rapid means of examining the genomes of *C. albicans* and *C. dubliniensis* using comparative genomic hybridisation studies. This process identified genes that were absent or divergent in *C. dubliniensis* which may help to explain the difference in virulence between the two species (Moran *et al.*, 2004).

This study attempted to identify an orthologue of *EED1*, a gene implicated in epithelial escape and dissemination. Following initial BLAST analysis of *EED1* no significant matches were identified, however the utilisation of ACT and CGOB analysis tools did result in the identification of a syntenic *C. dubliniensis* gene (Cd36_34980). Although regions either side of *EED1* on chromosome R were conserved between both species the intervening region was highly divergent and this was also evident among the other species identified in CGOB. Syntenic genes were identified in several yeast species and following Pfam searches a Myb-like DNA binding domain was identified in all these proteins. Myb-like DNA binding domains belong to the SANT domain family that specifically recognize the sequence YAAC(G/T)G. The interaction of regulatory proteins with DNA sequences plays an important role in the regulation of gene expression. Many DNA binding proteins are grouped into distinct classes based on their conserved DNA binding domains, characterised by particular structural motifs (Pabo & Sauer, 1992). Myb-like DNA binding domains are generally located at the N-terminal part of the protein. Each repeat of the Myb-domain has been proposed to fold into a

basic helix-loop-helix motif (bHLH). This bHLH motif is approximately 60 amino acids long and comprises of a DNA binding region followed by two α-helices separated by a loop region (Ledent & Vervoort, 2001). In Myb DNA binding proteins, one of the most conserved regions consisting of three tandem repeats has been shown to be involved in DNA-binding (Schultz *et al.*, 1998). Interestingly a study by Baranowskij *et al.*, (1994), found that Myb and Myb-related proteins have characteristic activation domains. These activator domains can be divided into three classes according to their amino acid composition, they are either rich in acidic amino acids, prolines or glutamines (Baranowskij *et al.*, 1994; Latchman, 1990). Interestingly all syntenic genes in this *EED1* region have a high proline and glutamine content (Table 3.2) indicating that these proteins have activation domains typical of Myb-like proteins. Although a Myb-like DNA binding domain was not identified in Eed1p, a partially conserved domain was found in Eed1p. In addition Eed1p is rich in both proline and glutamine. Also, all syntenic genes including *EED1* have a conserved C- terminal motif (IXXLLN), which could play a role in localization of the protein in the nucleus. Trafficking of proteins and RNAs between cytoplasm and nucleus is essential for the maintenance of cell function and crucially involved in adaptation to altered cellular conditions. This study has identified a novel family of Myb-like DNA binding domain proteins in, *C. parapsilosis, C. dubliniensis, L. elongisporus, C. lusitaniae, P. guilliermondii, P. stipitis* and *D. hansenii*. Although divergent at the amino acid sequence level these species do share similar characteristics. They posses a Myb-like DNA binding domain, they all possess a high proline and glutamine content and share a C-terminal motif and they were identified using the CGOB as being syntenic.

The *EED1* sequence was examined in different *C. albicans* strains and with the exception of the 8324 clinical isolate the sequences was 100% similar. Also, the *MDP1* sequence was examined in different strains of *C. dubliniensis* and again the sequences were very similar confirming the presence of these genes in both species.

3.2.2 *MDP1* is expressed at very low levels when compared with *EED1*

Quorum sensing gene expression is a well-known cell signalling mechanism in bacteria and is also thought to play an important role in biofilm formation. *C. albicans* was the first eukaryotic microorganism shown to exhibit quorum sensing (Alem *et al.*, 2006; Hornby *et al.*, 2001). The quorum-sensing molecule identified in *C. albicans* was

farnesol. Farnesol blocks the morphological shift from yeast to hyphae at high cell densities(Chen *et al.*, 2004). Chen *et al.*, (2004) identified Tyrosol as a second quorum sensing molecule in *C. albicans*. Like farnesol this compound is released continuously during growth and has the ability to abolish the lag phase normally seen when overnight cultures are diluted into fresh medium. Tyrosol also accelerates the formation of hyphae. Therefore, it appears that morphogenesis in *C. albicans* is under positive and negative control of tyrosol and farnesol respectively (Chen *et al.*, 2004). Interestingly, Chen *et al.*, (2004), also identified genes regulated by tyrosol by comparing gene expression profiles of *C. albicans* cells grown at high cell density (10^8 cells per ml) with those at low cell density (10^5 cells per ml) with or without tyrosol. These data identified genes whose expression increased or decreased in proportion to cell density and gene expression levels that were affected by tyrosol at low cell density. One such gene identified was *C. albicans EDT1 (EED1)*.

Real-time PCR data generated in this study showing the expression of *EED1* in response to cell density agreed with the results of Chen *et al.* (2004), that expression of *EED1* increased with increasing cell densities. However, expression results of *MDP1* found that expression of this gene decreased with increasing cell densities. This could indicate that tyrosol is a negative regulator of cell density in *C. dubliniensis*. From these initial results it would appear that *EED1* and *MDP1* do not have the same or a similar function and that these genes are differentially regulated. Another interesting result from this experiment is that the expression of *MDP1* was 100 times lower than that of *EED1*. A possible reason for this is that the *MDP1* transcript is present in one or fewer copies per cell and this could account for the very low levels of expression observed for *MDP1* in the conditions tested. In a paper by Holland (2002), the range of intracellular transcript abundance in yeast was kinetically monitored and this study found that yeast transcript abundance ranged from a few hundred copies per cell for glycolytic mRNAs to one-thousandth transcript per cell for transcripts encoding some of the transcription factors. This theory could be further tested by carrying out further real-time experiments where tyrsosol is added to the growth medium. This would give a definitive answer as to whether tyrosol acts as a negative regulator of cell density in *C. dubliniensis*.

Hypha development of *C. albicans* is under both positive and negative control. The *TUP1/NRG1* complex is responsible for the maintenance of the yeast phase through repression of genes required for filamentous growth. *EFG1* is involved in partial regulation of hypha development, deletion of *EFG1* results in rod-like, elongated cells

and the inability to form true hyphae under the conditions tested (Sharkey *et al.*, 1999). In figure 3.18 there was a 12.5-fold drop in expression of *EED1* in CAN33 (*Δefg1/Δefg1)* when compared with CAF2-1 (*EFG1)* suggesting that when expression of *EFG1* is switched off, expression of *EED1* is also switched off indicating that *EED1* could have a potential role in *EFG1*-dependent filamentation. Additional positive control of hyphae development is mediated through the mitogen activated protein kinase (MAPK) cascade (Liu *et al.*, 1994). Mutation in kinase components or the terminal transcription factor encoded by *CPH1* results in a medium-conditional defect in hypha development. Interestingly *EED1* is not expressed at all in CAN6 (*Δcph1. Δcph1)* and this also indicates that *EED1* could be dependent on *CPH1* or somehow implicated in the MAPK cascade.

We examined expression of *EED1* and *MDP1* under hypha inducing conditions in media containing 10 % newborn calf serum. We also examined the expression of *HWP1* as this gene is a good marker of hypha-specific gene expression. Expression of *HWP1* is dependent upon *EFG1* but does not require *CPH1* indicating that activation of *HWP1* is through the cAMP pathway. Stokes *et al.* (2007), found that *C. dubliniensis* expressed a *HWP1* homologue. *HWP1* in *C. dubliniensis* is expressed at 3h in this study when cells are inoculated in 10 % newborn calf serum following pre-culture in both YPD and Lee's medium, indicating that *HWP1* is expressed during hypha formation in *C. dubliniensis*. *MDP1* was also expressed under hypha inducing conditions. Although *MDP1* was expressed following pre-culture in YPD, there was a 27-fold drop at 1 h, 3 h and 5 h post inoculation in 10 % newborn calf serum. These low levels of expression could be attributed to a lack of hypha present in the medium. However, following pre-culture in Lee's medium there was a 1.5-fold increase in expression from T_0 to 1 h. However, expression drops again at 3 h before increasing again 2.3-fold at 5 h. Although in *MDP1* expression levels were low, the gene was expressed under hypha inducing conditions, indicating that in *C. dubliniensis MDP1* does play a role in filamentation.

Analysis of *EED1* expression in *Δnrg1/Δnrg1* mutant cells produced unexpected results. In *C. albicans* when *NRG1* is switched on, it results in the repression of hypha specific genes. However, in the absence of *NRG1*, these hypha specific genes are switched on. However, this was not the case in MMC3 (*Δnrg1/Δnrg1*) when *EED1* expression was examined. We found a decrease in *EED1* expression in the *Δnrg1/Δnrg1*

mutant MMC3. Although this result was unexpected it could suggest that *EED1* may have another function and not just as a hypha specific gene.

Finally expression analysis of both *EED1* and *MDP1* was carried out and it was observed that *MDP1* was expressed at very low levels. Further investigation will be carried out to determine the role of both *EED1* and *MDP1* in morphology and virulence.

Chapter 4

Deletion of *MDP1* and comparative analysis of the *Candida albicans EED1* and the *Candida dubliniensis MDP1* genes

4.0 Introduction

One of the most powerful methods of identifying the function of a specific gene is to investigate the phenotypic consequence when deleting the gene. Previously, targeted mutagenesis was the most commonly used method to assess the function of a gene in *C. alibcans*. The *URA3* blaster approach was previously used to construct knockout mutants in *C. albicans*. However, the use of the *URA3* marker can cause problem with interpreting mutant phenotypes as the expression of the *URA3* gene depends on the integration locus (Lay *et al.*, 1998) and uridine auxotrophy renders *C. albicans* avirulent the could potentially affect virulence (Bain *et al.*, 2001). Construction of knock-out mutants directly from *C. albicans* wild type strains by the use of dominant selective markers eliminates potential problems related to the use of auxotrophic markers. In this study, the dominant selection marker *caSAT1*, which confers resistance to nourseothiricin, was used. Following the identification of syntenic *EED1* gene in *C. dubliniensis*, the *SAT1*- flipper cassette was used to delete the *C. dubliniensis MDP1* gene in order to determine the function of this gene. This method of gene disruption was first described by Reuss *et al.* (2004). It is a highly efficient way to construct homozygous knockout mutants in *C. albicans* wild type strains. and it has advantage that mutants differ from the parental wild type strain only by the absence of the target gene. This study was one of the first studies to carry out gene deletions in *C. dubliniensis* using the *SAT1*-flippper cassette.

A variety of environmental conditions stimulate the yeast to hypha transition in *C. albicans*. This includes growth at elevated temperatures (e.g. 37°C or higher) and media containing special components (serum or a particular combination of amino acids) that promote hypha growth (Gow, 1997). In a paper by Gilfillan *et al.* (1998) the authors showed that hypha production in *C. dubliniensis* was slower than *C. albicans* when hyphae were induced in water with 10 % (v/v) serum. These results indicate that serum has a significant effect on hypha production in *C. albicans* and *C. dubliniensis*, however, the level of hypha production of *C. dubliniensis* does not approach that of *C. albicans*.

4.1 Results

4.1.1 Deletion of *C. dublinensis MDP1*

The *C. dublinieinsis MDP1* gene was deleted using the *SAT1*-flipper cassette (Chapter 2, 2.6.1). The SAT1 flipping method relies on the use of a cassette that contains a dominant nourseothricin resistance marker for the selection of integrative transformants and a adapted *FLP* gene that allows the subsequent excision of the cassette, which is flanked by FLP target sequences, from the genome. Two rounds of integration/excision generate homozygous mutants that differ from the wild-type parent strain only by the absence of the target gene, and reintegration of an intact gene copy for complementation of mutant phenotypes is performed in the same way. In order to create a disruption cassette, the 5' and 3' ends of *MDP1* were cloned into the *Kpn*I/*Xho*I and *Sac*II/*Sac*I sites respectively of pFS2A. In order to achieve the disruption of *MDP1* a 315 bp fragment of the 5' end of the *C. dubliniensis MDP1* gene was amplified using the primer pair 5F/5R (Table 2.2). This fragment was digested with *Kpn*I and *Xho*I and cloned into the vector pBluescript II KS [-] (Norrander *et al.*, 1983). At the 3' end of *MDP1* a 478 bp fragment of the gene was amplified using the primer pair 3F/3R (Table 2.2) and this was digested with *Sac*II and *Sac*I and cloned into the vector pBluescript II KS [-]. Both of these fragments were subcloned from pBluescript II KS [-] into the *Kpn*I/*Xho*I and *Sac*II/*Sac*I sites flanking the *SAT1* gene in pFS2A to generate pLOC1 (Fig. 2.1). The *C. dubliniensis* strain CD36 was transformed with the *Kpn*I/*Sac*I fragment from pLOC1. In order to detect correct integration of the cassette at the *MDP1* locus, nourseothricin resistant transformants were screened using the primer pair EDTP/FLP and 5F/3R (Table 2.2). The EDTP primer annealed to upstream chromosomal DNA and the FLP primer annealed to the cassette region. Thus amplification products obtained from this primer pair were specific for transformants with integration at the *MDP1* locus (Fig. 4.1). Six positivie transformants were identified. In order to disrupt the second copy of *MDP1*, excision of the cassette was induced by expressing the FLP recombinase in the presence of maltose (Chapter 2, 2.6.2). In order to do this positive transformants were grown in yeast peptone maltose (YPM) overnight at 30°C so that the *MAL2* promoter would induce FLP expression (Titz *et al.*, 2006). Putative revertants were spread onto plates with a drug concentration of 4 μg/ml and 2 μg/ml, which allowed for nourseothricin sensitive revertants to be

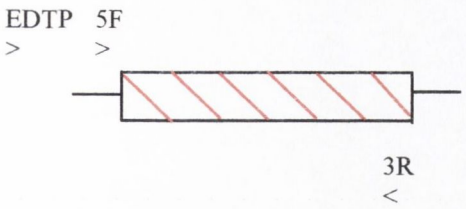
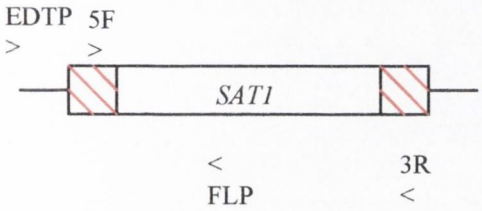
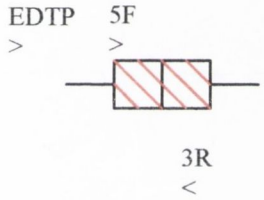
Genotype	Primer Pair	Expected Product Size
WT *MDP1*		
	EDTP/FLP 5F/3R	No Product 3,700 bp
***MDP1*::SAT**		
	EDTP/FLP 5F/3R	1,950 bp 5,100 bp
***MDP*Δ::FRT**		
	EDTP/FLP 5F/3R	No product 982 bp

Figure 4.1. PCR based strategy to generate allelic genotypes of *MDP1* transformants are shown in column 1. Primer pairs used to detect these genotypes are in column 2 and the expected PCR size is in column 3.

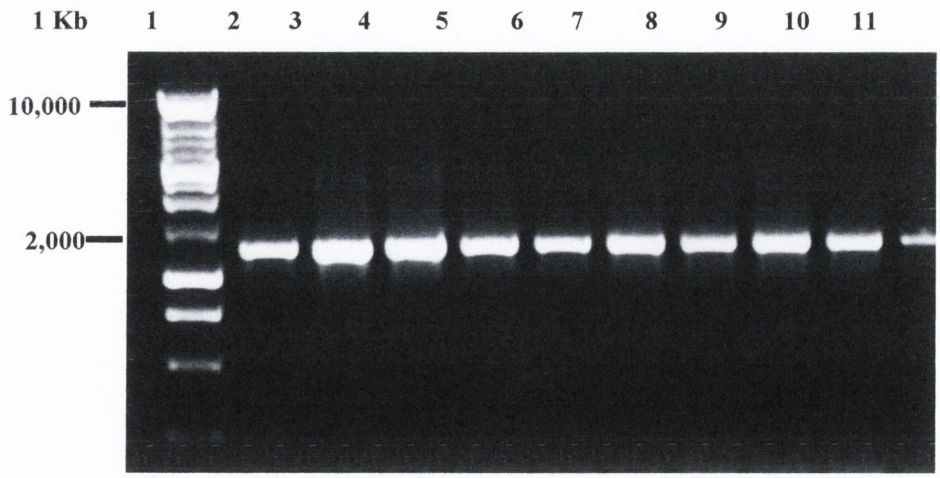


Figure 4.2. Examples of a PCR-based screening of CD36 transformants using the primer pair EDTP/FLP. Lane 1, 1Kb DNA Ladder; Lane 2-11 PCR product of CD36 transformants using the primer pair EDTP/FLP. The presence of this 1950 bp product indicated that the *SAT1* cassette had inserted into the *MDP1* gene.

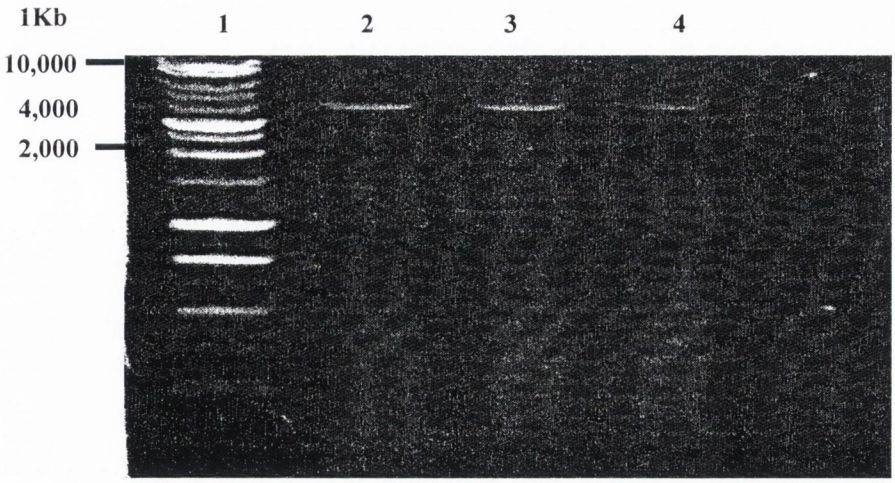


Figure 4.3. PCR using the primer pair 5F/3R to detect an intact copy of the *MDP1* gene (3,700 bp). Lane 1 1Kb DNA Ladder; Lane 2-4, CD36 positive 5F/3R PCR product. If the *MDP1* was correctly knocked out, no product would be obtained.

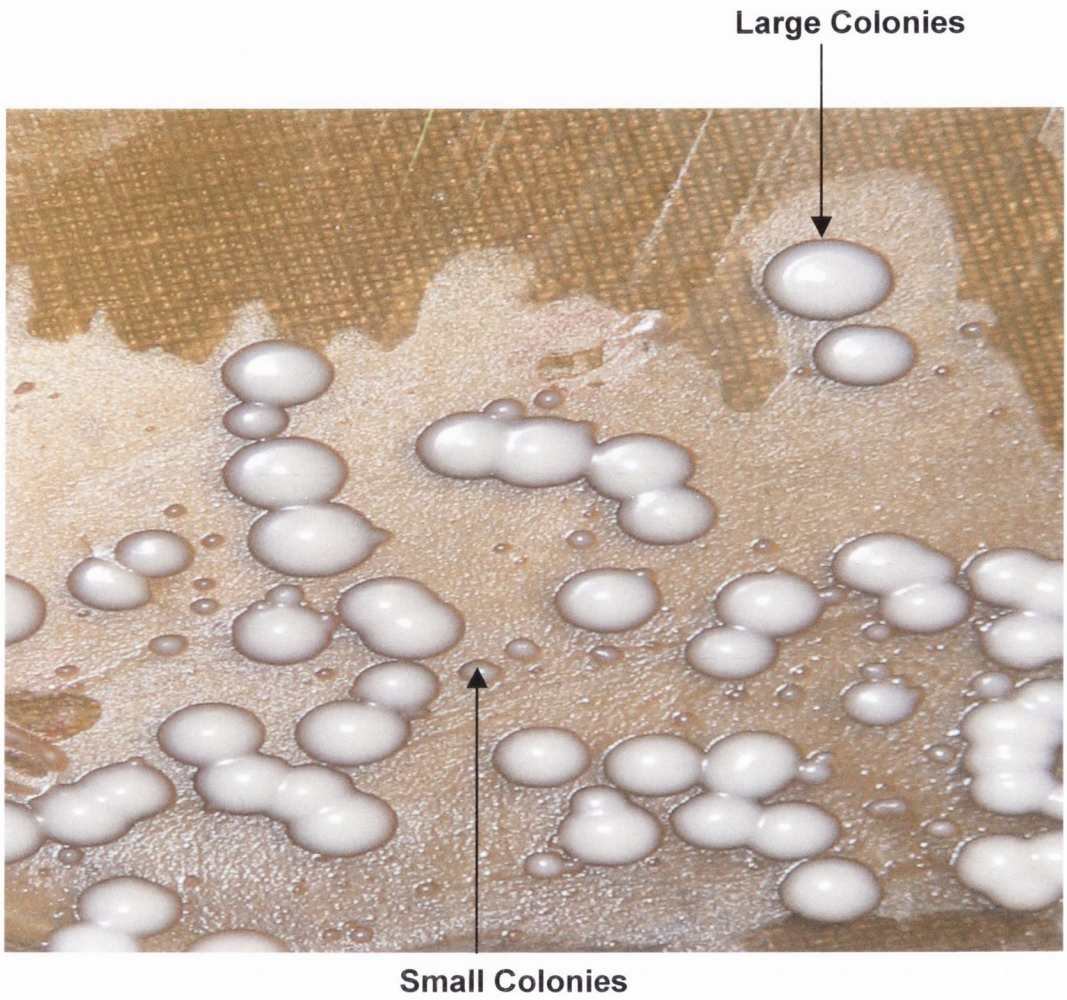


Figure 4.4. Light microgrpahs of *C. dubliniensis* Wü284 transformants on plates containing 2 µg/ml nourseothricin (the *SAT1* antibiotic). The small colonies correspond to those cells that have spontaenously lost the *SAT1* flipper cassette.

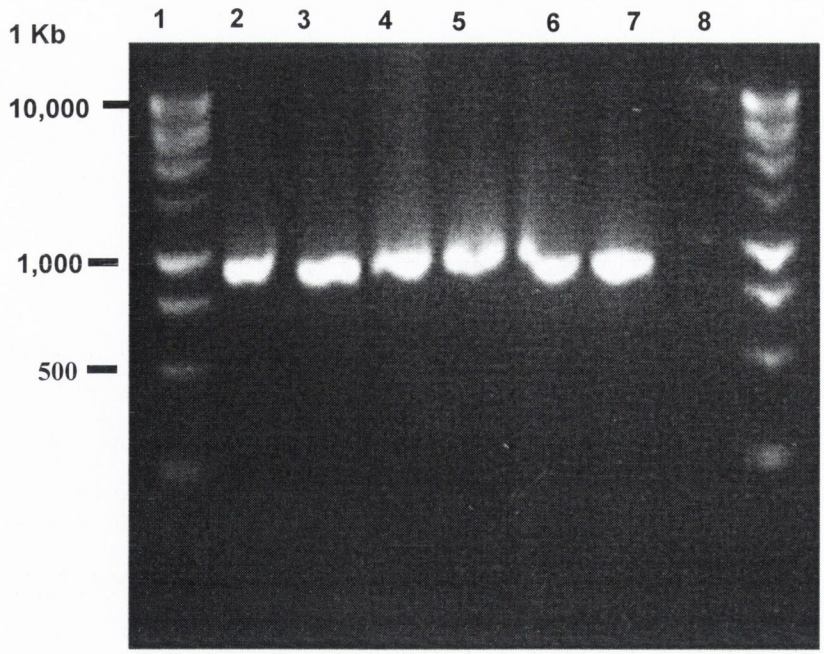


Figure 4.5 PCR using the primer pair 5F/3R to detect loss of the *SAT1*-cassette (982 bp). Lane 1 1Kb DNA Ladder; Lane 2-7 Δ*mdp1*/Δ*mdp1* double disruptants identified (982 bp) using the 5F/3R PCR. Lane 8 Negative control (wild-type *MDP1*).

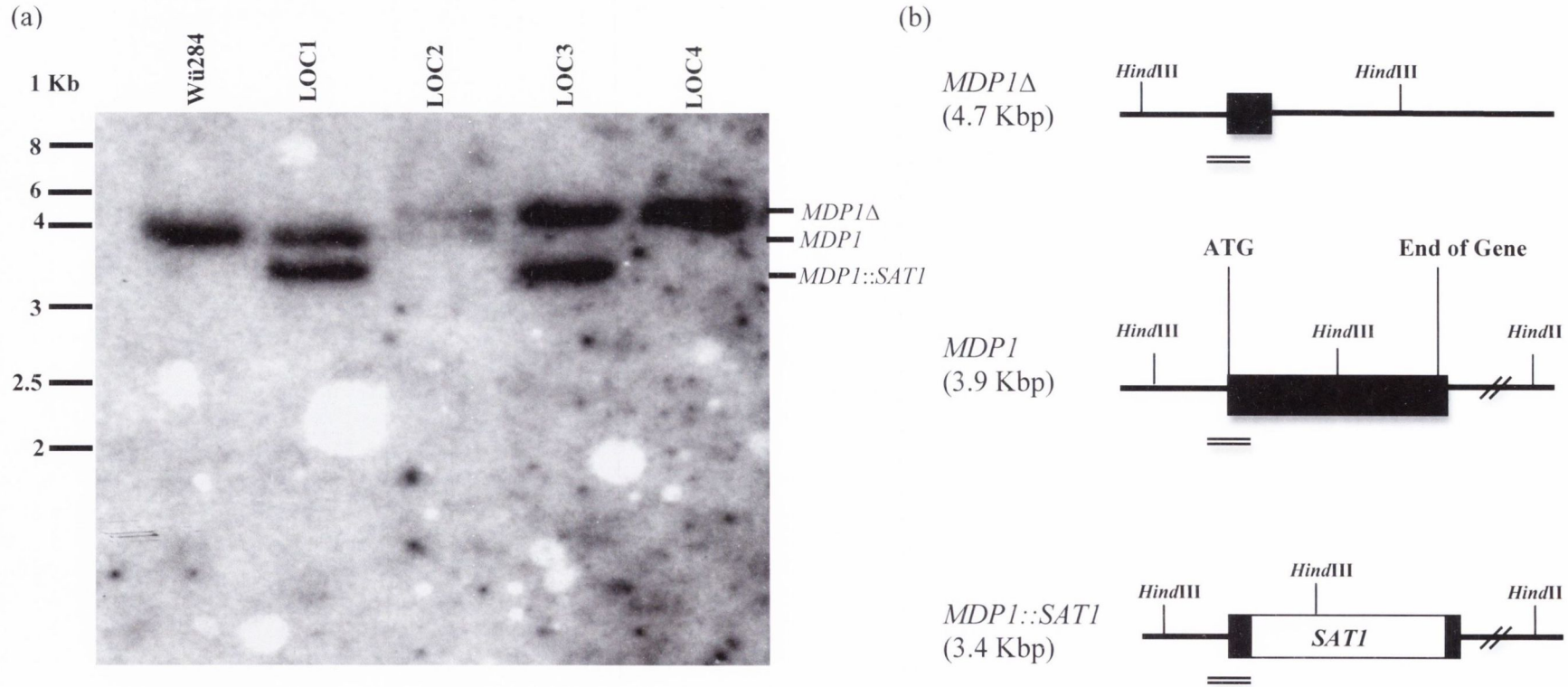


Figure 4.6. Transformants were selected on medium containing nourseothricin and were analysed by Southern blot analysis. (a) The *Hind*III-digested genomic DNA was electrophoresed on a 0.8 % (w/v) agarose gel, transferred to a nylon membrane and hybridised with a radiolabelled probe homologous to the *C. dubliniensis MDP1* gene. (b) Cartoon showing the structure and relative sizes of Hin*dIII* generated restriction fragments from the genomic DNA of the wild-type *MDP1* locus, the *SAT1* transformed locus (*MDP1::SAT1*) and the deleted locus (*MDP1*Δ). The double line below each locus shows the size ans location of the fragment used in Southern hybridisations.

4.1.4 Spot Plate Assays

Susceptibility of *C. dubliniensis* Wü284, LOC2 (*Δmdp1/MDP1*) and LOC4 (*Δmdp1/Δmdp1*) and *C. albicans* SC5314, BWP17 and the *Δeed1/ Δeed1* mutant was tested qualitatively by spotting serial dilutions of yeast cultures on inhibitor containing media (Chapter 2, 2.8.3). Overnight broth cultutres were firstly diluted to 1 X 10^7 cells/ ml. Four serial 1:10 dilutions of this first dilution were made and 10 µl of each dilution were spotted onto each plate and were incubated at 37°C, unless otherwise stated, for 48 h (Table 2.4). It was observed that the *C. dubliniensis Δmdp1/Δmdp1* mutant grew more efficiently than either Wü284 or LOC2 (*Δmdp1/MDP1*) mutant when grown on media containing 1 M, 1.5 M and 1.6 M NaCl, 0.5 M $CaCl_2$, and on YPD grown at 42°C (Fig. 4.11). The *C. dubliniensis* wild type and LOC2 (*Δmdp1/MDP1*) mutant both were found to be more sensitive than either the homozygote mutant or *C. albicans* SC5314. All of the *C. dubliniensis* strains grew poorly on YPD supplemeneted with the cell wall damaging agent CongoRed (Fig. 4.11).

In *C. albicans* however, the wild type, *Δeed1/EED1* and the *Δeed1/Δeed1* showed no difference in growth on any of the inhibitors (Fig. 4.12). Although on higher concentrations of NaCl (1.5 M and 1.6 M) all grew poorly, this was also observed on media containing 0.5 M $CaCl_2$. However, unlike *C. dubliniensis*, all of the *C. albicans* strains grew well on media containing Congo Red, and on media grown at 42°C (Fig. 4.12).

4.1.5 Hypha Induction

The ability to exist in both the yeast and hypha phase is thought to be crucial for virulence in *C. albicans* as it enables it to colonise and disseminate within host tissues thereby promoting infection. Gilfillan *et al.*, (1998) and Stokes *et al.*, (2007), previously compared hypha production between *C. albicans* and *C. dubliniensis* under a limited set of induction conditions and found that hypha production is less efficient in *C. dubliniensis*. The effects of a number of media known to induce hypha in *C. albicans* was examined with *C. albicans* BWP17, *Δeed1/Δeed1* and *C. dubliniensis* Wü284, LOC2 (*Δmdp1/MDP1*) and LOC4 (*Δmdp1/Δmdp1*). Intially hyphae induction was examined in liquid media by inoculating 10 % newborn calf serum at 37°C with an inoculum of 2 X 10^6 CFU/ml of yeast cell (Chapter 2, 2.8.1) of overnight cultures of each strain grown in YPD or Lee's pH 4.5 broth (Table 2.3).

In *C. dubliniensis* both the wild type Wü284 and LOC4 (*Δmdp1/Δmdp1*) mutants produced hyphae less efiiciently than *C. albicans* when grown in YPD overnight at 30°C and 37°C. Two different temperatures were examined as it has been shown previously that *C. dubliniensis* grows better at 30°C. However, following preculture at 30°C Wü284 did produce hyphae more efficiently than the LOC4 (*Δmdp1/Δmdp1*) mutant (Fig. 4.13). Following preculture at 37°C hypha formation was very poor in both strains with less than 10 % of hyphae produced during the course of the experiment. Despite this Wü284 did produce slightly more hypha than the LOC4 (*Δmdp1/Δmdp1*) mutant, indicating that although hypha formation is far less in *C. dubliniensis*, *MDP1* is essential for filamentation in response to temperature and serum (Fig. 4.13).

In *C. albicans*, following preculture at 30°C, hyphae are produced rapidly in the wild type BWP17 strain, with 90 % of cells growing as hypha by the third hour. However, the *Δeed1/Δeed1* mutant produced hypha poorly, with less than 5 % of cells producing hyphae during the course of the experiment (Fig. 4.14). Following preculture at 37°C the wild type strain BWP17 produced hypha rapidly although without the temperature shift, hypha formation is slightly slower. However, by the third hour, cells are 80 % hypha. Following preculture at 37°C, the *Δeed1/Δeed1* mutant again produced less than 5 % hyphae indicating that *EED1* is required for filamentation in *C. albicans* in response to temperature and serum (Fig. 4.14).

Hypha induction in DMEM with and without CO_2 was also examined to determine whether either of these conditions could affected hypha growth in either *C. albicans* or *C. dubliniensis*. *C. dubliniensis* Wü284 and LOC4 (*Δmdp1/Δmdp1*) did not form hyphae when inoculated into DMEM at 37°C. Also, no hyphae were produced in either the wild type or the mutant when inoculated with DMEM at 37°C with 5 % CO_2. *C. albicans* wild type strain BWP17 produced hyphae rapidly when inoculated with DMEM at 37°C and with DMEM at 37°C with 5 % CO_2. The *Δeed1/Δeed1* mutant also failed to produce hyphae under either of these conditions.

Table 4.1. Strains used during this study

Strain	Genotype	Parent	Reference
Wü284	*MDP1/MDP1*	-	Staib *et al.*, (2001)
LOC1	*MDP1/MDP1::SAT1*	Wü284	This study
LOC2	*MDP1/MDP1Δ::FRT*	LOC1	This study
LOC3	*MDP1::SAT1/MDP1Δ::FRT*	LOC2	This study
LOC4	*MDP1Δ::FRT/MDP1Δ::FRT*	LOC3	This study
BWP17 + *CIp30*	*EED1/EED1*	-	Zakikhany *et al.*,(2007)
EED1 Heterozygote	BWP17, *eed1Δ::URA3/EED1*, *CIp30*	BWP17, *CIp30*	Zakikhany *et al.*,(2007
Δeed1	BWP17, *eed1Δ::HIS1/eed1Δ::ARG4*, *CIp10*		Zakikhany *et al.*,(2007

identified as small colonies (Fig. 4.2). Following the second round of transformation to disrupt the second copy of *MDP1*, PCR screening using the primer pair 5F/3R revealed that one intact allele still remained in all transformant screened (Fig. 4.3) and Southern blot analysis was carried out to confirm this (data not shown). Despite multiple attempts it was not possible to knockout both alleles in CD36. Although unusual, other investigators in our laboratory have also encountered this problem when cloning other genes using this reference strain CD36 (Dr. Gary Moran, personal communication). In these cirucmstances it appears that the cassette reintegrates into the first disrupted allele.

As a result of this, it was decided that a number of *C. dubliniensis* strains would be used as transformation recpients. The strains used were CD33, CD38, and Wü284. As before, transformed cells were streaked onto plates containing 100 μg/ml nourseothricin to select for transformants and incubated overnight at 30°C. The strain Wü284 yielded the largest number of transformants. These were again screened by PCR using the primer pair EDTP/FLP. Following the second round of insertion/excision putative revertants were spread onto plates with a drug concentration of 4 μg/ml and 2 μg/ml, which allowed for nourseothricin sensitive revertants to be identified as small colonies (Fig. 4.4). These were then screened by PCR using the primer pair 5F/3R (Fig. 4.1). Forty transformants were screened and six double disruptants were identified. A single PCR product of 982 bp was detected corresponding to the deleted gene, and no products corresponding to the intact gene (3,700 bp) could be detected. (Fig. 4.5). Southern hybridisation was carried out to confirm a Δ*mdp1*/Δ*mdp1* genotype (Fig. 4.6). As mentioned previously, since this study began, the *C. albicans* orthologue has been described as *EED1* (epithelial escape and dissemination), a regulator of filamentation, which is essential for virulence during infection of Reconstituted Human Oral Epithelium (RHE) (Zakikhany *et al.*, 2007). At this stage in the study, Bernhard Hube's Laboratory very kindly gave us the *EED1* wild type, heterozygous and homozygous mutants in order to carry out comparative analysis between the two species. A list of all strains used in this study are available in table 4.1.

4.1.2 Growth curve analysis of the *C. dubliniensis MDP1* mutant

Phenotypic analysis of the *C. dubliniensis MDP1* wild type, LOC2 (*Δmdp1/MDP1*) and LOC4 (*Δmdp1/Δmdp1*) was examined by growth curve analysis (Chapter 2, 2.8.2) as was the *C. albicans EED1* wild type and the *Δeed1/Δeed1* mutant. At specific time points, aliquots of each culture were removed and optical densities at 600 nm measured using a spectrophotometer. The growth temperatures examined were 30°C, 37°C and 42°C. These values were then used to plot a growth curve for each isolate. Experiments were performed on three separate occassions. There was no noticeable difference in doubling times between *C. dubliniensis* wild type, LOC2 (*Δmdp1/MDP1*) and LOC4 (*Δmdp1/Δmdp1*) at 30°C, 37°C and 42 °C (Fig. 4.7). In *C. albicans* there was also no difference in doubling time between the *C. albicans* wild type and the *Δeed1/Δeed1* at 30 °C and 37 °C (Fig. 4.8). However when grown at 42 °C the *C. albicans Δeed1/Δeed1* mutant grew better than the wild type and the difference in doubling times were 2.159 h and 3.240 h respectively (Fig. 4.8).

4.1.3 Osmotic Stress

Growth curve analysis was also carried out with *C. dubliniensis* Wü284 and the *Δmdp1/Δmdp1* mutant in YPD supplemented with 0.6 M, 1 M, 1.6 M NaCl (Fig. 4.9). As before, these experiments were carried out on three separate occasions. Both *C. dubliniensis* Wü284 and LOC4 (*Δmdp1/Δmdp1)* grew similarly in YPD supplemented with 0.6 M and 1 M NaCl (Fig. 4.9). However, at higher concentrations of salt (1.2 M) the *C. dubliniensis* mutant LOC4 grew better than Wü284 in higher concentrations of salt. There was a signifcant difference in doubling time observed between Wü284 (7.905 h) and LOC4 (2.925 h) (*P* 0.0057; Fig. 4.9).

Growth curve analysis in YPD supplemented with 0.6 M, 1 M, 1.6 M NaCl was also carried out with *C. albicans* BWP17 and the *Δeed1/Δeed1* mutant. Both the wild type and the *Δeed1/Δeed1* mutant grew well at the 0.6 M and 1 M concentrations of NaCl and there was no noticeable difference in doubling time observed (Fig. 4.10). However, at 1.2 M NaCl concentrations both strains grew more slowly and there was a small difference in doubling time observed between BWP17 (2.529 h) and the *Δeed1/Δeed1* mutant (2.448 h) (Fig. 4.10).

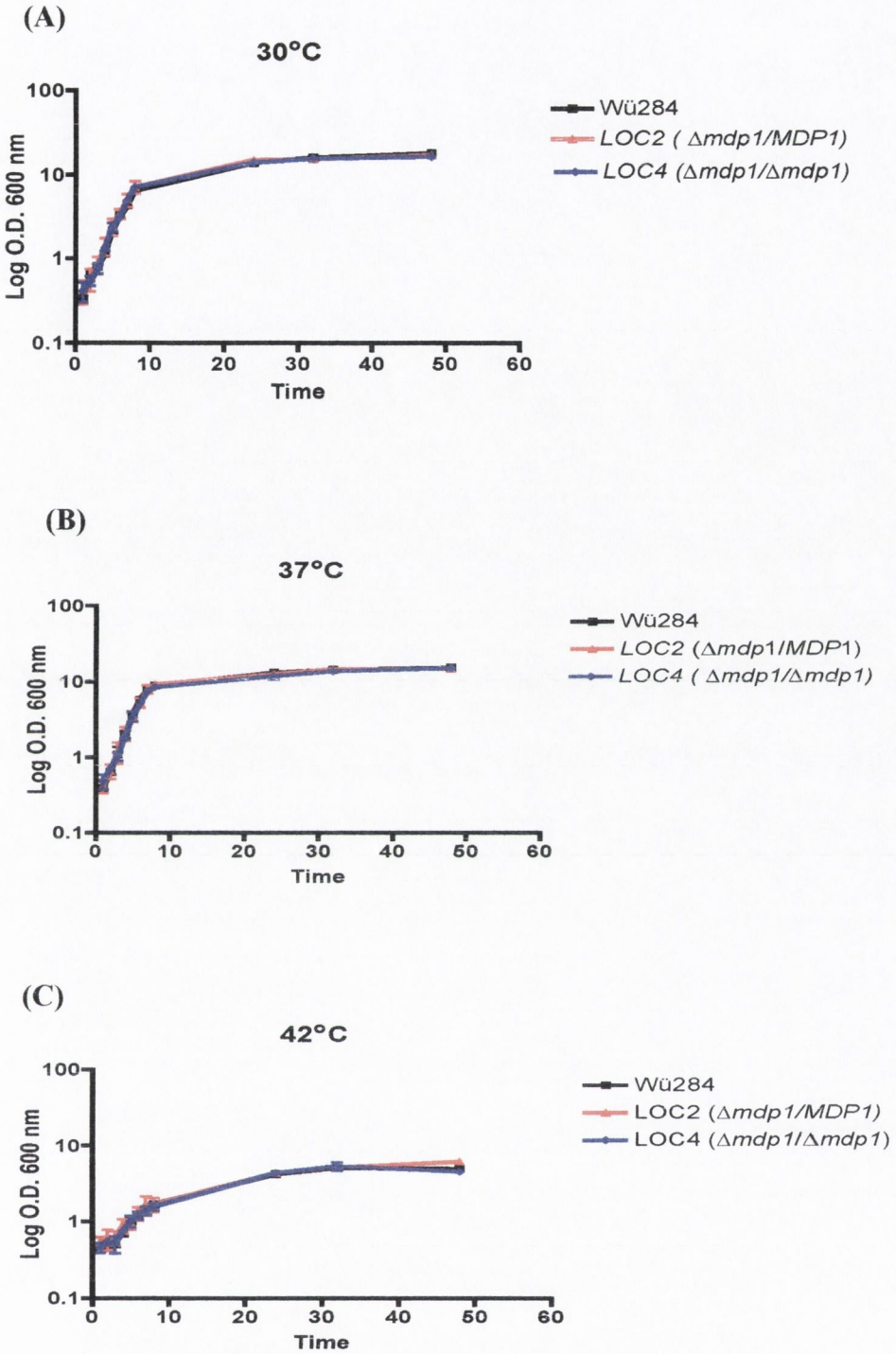


Figure 4.7. Logarithmic growth curves of *C. dubliniensis* Wü284, LOC2 and LOC4 in YPD broth at (A) 30°C, (B) 37°C and (C) 42°C. Each experiment was repeated on three separate occassions.

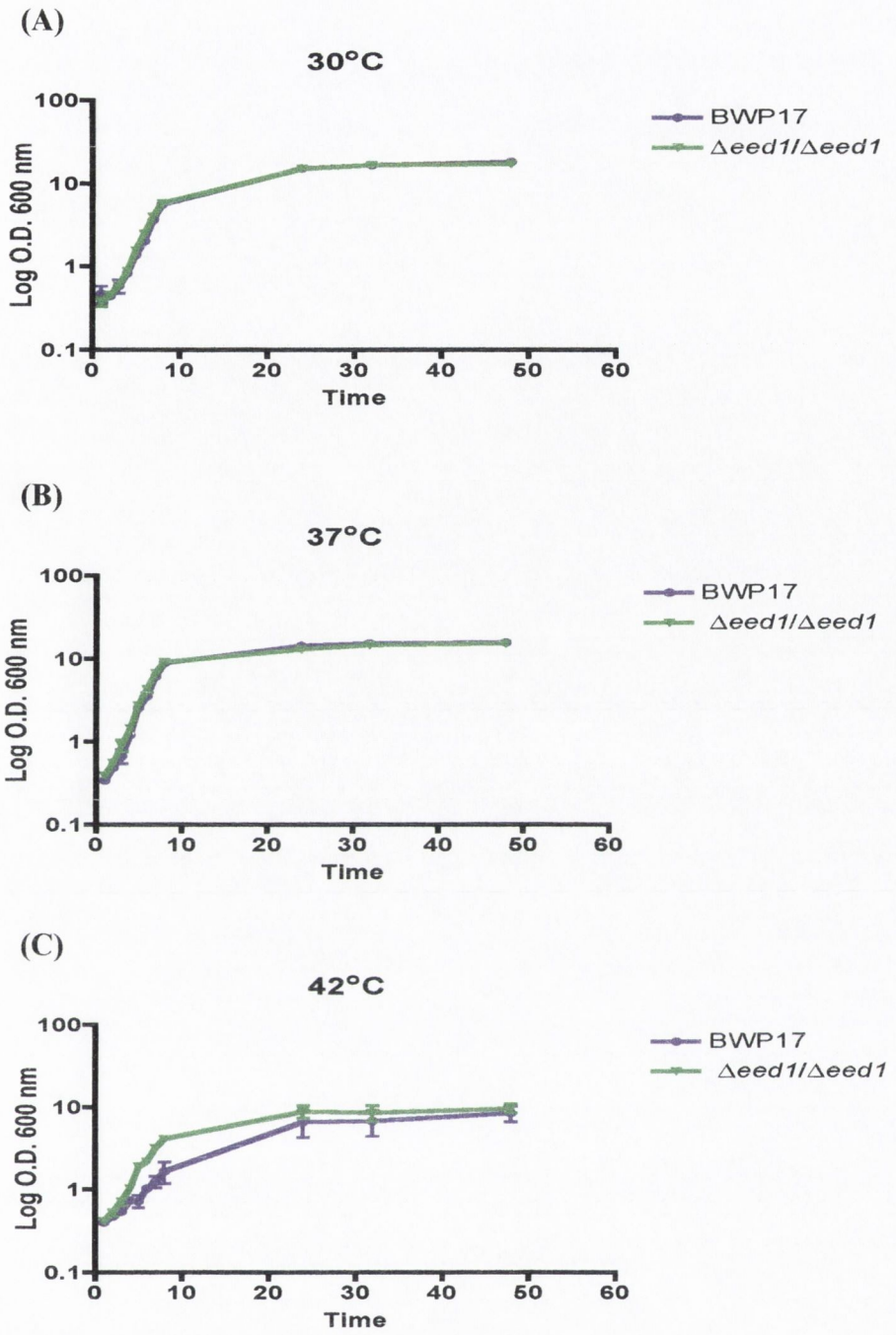


Figure 4.8 Logarithmic growth curves of *C. albicans* BWP17 and the *Δeed1/Δeed1* mutant in YPD broth at (A) 30°C, (B) 37°C and (C) 42°C. Experiments were repeated on three spearate occassions.

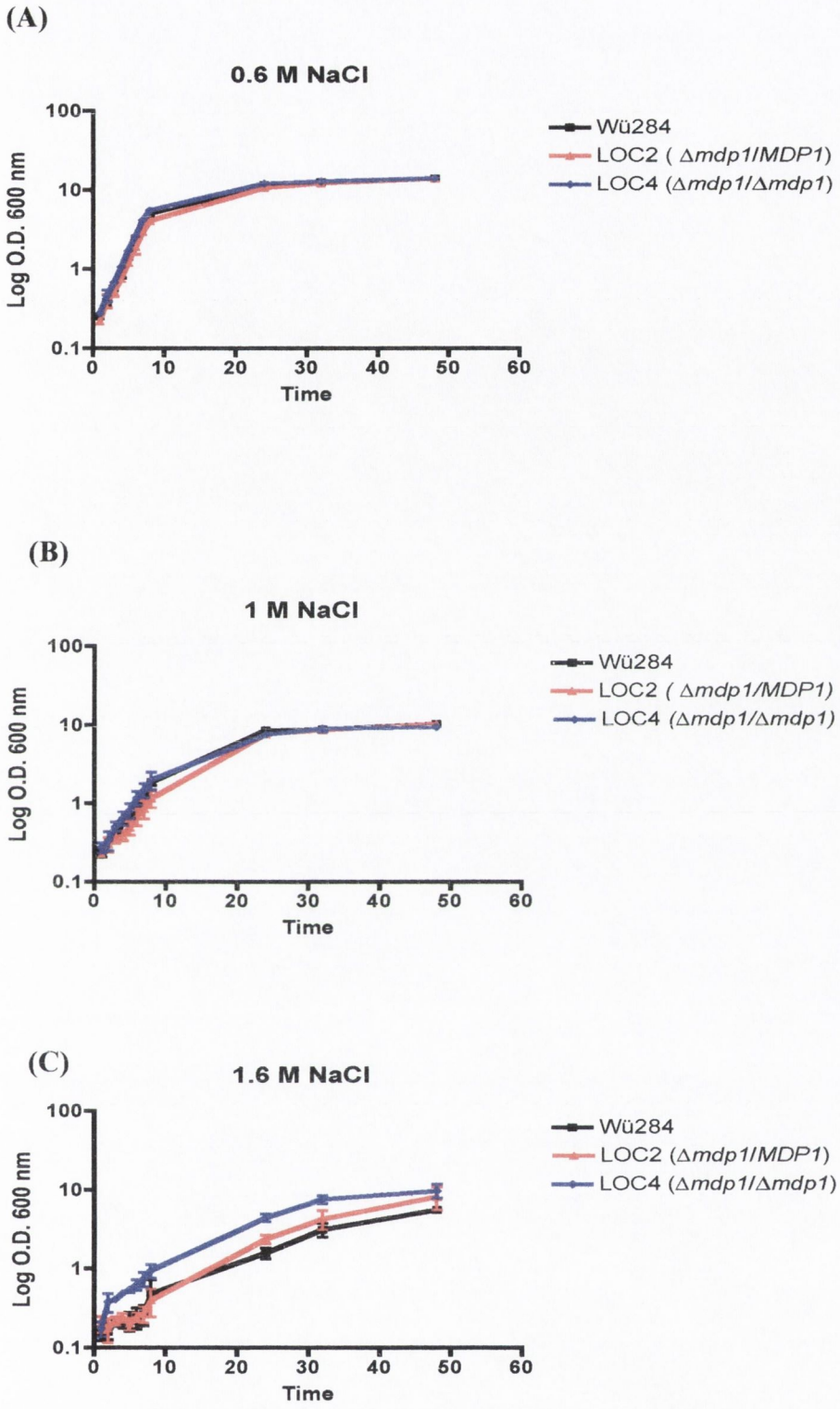


Figure 4.9. Logarithmic growth curves of *C. dubliniensis* Wü284, LOC2 (*Δmdp1/MDP1)* and LOC4 (*Δmdp1/Δmdp1*), grown in YPD broth at 37°C supplemented with the following concentrations of NaCl, (A) 0.6 M NaCl (B) 1 M NaCl and (C) 1.6 M NaCl. Experiments were repeated on three separate occasions.

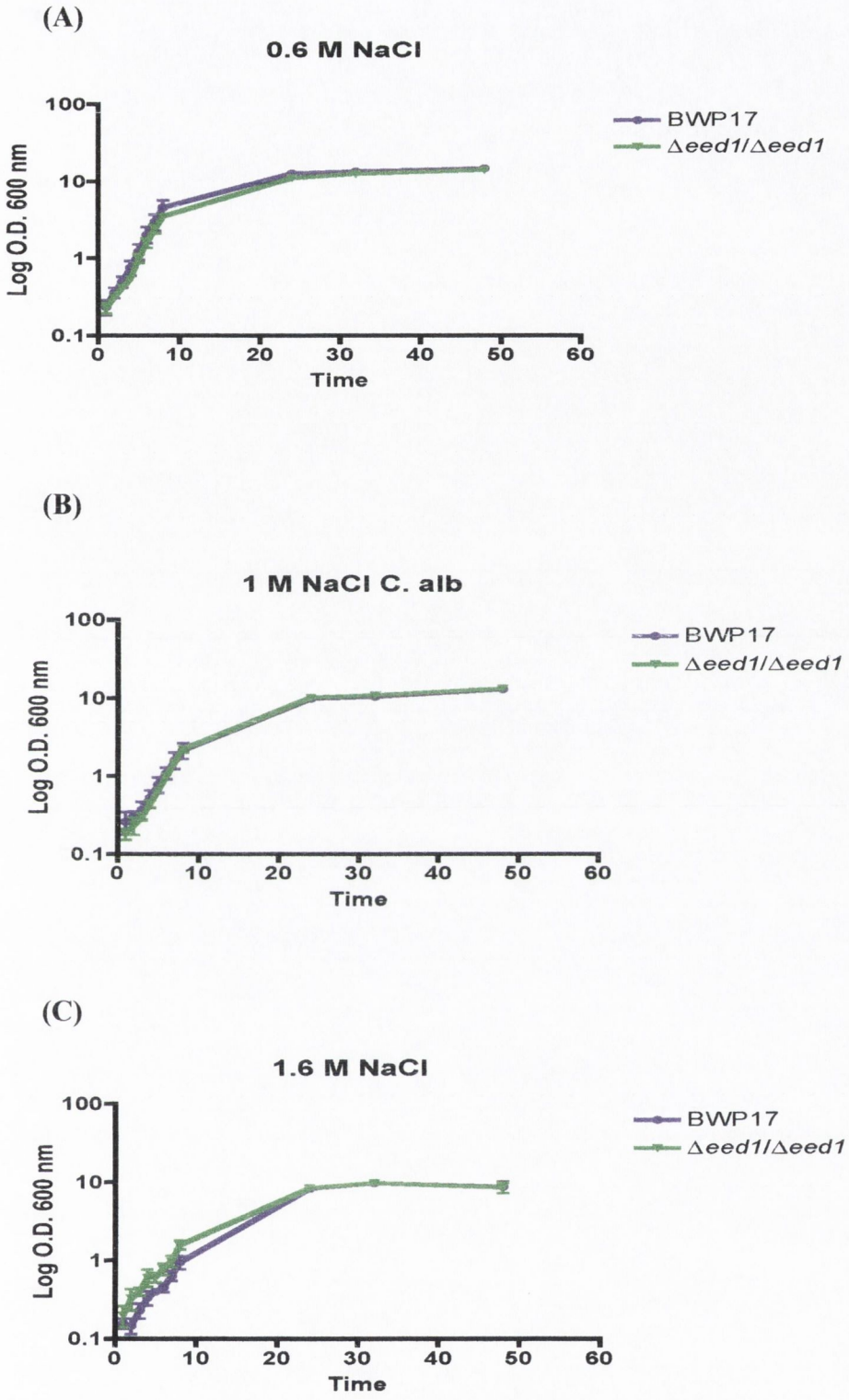
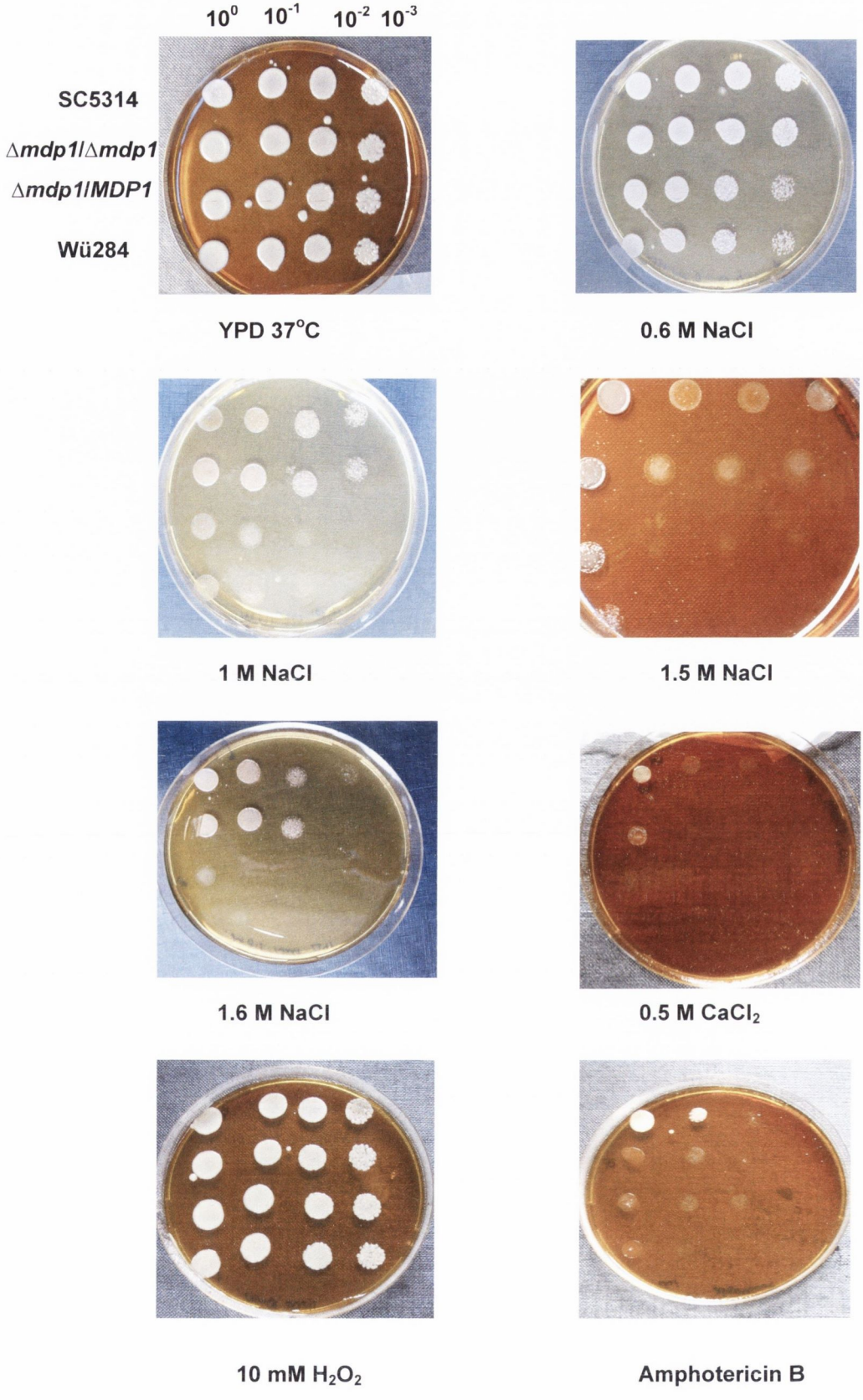


Figure 4.10. Logarithmic growth curves of *C. albicans* BWP17 and the *Δeed1/Δeed1* mutant, grown in YPD broth at 37°C supplemented with the following concentrations of NaCl, (A) 0.6 M NaCl (B) 1 M NaCl and (C) 1.6 M NaCl. Experiments were repeated on three separate occasions.


10^0 10^{-1} 10^{-2} 10^{-3}
SC5314
Δmdp1/Δmdp1
Δmdp1/MDP1
Wü284
YPD 37°C
0.6 M NaCl
1 M NaCl
1.5 M NaCl
1.6 M NaCl
0.5 M $CaCl_2$
10 mM H_2O_2
Amphotericin B

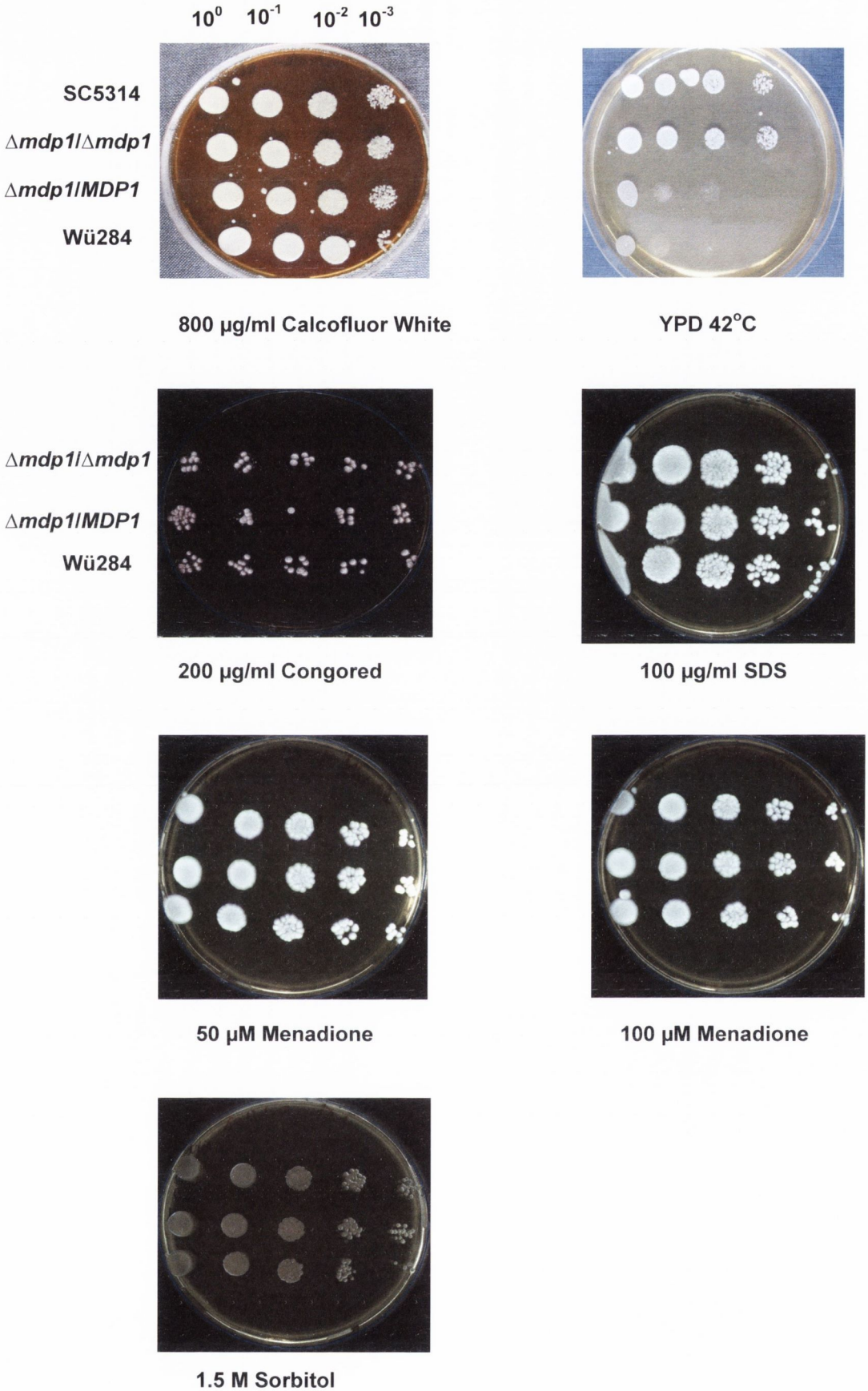
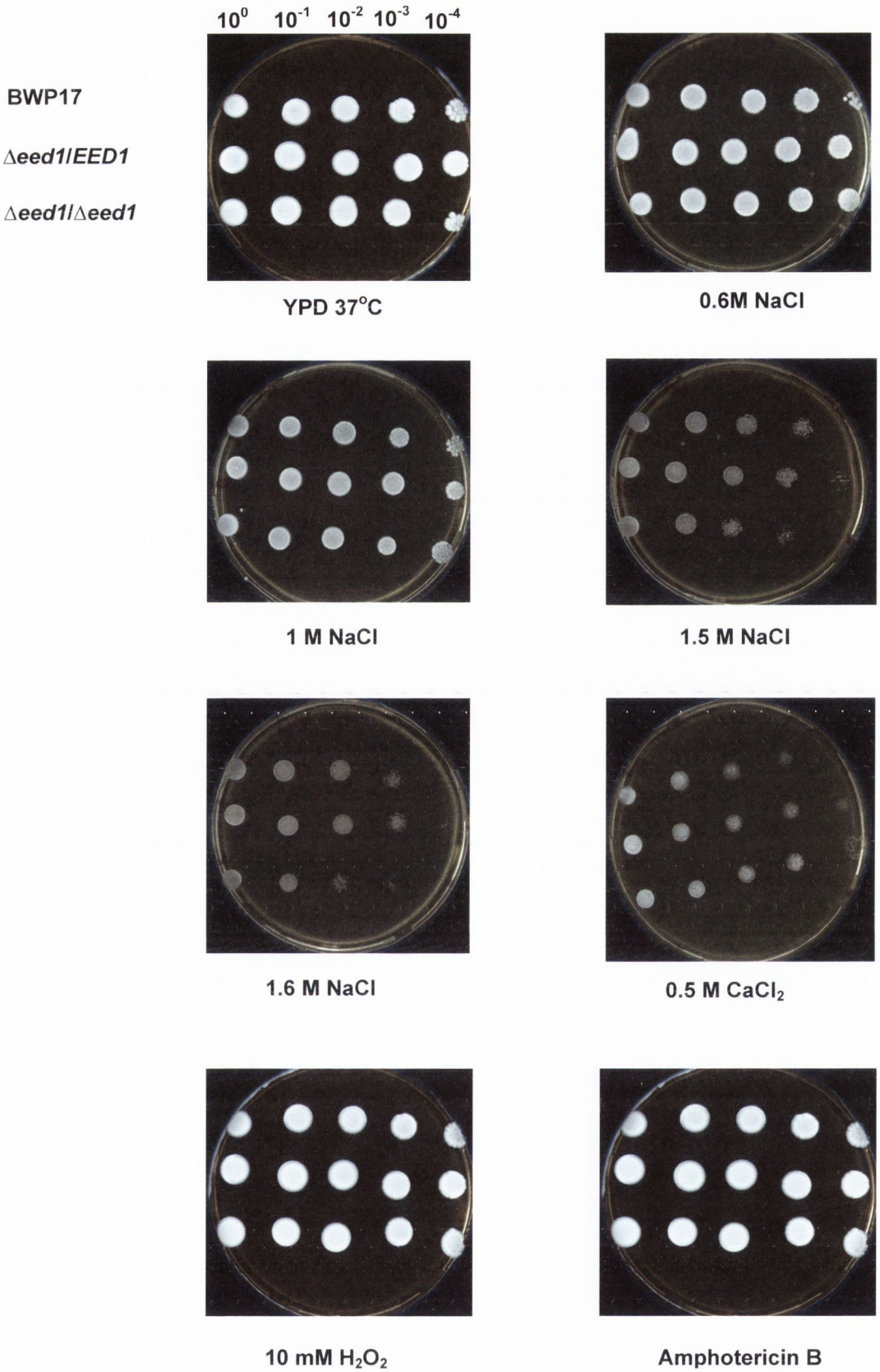


Figure 4.11. Susceptibility of *C. dubliniensis* Wü284, *Δmdp1/MDP1*, *Δmdp1/Δmdp1* and *C. albicans* SC5314 to increasing concentrations of inhibitors. Each strain was grown to exponential phase to a density of 1 x 10^7 c.f.u./ml in YPD. Serial dilutions of each were prepared and 10 µl of each dilution was spotted onto YPD agar containing inhibitors, which were then incubated for 48 h at 37°C unless otherwise stated.


10^0 10^{-1} 10^{-2} 10^{-3} 10^{-4}
BWP17
Δeed1/EED1
Δeed1/Δeed1
YPD 37°C
0.6M NaCl
1 M NaCl
1.5 M NaCl
1.6 M NaCl
0.5 M $CaCl_2$
10 mM H_2O_2
Amphotericin B

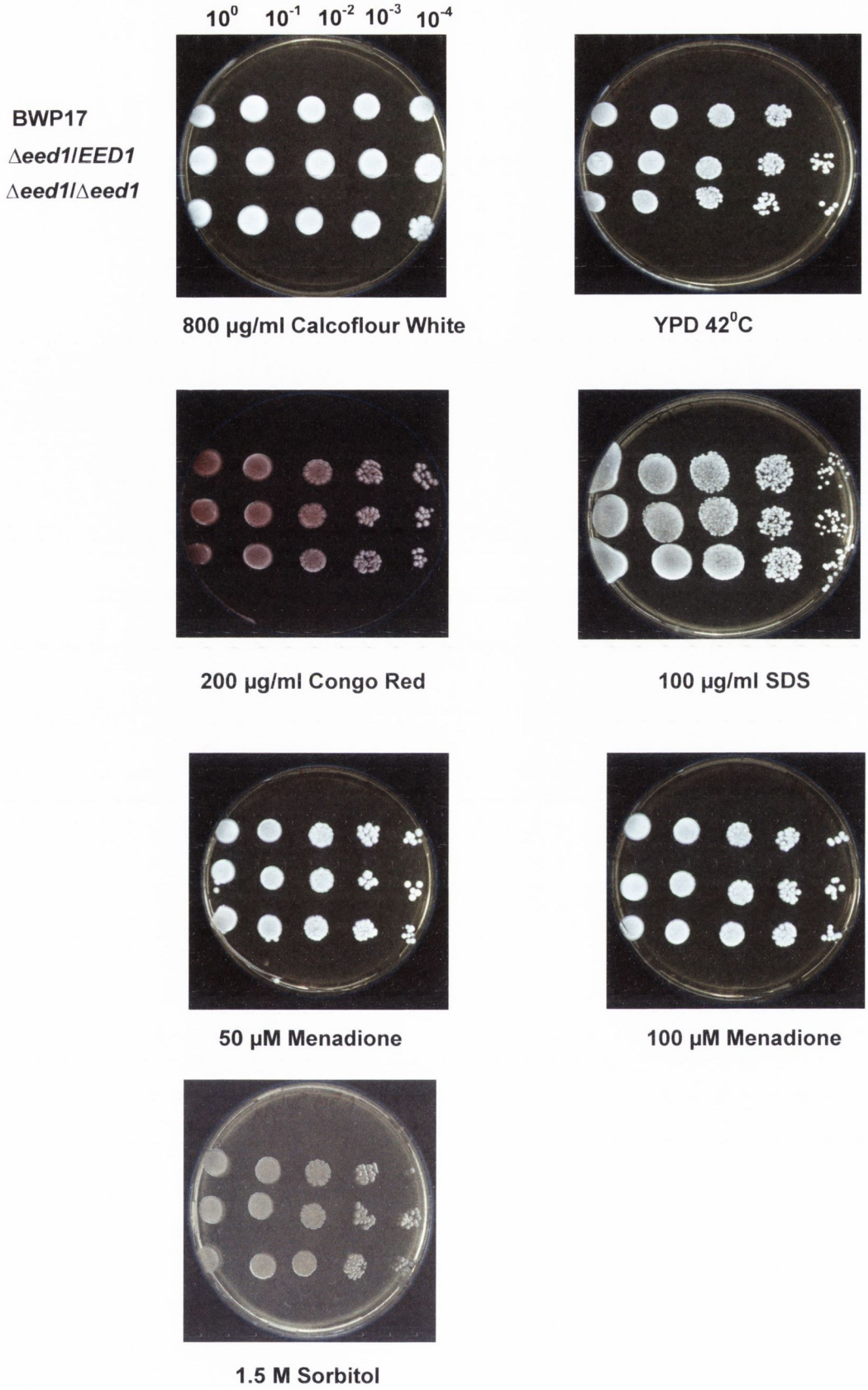


Figure 4.12. Susceptibility of *C. albicans* BWP17, *Δeed1/Δeed1* and *Δeed1/EED1* mutant to increasing concentrations of inhibitors. Each strain was grown to exponential phase to a density of 1 x 10^7 c.f.u./ml in YPD. Serial dilutions of each strain were prepared and 10 µl of each dilution was spotted onto YPD agar containing inhibitors, which were then incubated for 48 h at 37°C unless otherwise stated.

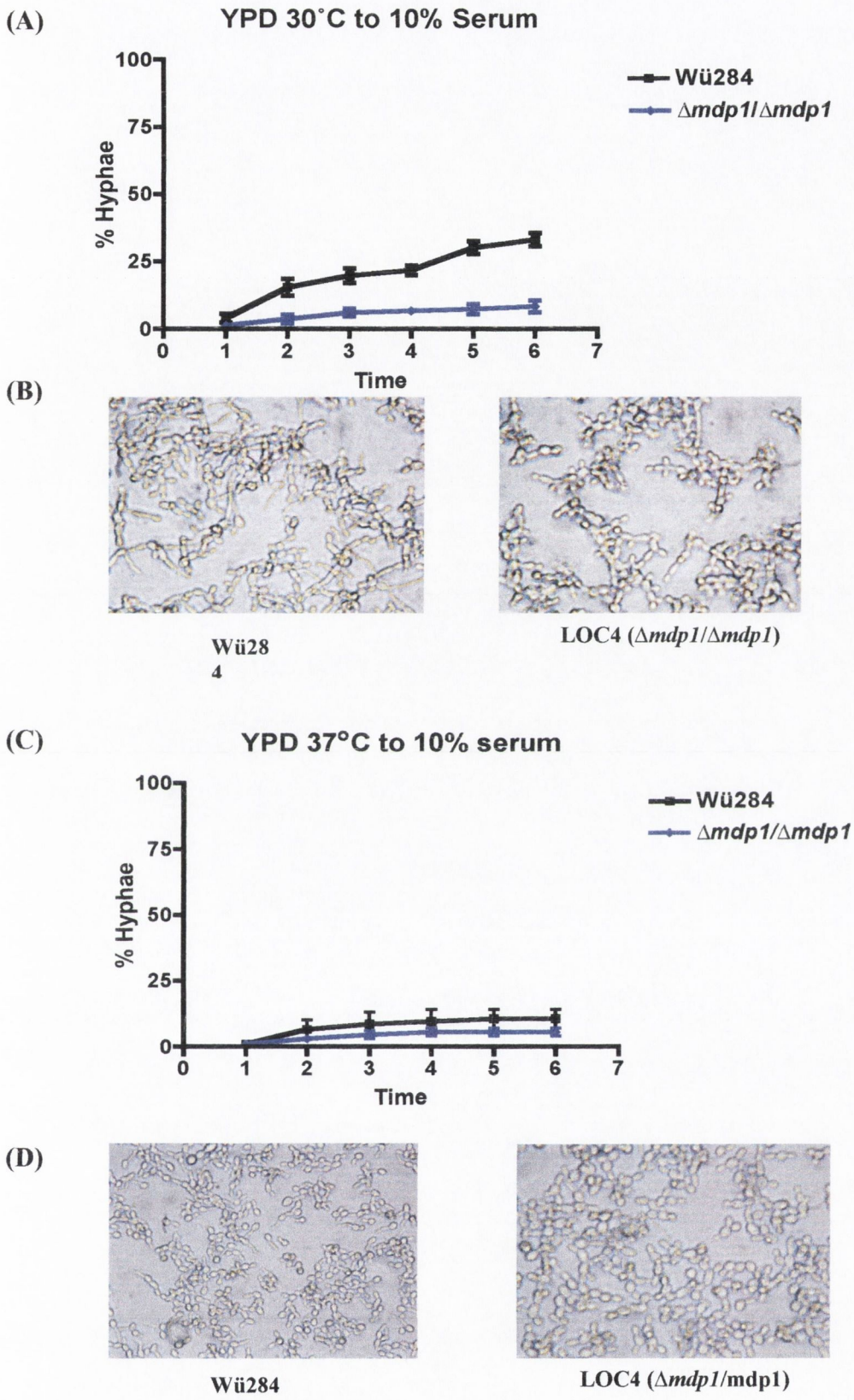


Figure 4.13. (A) Hyphal formation of Wü284 and LOC4 (*Δmdp1/Δmdp1*) following growth in YPD at 30°C and inoculation into 10 % new born calf serum. **(B)** Light micrographs of each strain following growth in YPD at 30°C and inoculation into 10 % new born calf serum after 3 h. **(C)** Hyphal formation of Wü284 and LOC4 (*Δmdp1/Δmdp1*) following growth in YPD at 37 °C and inoculation into 10 % new born calf Serum. **(D)** Light micrograpahs of each strain following growth in YPD at 37 °C and inoculation into 10 % new born calf serum after 3 h.

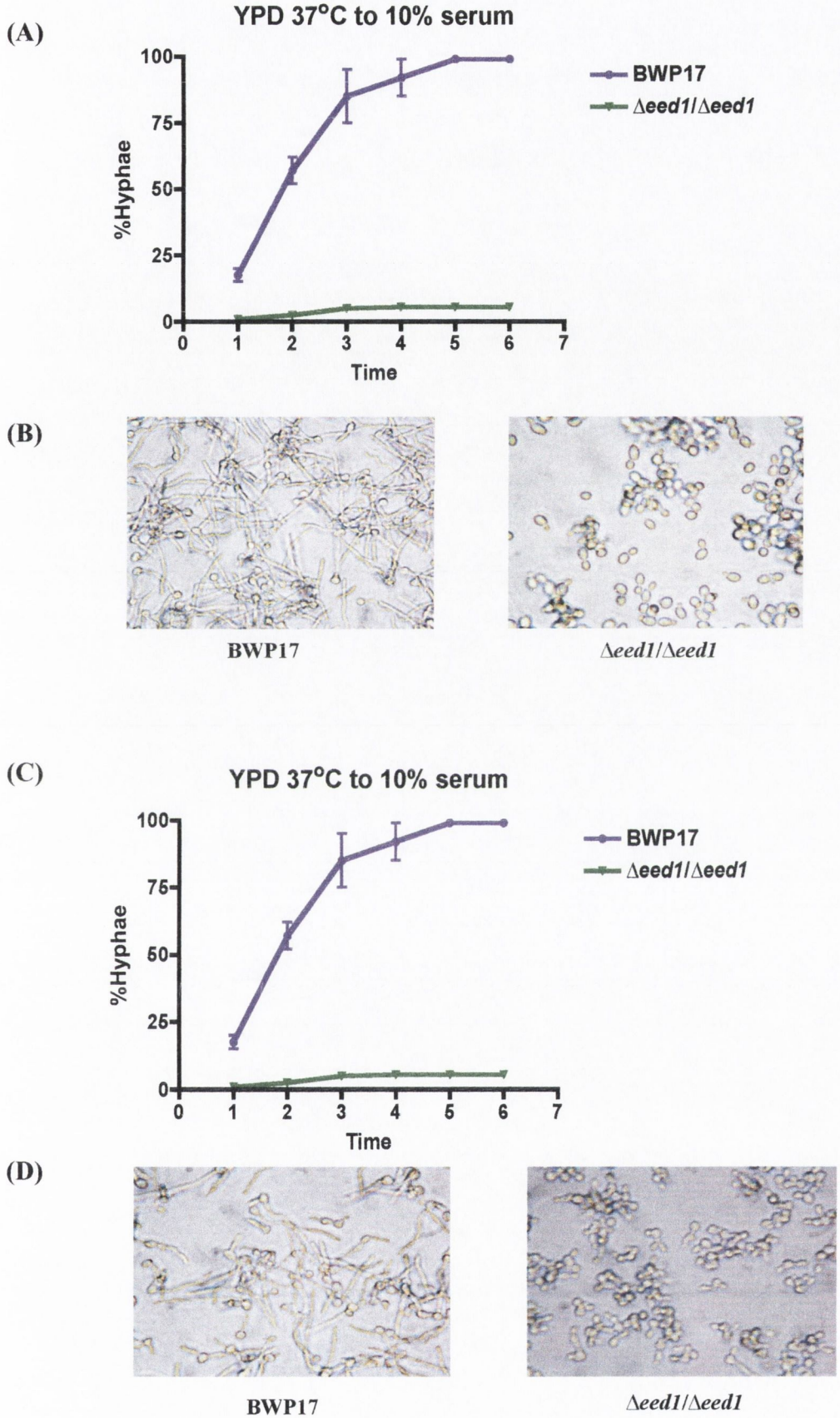


Figure 4.14. (A) Hyphal formation of *C. albicans* BWP17 and *Δeed1/Δeed1* following growth in YPD at 30°C and inoculation in 10% new born calf Serum. **(B)** Light micrographs of each strain following growth in YPD at 30°C and inoculation into 10 % new born calf serum, taken after 3 h. **(C)** Hyphal formation of *C. albicans* BWP17 and *Δeed1/Δeed1* following growth in YPD at 37°C and inoculation in 10% new born calf serum. **(D)** Light micrograpahs of each strain following following growth in YPD at 37 °C and inoculation into 10 % new born calf serum, taken after 3 h.

4.1.6 A pH Shift affects filamentation

In *C. albicans* pH and temperature are among the external signals that trigger morphogenesis. Therefore, it was decided to examine the effect of a pH shift on filamentation in both. *C. dubliniensis* Wü284 and LOC4 (*Δmdp1/mdp1*) and *C. albicans* BWP17 and the *Δeed1/Δeed1* mutant by growing the cells overnight in Lee's medium pH 4.5 at 30°C, followed by subsequent inoculation into 10 % newborn calf serum at 37°C. In previous experiments where the cells were grown overnight in YPD, the pH of this medium was pH 5.6.

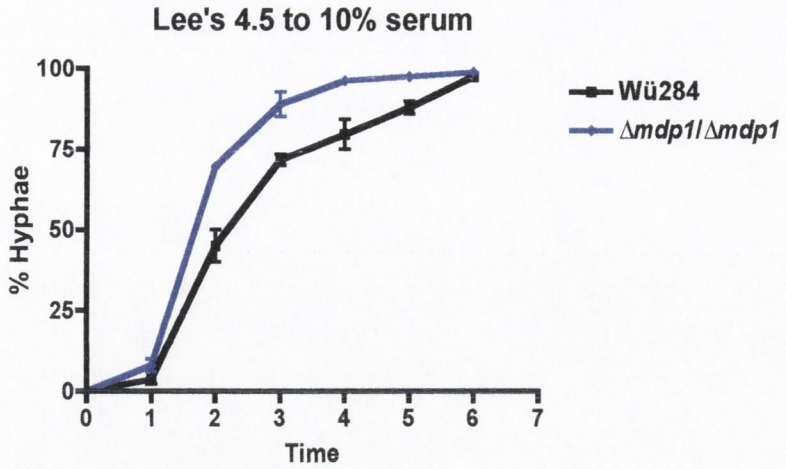
In *C. dubliniensis* the pH shift from pH 4.5 to pH 7.5 enhanced filamentation in in both Wü284 and LOC4 (*Δmdp1/Δmdp1*) (Fig. 4.15). Both strains produced hyphae rapidly and by the third hour of the experiment, Wü284 were ~ 60 % hyphal and LOC4 (*Δmdp1/Δmdp1*) were over 75 % hyphal. The level of hyphae produced was similar to levels observed in a *C. albicans* wild type strain (Fig. 4.15). In *C. albicans* a pH shift from 4.5 to 7.5 enhanced hypha formation in serum in both BWP17 and the *Δeed1/Δeed1* mutant (Fig. 4.16). However, in the *Δeed1/Δeed1* mutant, the pH shift only partially restored its ability to produce filaments compared to the wild type (Fig. 4.16). Following the discovery that a pH shift from 4.5 to 7.5 could enhance filamentation in both *C. albicans* and *C. dubliniensis* mutants, the role of this pH shift was confirmed by preculturing the cells overnight in Lee's medium pH 6.0 and pH 8.0 at 30°C, followed by inoculation into 10 % newborn calf serum at 37°C. This experiment was carried out to determine that the pH shift and not the compostion of the growth media was responsible for the increased hyphal growth. Following preculture in Lee's pH 6 *C. dubliniensis* Wü284 produced hyphae, however, this was less efficient than preculture at pH 4.5 and resembled the level of filamentation observed following preculture in YPD (pH 5.6). Following preculture at pH 6.0, LOC4 (*Δmdp1/Δmdp1*) failed to produce hyphae demonstrating that a pH shift from 4.5 to 7.5 is essential for filamentation in a *Δmdp1/Δmdp1* mutant (Fig. 4.17). Following pre-culture in Lee's medium pH 8.0, both the wild type and mutant fail to produce hyphae when inoculated into 10 % serum (Fig. 4.17). *C. ablicans* BWP17 produced hyphae rapidly, under all conditions tested. However, this pH shift from 6.0 to 7.5 could not restore the ability of *Δeed1/Δeed1* mutant to produce filaments (Fig. 4.18). Following pre-culture on Lee's medium pH 8.0 the wild type *C. albicans* produced hyphae rapidly as expected and again the *Δeed1/Δeed1* mutant failed to produce hyphae (Fig.4.18).

A pH shift gratly enhanced hypha formation in both the *C. dubliniensis* wild-type and homozygous mutant. It was therfore decided to carry out an experiment using 4 different strains of *C. dubliniensis* to see whether a pH shift from 4.5 to 7.5 also enhanced filamentation in these strains. The following strains were MAN 448, LP3, 96.54 and William II, these strains were randomly selected. Firstly, strains were grown in YPD overnight at 30°C and inoculated into 10 % newborn calf serum at 37°C. All strains produced less than 25 % hyphae during the course of the experiment (Fig. 4.19). Following preculture in Lee's pH 4.5, hypha induction increased greatly. The *C. dubliniensis* strains MAN 448 and 96.54 were grew in the hyphal form by the first hour. The *C. dubliniensis* strains LP3 and William II produced hypha at a slower rate to the other two strains. Nonetheless, preculture in Lee's medium pH 4.5 did result in an increase in hyphae formation compared to pre-culture in YPD. (Fig. 4.19).These results show that the increase in filamentation observed in Wü284 is also observed in other *C. dubliniensis* isolates and that a pH shift from 4.5 to 7.5 enhances filamentation in *C. dubliniensis*.

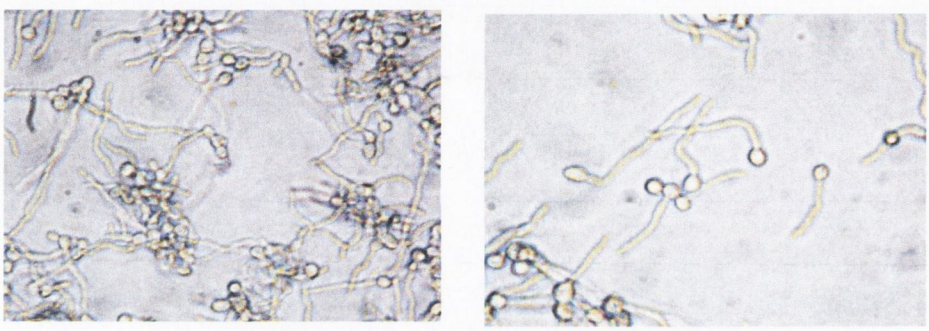
4.1.7 Do cell density, temperature and nutrient starvation have an effect on Δ*eed1*/Δ*eed1*?

The results of the previous experiments demonstrate that the *C. albicans* Δ*eed1*/Δ*eed1* mutant can still form true hyphae in response to a pH shift from 4.5 to 7.5. However, in the absence of this shift, when precultured at pH 6.0, the Δ*eed1*/Δ*eed1* mutant failed to form hyphae, whereas the wild type could still form true hyphae. Therefore, the wild type strain may form hyphae in response to non-pH related signals by a mechanism which requires *EED1*. In order to determine what effect causes the increase in filamentation of the *C. albicans* wild type observed when precultured in pH 6, it was decided to carry out a series of experiments which examined a shift in cell density, a temperature shift and nutrient starvation. Firstly, *C. albicans* BWP17 and the Δ*eed1*/Δ*eed1* mutant were subjected to a shift in temperature by growth in YPD overnight at 30°C followed by a 1 in 500 dilution into fresh YPD at 37 °C and grown for 3 h. In *C. albicans* BWP17, the yeast cells were round in appearance and although true hypha cells were present, the majority grew as pseudohypha. (Fig. 4.20). The Δ*eed1*/Δ*eed1* mutant cells grew in the yeast phase after 3 h although the cells appeared to be slightly oval in appearance (Fig. 4.20). Next, a shift in cell density was examined

(A)



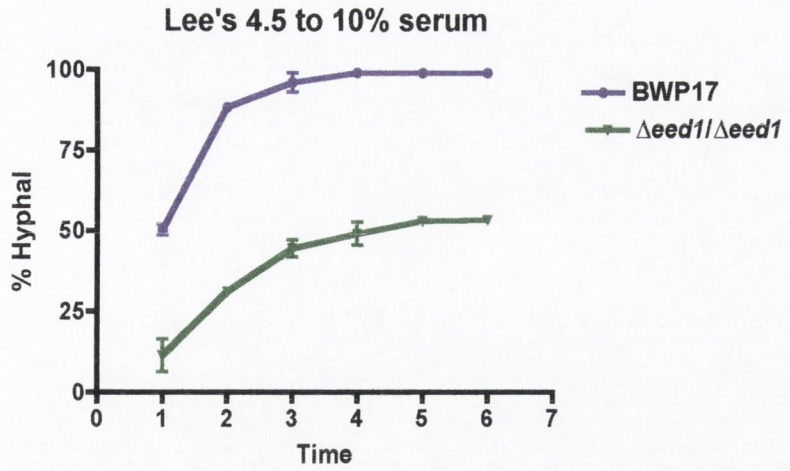
(B)



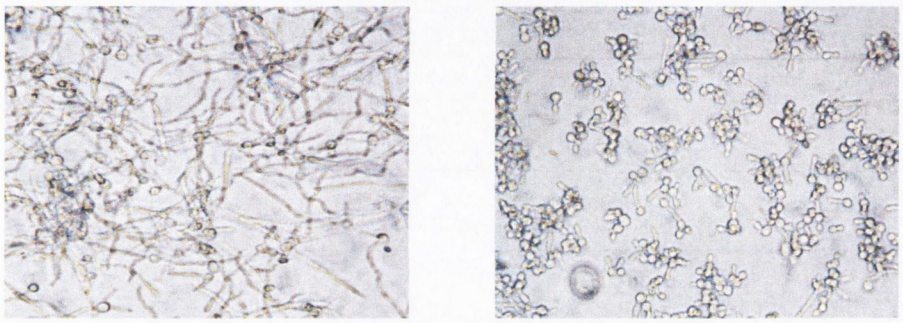
Wü284 **LOC4 (*ΔCdMDP1*)**

Figre 4.15. (A) Hyphal formation of Wü284 and LOC4 (*Δmdp1/Δmdp1*) following growth in Lee's Medium pH 4.5 followed by inoculation into 10 % new born calf serum.**(B)** Light micorgrpahs of each strain grown overnight in Lee's medium pH 4.5 followed by inoculation into 10 % new born calf serum, taken after 3 h.

(A)



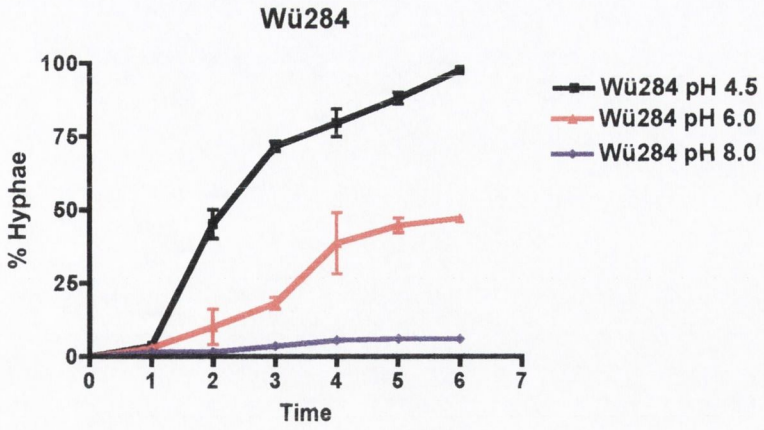
(B)



BWP17 ***Δeed1/Δeed1***

Figure 4.16. (A) Hyphal formation of BWP17 and *Δeed1/Δeed1* following growth in Lee's Medium pH 4.5 followed by inoculation into 10 % new born calf serum. **(B)** Light micorgraphs of each strain grown overnight in Lee's medium pH 4.5 followed by inoculation into 10 % new born calf serum, taken after 3 h.

(A)



(B)

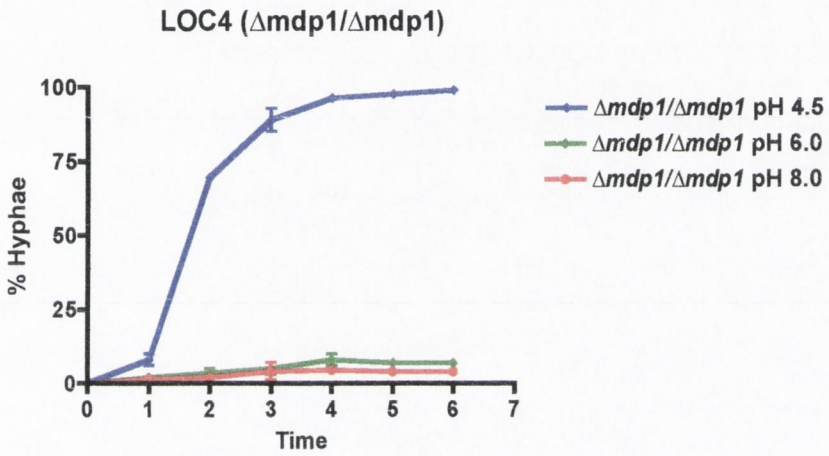
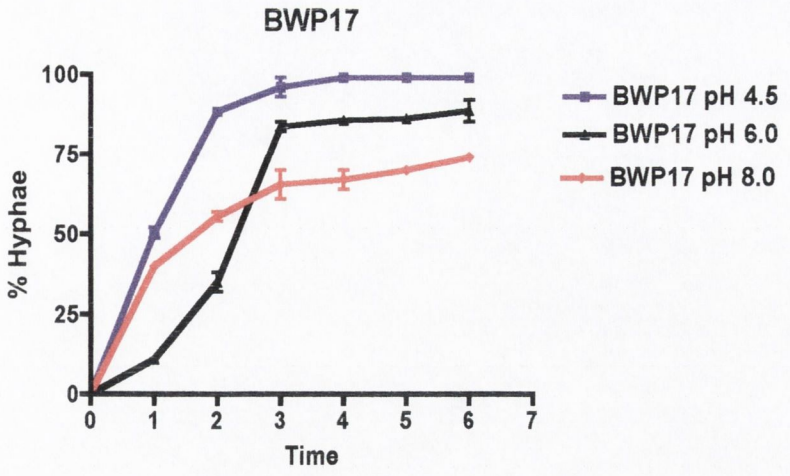


Figure 4.17. (A) Hyphal formation of Wü284 following growth in Lee's edium pH 4.5, pH 6.0 and pH 8.0 followed by inoculation into 10 % new born calf serum. **(B)** Hyphal formation of LOC4 (*Δmdp1/Δmdp1*) following growth in Lee's Medium pH 4.5, pH 6.0 and pH 8.0 followed by inoculation into 10 % new born calf serum.

(A)



(B)

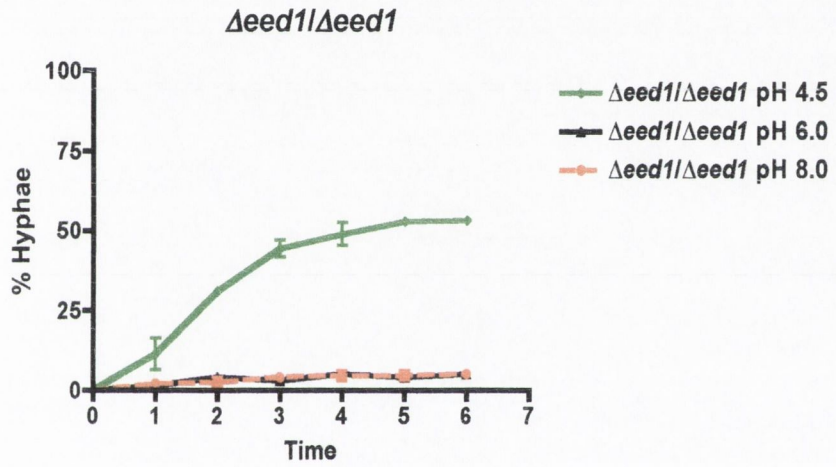
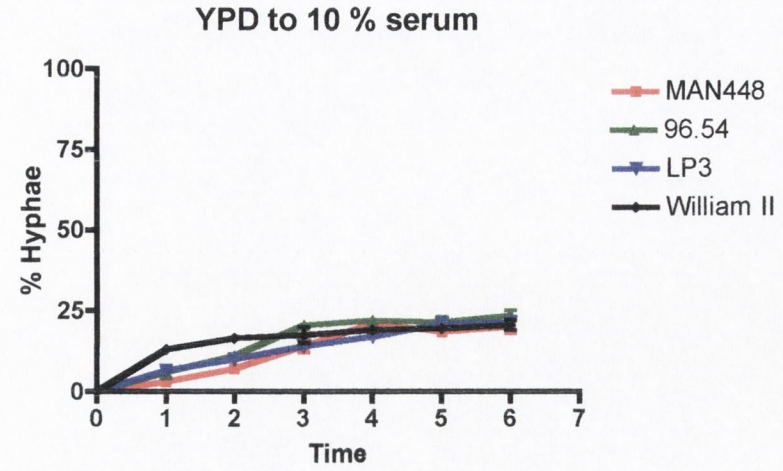


Figure 4.18. (A) Hyphal formation of BWP17 following growth in Lee's Medium at pH 4.5, pH 6.0 and pH 8.0 followed by inoculation into 10 % new born calf serum.**(B)** Hyphal formation of *Δeed1/Δeed1* following growth in Lee's medium pH 4.5, pH 6.0 and pH 8.0 and inoculation into 10 % new born calf serum.

(A)



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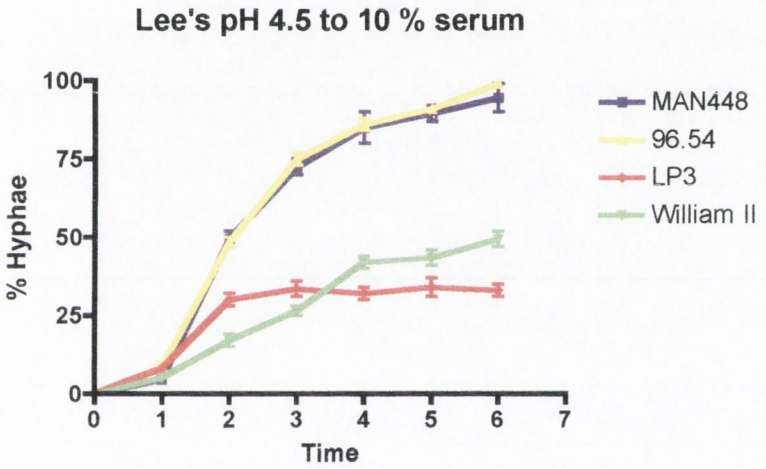


Figure 4.19. Hypha formation of four *C. dubliniensis* clinical isolates. **(A)** Hyphal formation of MAN448, 96.54, LP3, William II (clinical isolates) following growth in YPD Medium at 30°C followed by inoculation into 10 % new born calf serum. **(B)** Hyphal formation of MAN448, 96.54, LP3, William II (clinical isloates) following growth in Lee's medium pH 4.5 followed by inoculation into 10 % new born calf serum.

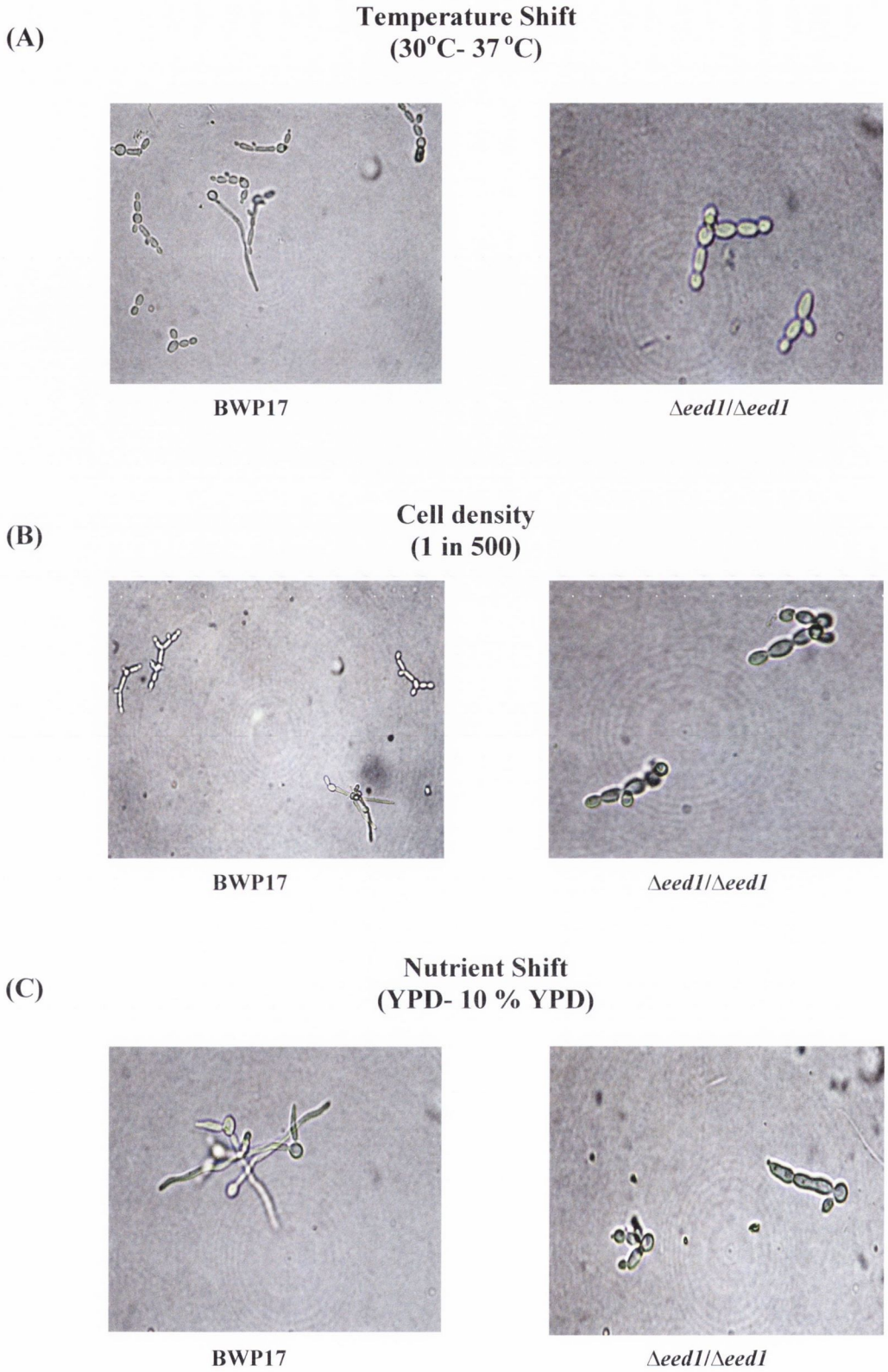


Figure 4.20. **(A)** BWP17 and the Δ*eed1*/Δ*eed1* mutant following preculture in YPD at 30°C and subsequent inoculation into YPD at 37°C. **(B)** BWP17 and the Δ*eed1*/Δ*eed1* mutant following preculture in YPD at 37°C and subsequent inoculation into YPD at 37°C. **(C)** BWP17 and the Δ*eed1*/Δ*eed1* mutant following preculture in YPD at 37°C and subsequent inoculation into 10 % YPD at 37°C.

by growing both *C. albicans* BWP17 and the *Δeed1/Δeed1* mutant in YPD at 37°C overnight, followed by dilution of 1 in 500 into fresh YPD and grown for 3 h at 37°C. In *C. albicans* BWP17 the cells grew predominantly in the yeast phase but there were pseudohyphae present, whereas the *Δeed1/Δeed1* mutant cells were predominantly yeast phase but exhibited clumping (Fig. 4.20). The final condition analysed was one of starvation where *C. albicans* BWP17 and the *Δeed1/Δeed1* mutant were grown overnight at 37°C and diluted 1 in 500 into fresh 10 % YPD and grown for 3 h at 37°C. Under these conditions, BWP17 formed abundant true hyphae, whereas the *Δeed1/Δeed1* mutant again formed elongated yeast cells which resembled pseudohyphae (Fig. 4.20). These results indicate that *EED1* may be involved in regulating different morphological shifts depending on the nutritional content of the media.

4.1.8 Analysis of *NRG1* expression in response to a pH shift

The data obtained from the pH shift experiment indicated that the increase in filamentation observed when *C. dubliniensis* strains were precultured in a low pH medium was occuring independent of *MDP1*. The mechanism for this pH shift could involve the repression of *NRG1*, which would result in the increase in hyphae formation (Fig. 4.15). In order to assess this, real-time analysis was carried out using *C. dubliniensis* Wü284 to determine whether expression of *NRG1* was up-regulated or down-regulated in response to pH. The experimental conditions tested were pre-culture of Wü284 in YPD (pH 5.6) or Lee's medium (pH 4.5) grown at 30°C and subsequent inoculation into 10 % serum. Following pre-culture in Lee's pH 4.5, expression of *NRG1* relative to *ACT1* at T_0 was 4-fold less than the expression of *NRG1* following pre-culture in YPD. At the 1 h time point expression of *NRG1* dropped 7-fold from T_0 following pre-culture in YPD and the expression dropped further at 3h (Fig. 4.21). However, when the inoculum was pre-cultured in Lee's pH 4.5, at 1 h there was negligible expression of *NRG1* relative to *ACT1* (Fig. 4.21). It is clear from these results that *NRG1* is expressed at very low levels following pre-culture in Lee's medium pH 4.5, indicating at this low level of *NRG1* expression may in turn result in increased filamentation in *C. dubliniensis* Wü284 following inoculation into 10 % serum.

4.1.9 Hypha induction on solid media

Solid media were also used to compare hypha production of *C. albicans* BWP17 and the *Δeed1/Δeed1* mutant and *C. dubliniensis* Wü284 and the *ΔMDP1* mutant (Chapter 2, 2.8.1).

Initially, *C. dubliniensis* Wü284 and the *Δmdp1/Δmdp1* mutant and *C. albicans* SC5314 (as a control) were examined on Spider medium (Table 2.3). Spider medium was used as it has previously been shown to induce hypha formation in *C. albicans* (Liu *et al.*, 1994). *C. albicans* SC5314 produced rough filamentous colonies, whereas both *C. dubliniensis* Wü284 and the *Δmdp1/Δmdp1* mutant produced smooth colonies (Fig. 4.22). When the *C. dubliniensis* Wü284 and the *Δmdp1/Δmdp1* mutant were grown on Lee's medium and on YPS embedded medium both produced smooth colonies, whereas the *C. albicans* control strain SC5314 produced rough filamentous colonies on both these agars (Fig. 4.22). Yeast nitrogen base (YNB) agar with and without 10 % serum was also examined. *C. dubliniensis* Wü284 and the *Δmdp1/Δmdp1* mutant did not produce hyphae on YNB without 10% newborn calf serum. However, when 10 % newborn calf serum was present in the medium, hypha fringes appeared to be visible under the microscope on both Wü284 and the *Δmdp1/Δmdp1* mutant (Fig. 4.22).

Phenotypic analysis of *C. albicans* BWP17 and the *Δeed1/Δeed1* mutant was also carried out. On Spider medium BWP17 produced rough filamentous colonies, whereas the *Δeed1/Δeed1* mutant produced smooth colonies (Fig. 4.23). The *C. albicans* BWP17 produced rough filamentous colonies on Lee's medium and on YPS embedded medium and the *Δeed1/Δeed1* again produced smooth round colonies (Fig. 4.23) indicating that in *C. albicans* the *EED1* gene is essential for hypha formation to occur. Yeast nitrogen base (YNB) agar with and without 10 % serum was also examined. BWP17 did produce hyphae on YNB agar without 10 % serum, however on YNB containing 10 % serum, hyphae were clearly visible within the colony. The *Δeed1/Δeed1* mutant produced smooth round colonies on YNB without serum. When 10 % serum was added to YNB hypha fringes were visible

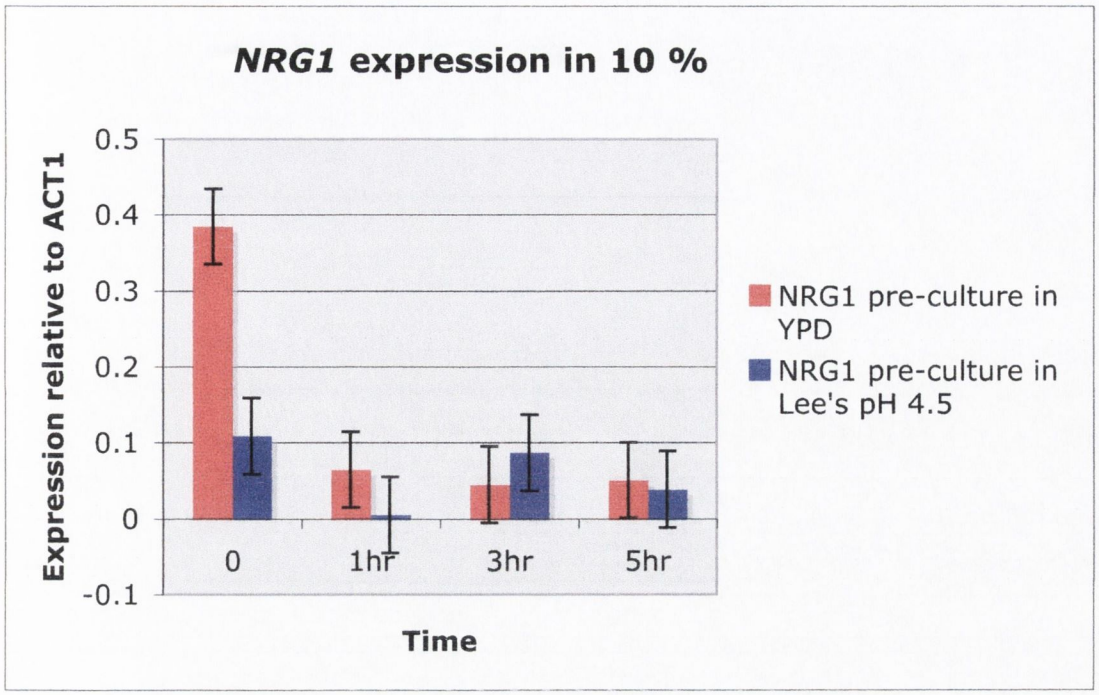


Figure 4.21. Relative expression of *C. dubliniensis NRG1* following pre-culture in YPD (Pink) and relative expression of *NRG1* following pre-culture in Lee's pH 4.5 (Navy) and subsequent inoculation into 10% serum. Experiment was repeated on more than three occasions.

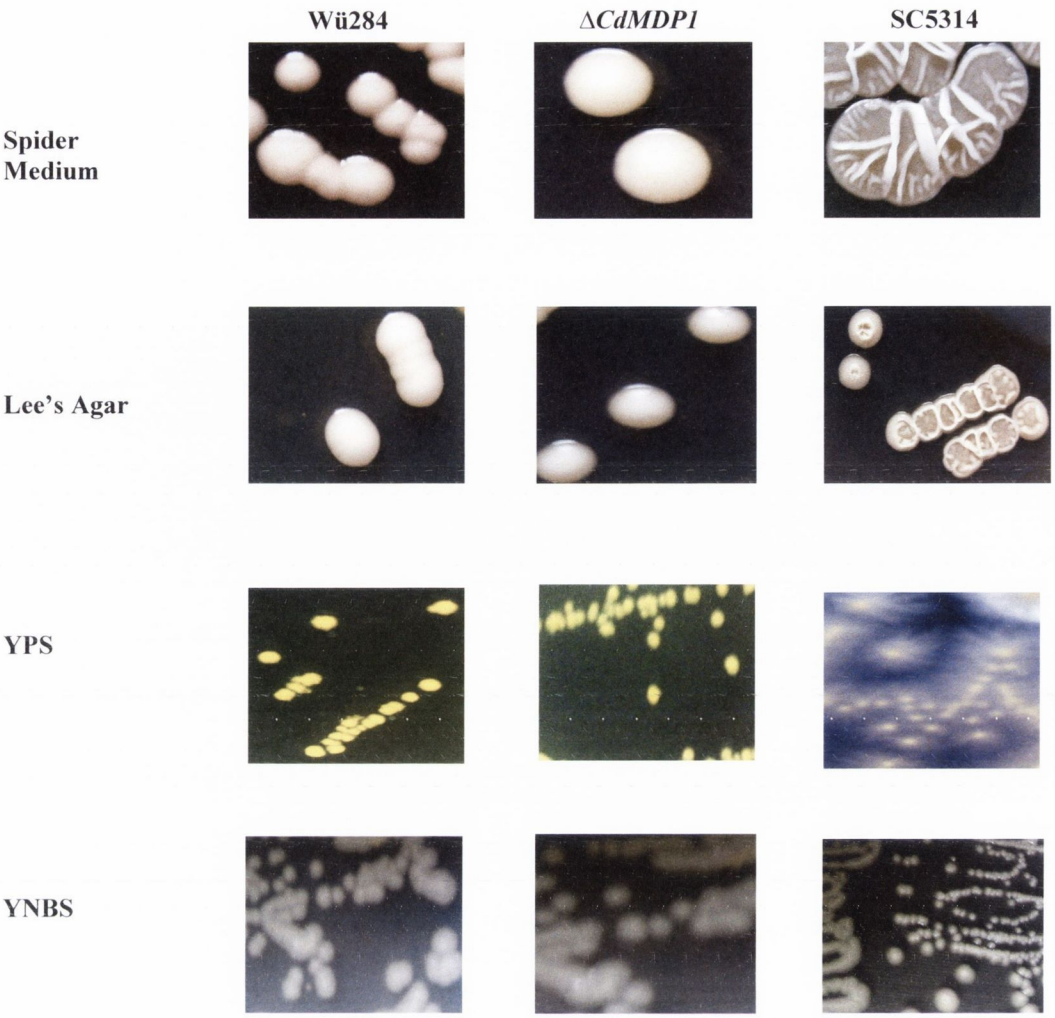


Figure 4.22. Phenotypic analysis of the Δ*mdp1*/Δ*mdp1* mutant on filament inducing media. *C. dubliniensis* Wü284 and the Δ*mdp1*/Δ*mdp1* mutant produced smooth colonies on Spider, Lee's and YPS, whereas the *C. alibcans* strain SC5314 produced rough filamentous colonies on Spider and Lee's. The presence of hyphae in *C. albicans* SC5314 is also visible on YPS media. On YNBS Wü284, the Δ*mdp1*/Δ*mdp1* mutant and SC5314 all produced hyphae on this agar.

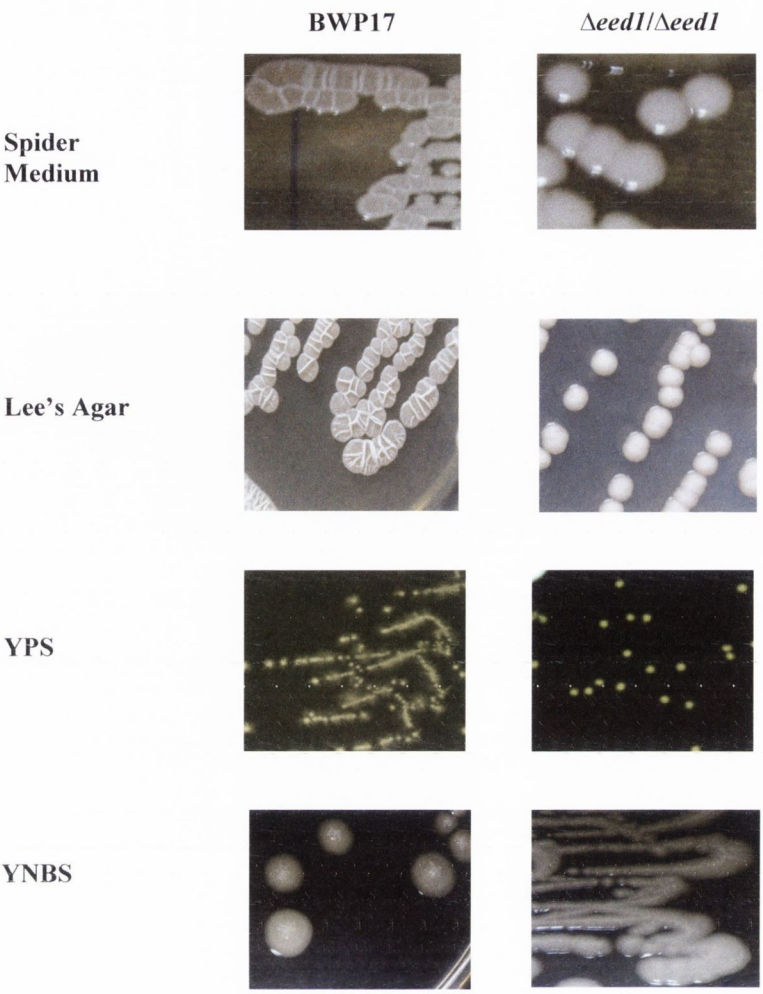


Figure 4.23. Phenotypic analysis of the *Δeed1/Δeed1* mutant on filament inducing media. *C. albicans* BWP17 produced rough filamentous colonies on Spider, Lee's, YPS and YNBS agar. The hyphal fringe can be seen clearly for BWP17 on YPS. The *Δeed1/Δeed1* mutant produced smooth colonies on Spider, Lee's and YPS. On YNBS both BWP17 and the *Δeed1/Δeed1* mutant prodcued hyphae.

4.1.10 Complementation studies

In order to investigate further the function of the *C. dubliniensis MDP1* gene and the *C. albicans EED1* gene, it was decided to carry out complementation of both mutants with their respective parental wild type gene to determine whether the phenotype could be rescued.

This was initially attempted by amplifying a 5414 bp fragment from *C. dubliniensis* Wü284 using the primer pair CdCEDTF and CdCEDTR (Table 2.2) and also a 5211 bp fragment from *C. albicans* BWP17 was amplified using the primer pair CaCEEDF/ CaCEEDR and gel purified prior to carrying out ligation(Table 2.2). These fragments were then ligated to pBluescript II KS (Chapter 2, 2.4.3) and transformed into DH5α (Chapter 2, 2.4.4). Transformants were screened by digesting with *Sac*II and *Not*I restriction enzymes. One positive transformant with the correct size insert was obtained. From *C. dubliniensis*, 18 transformants were obtained and these transformants were screened by digesting with *Sac*II and *Not*I restriction enzymes. However, none of these clones yielded inserts of the correct size. Due to this it was decided to repeat the PCR and gel purification steps and to ligate the fragments to pGem-T Easy (Chapter 2, 2.4.3, 2.4.4). Ligations were carried out using both pBluescript and pGEM-T Easy. Multiple transformants were obtained for *C. dubliniensis*, and initially 19 were screened by digestion with the restriction enzymes *Sac*II, *Not*I and *EcoR*I and again multiple fragments were obtained from both sets of transformants, none of which were of the correct size. From *C. albicans* a further two positive clones were obtained. So three positive clones in total were sent for commercial sequencing (Chapter 2, 2.5). However on further anaylsis of the sequences from the three positive clones it appears that the sequences are scrambled at either end of the gene and the middle region could not be sequenced. These findings led to the suggstion that the *EED1* gene of *C. albicans* and the *MDP1* gene of *C. dubliniensis* could be toxic to *E. coli.* This means that these genes possibly severely interfere with the survival of *E. coli* cells, this results in the death of the bacteria or a dramatic decrease in the expression capabilities. Toxicity can occur if the gene cloned is expressed in *E. coli* and is toxic to the cell. This tends to occur more commonly in high copy number vectors and templates with low a G+C ratio and also where4 the genes have undergone sequence rearrangements.

Following this it was decided to use ABLE®K competent cells as ABLE®K strains were designed to enhance the probability of retrieving clones that are toxic to *E.*

coli through the reduction of plasmid copy number by ~ 10-fold. This copy number reduction decreases the levels of cloned gene products and enhances the probability that a toxic clone will be propagated. Transformation was carried out as described (Chapter 2, 2.3.5). From this transformation four putative *C. albicans* clones were obtained and three putative *C. dubliniensis* clones were obtained. These were sent for DNA sequencing (Chapter 2.5). Again the clones for both *C. albicans* and *C. dubliniensis* had scrambled sequences. After discussing this with other investigators who had also encountered problems when trying to clone the *EED1* gene we attempted to use TOPO XL PCR Cloning Kit (B. Hube, personal communication). This method was carried out according to manufacturers instructions. From *C. albicans,* three positive transformants were identified and from *C. dubliniensis* two positive transformants were identified. These were then sent for sequencing. In *C. dubliniensis* none of the sequences aligned to *MDP1* and BLAST searches found similarity to an unrelated gene on Chromsome 2. In *C. albicans* the sequence quality was very poor and BLAST searches were carried out on part of the sequence and this found no significant match to any species. Following the failure of this method and discussion with other investigators who have encountered the same problems, it was decided to explore other methods which would enable further analysis of the function of *MDP1* in *C. dubliniensis* and *EED1* in *C. albicans* (Chapter 6).

4.2 Discussion

4.2.1 Deletion of *C. dubliniensis MDP1* using the *SAT1* flipper cassette

In this study the *SAT1* flipping method was used to carry out gene knockout studies in *C. dubliniensis*. This method of gene disruption was described by Reuss *et al.*, (2004). It is a highly efficient method to construct homozygous knockout mutants in both *C. albicans* and *C. dubliniensis* wild type strains and avoids problems related to the use of nutritional markers and auxotrophic host strains. This is one of the first studies to describe the use of the *SAT1* flipping method in *C. dubliniensis*. In this current study 100 μg/ml of nourseothricin were used for *C. dubliniensis* strains whereas higher concentrations were used previously for generating knockouts in *C. albicans*. This could be that *C. dubliniensis* is more sensitive to the antibiotic. The use of the *SAT1* marker is an excellent method for gene disruption because the mutants only differ from the parental wild type strain by the absence of the target gene. Another advantage is that the knockout mutants do not contain selection markers that are not present in the wild type control strain. This study demonstrates that the *SAT1* flipper is also a good method for gene disruption in *C. dubliniensis*. By successfully deleting both copies of the *C. dubliniensis MDP1*, phenotypic analysis of the mutant was carried out.

In the present study there were no noticeable differences in doubling times observed at 30°C, 37 °C or 42 °C between the *C. dubliniensis* wild type Wü284, *MDP1*/Δ*mdp1* and Δ*mdp1*/Δ*mdp1* mutants. Also in *C. albicans* there were no noticeable differences doubling times between the wild type BWP17 and the Δ*eed1*/Δ*eed1* mutant. However, when grown at 42°C the Δ*eed1*/Δ*eed1* mutant did grow faster than the wild type.

In the present study LOC4 was examined for its response to varying concentrations of NaCl. Under conditions of environmental stress (i.e. NaCl and elevated temperature) the growth rate of *C. dubliniensis* was significantly reduced in comparison to that of *C. albicans* when grown under identical conditions, suggesting that stress responses may be less efficient in *C. dubliniensis* (Alves *et al.*, 2002; Pinjon *et al.*, 1998). In this study, *C. dubliniensis* Wü284, *MDP1*/Δ*mdp1*, Δ*mdp1*/Δ*mdp1*, *C. albicans* BWP17 and the Δ*eed1*/Δ*eed1* mutant all grew well at 0.6 M NaCl. However at 1 M and 1.2 M NaCl *C. dubliniensis* Δ*mdp1*/Δ*mdp1* grew better than Wü284 at these higher concentrations of NaCl, suggesting that in high salt environments *MDP1* mutants

would have a selective advantage compared to the wildtype. In *C. albicans* both BWP17 and the *Δeed1/Δeed1* mutant grew slower at higher concentrations of salt (1.2 M), however there was only a slight difference in doubling times observed. Susceptibility of *C. dubliniensis* Wü284, *Δmdp1/Δmdp1* and *C. albicans* BWP17 and the *Δeed1/Δeed1* mutant was tested qualitatively by spotting serial dilutions of each culture on a variety of inhibitor containing media. It is evident here that at higher concentrations of salt (1.5 M, 1.6 M NaCl and 0.5 M $CaCl_2$) and at high temperatures (42°C) *Δmdp1/Δmdp1* grew far better than the wild type on agar plates, the results correlating with the growth curve results. In *C. albicans* both BWP17 and the *Δeed1/Δeed1* mutant grew all on agar which contains inhibitors, although BWP17 appears to grow slightly better that the *Δeed1/Δeed1* mutant of higher concentrations of salt (1.5 M and 1.6 M). In *C. dubliniensis* deletion of *MDP1* may lead to activation of some stress response mechanism due to the increased growth on NaCl and 42°C. Response to stress does not seem to be significantly effected in *C. albicans* when *EED1* is deleted.

4.2.2 *MDP1* and *EED1* are required for filamentation

A variety of environmental conditions stimulate the yeast to hypha transition in *C. albicans*. These include growth at elevated temperatures (37°C or higher) or media containing specific components (serum or a particular combination of amino acids) (Gow, 1997). Stokes *et al.* (2007) and Gilfillan *et al.* (1998), found that *C. dubliniensis* routinely produced hyphae in 10 % (v/v) serum, however, in this and other liqiuid media the rate of hypha induction was significantly lower compared to *C. albicans*. Results from this current study also correlated with the results of Stokes *et al.* (2007) and Gilfillan *et al.* (1998), in that *C. dubliniensis* Wü284 produced < 50 % hyphae following 6 h incubation in 10 % new born calf serum. A temperature shift from 30°C to 37°C did enhance hypha production in *C. dubliniensis* Wü284, however this shift in temperature did not affect hypha production in the *Δmdp1/Δmdp1* mutant. The *Δmdp1/Δmdp1* mutant produces < 5 % hyphae in 10 % (v/v) newborn calf serum following pre-culture at 30°C or 37°C. These results demonstrate that although the amount of hyphae produced in the wild type Wü284 is low, *MDP1* plays a role in filamentation in response to temperature and serum. *C. albicans* BWP17 produces hyphae rapidly under these conditions with 50 % hypha within 1 h and 90 % hypha by 3

h following pre-culture at 30°C. Lower levels of hypha production were observed following pre-culture at 37°C, although by 3 h BWP17 produced 90 % hypha. The Δ*eed1*/Δ*eed1* mutant did not produce any hyphae in 10 % serum following pre-culture at 30°C or 37°C. This result also demonstrates that *EED1* is essential for filamentation to occur in response to temperature and serum in *C. albicans*. These results indicate that serum has a significant effect on hypha production in both *C. albicans* and *C. dubliniensis* and that this effect requires *EED1* and *MDP1*. However the amount of hypha production in *C. dubliniensis* does not approach that of *C. albicans*.

pH and temperature are among the external signals that trigger morphogenesis in *C. albicans*. A temperature shift from 30 °C to 37 °C and a pH > 6.0 are necessary for the induction of pH regulated dimorphism. pH and temperature inducing conditions are found in the human body and alterations in both conditions maybe experienced by a pathogen during infection. *In vitro C. albicans* can thrive over a wide range of extracellular pH values (pH 2-10) (Odds, 1988). *C. albicans* causes infections in a broad range of host niches, which show significant differences in ambient pH. For example, the mouse systemic model has a pH of 7.3, whereas the pH in the rat vagina is 4.5. The ability of *Candida* to react to different pH environments is crucial for its pathogenicity. Under optimal temperature conditions (37°C), filamentation is favoured by ambient pH values close to neutral and is considerably reduced at pH values lower than 6. In contrast, the yeast form predominates almost exclusively at pH 4 (Fonzi, 2002). Lee's medium is a known inducer of the yeast to hypha transition in *C. albicans* (Lee *et al.*, 1975), and this medium allows us to control the pH shift. Thus it was decided to examine the effect of a pH shift from 4.5 to 7.5 (10 % serum) on the *C. dubliniensis MDP1* gene. The results were dramatic for both the *C. dubliniensis* wild type Wü284 and LOC4 (Δ*mdp1*/Δ*mdp1*) mutant in that the pH shift dramatically increased hypha production to levels similar to wild type *C. albicans*. In *C. albicans* this pH shift from 4.5 to 7.5 did result in an increase in hypha formation in the wild type BWP17, however in the Δ*eed1*/Δ*eed1* mutant the pH shift only partially restores its ability to produce hyphae. In the absence of the pH shift (pre-culture at pH 6 and pH 8) the Δ*eed1*/Δ*eed1* mutant failed to produce hyphae whereas the wild type still produced true hyphae. The inability of the *EED1* mutant to form true hyphae in the absence of a pH shift indicates that *EED1* is required for filamentation in response to other conditions (e.g. temperature, nutrients and cell density). Therefore experiments were designed to examine the effects of temperature, nutrients and cell density on filamentation. The

results suggest that a temperature shift has an effect on the morphology of *C. albicans* wild type BWP17, as it produced hyphae and pseudohyphae whereas the mutant remained totally in the yeast phase. However, the most dramatic effect observed between the *C. albicans* wild-type and mutant was when nutrient starvation experiments were was carried out. BWP17 readily produced true hyphae whereas the *Δeed1/Δeed1* mutant remained in the yeast phase; indicating that in *C. albicans EED1* may also play a role in regulation of the morphological shift under nutrient poor conditions. These data suggest that nutrient and temperature induced filamentation requires *EED1*, whereas pH signals are transduced by a different mechanism (Fig. 4.24). This evidence would also indicate that in *C. dubliniensis* the dramatic effect of this pH shift (pH 4.5 to pH 7.5) observed in *C. dubliniensis* Wü284 and LOC4 is occurring independently of *MDP1*.

Moran *et al.* (2007), found that *CdNRG1* is down-regulated less rapidly than *CaNRG1* when both species are inoculated in 10 % serum. *NRG1* is a DNA-binding repressor that functions as a strong repressor of a set of filament-specific, serum inducible genes (Braun *et al.*, 2001). Moran *et al.* (2007) found that by deleting both copies of *NRG1* in *C. dubliniensis* this could increase the rate of filamentation. This suggests that perhaps the increase in filamentation observed in Wü284 and *Δmdp1/Δmdp1* is a direct result of a repression of *NRG1* and hence an increase in filamentation. In this study, real-time analysis of *NRG1* in *C. dubliniensis* demonstrated that when pre-cultured at low pH (Lee's medium pH 4.5) the expression levels of *NRG1* are extremely low, thus, the low levels of *NRG1* expression at pH 4.5 may allow filamentation to occur more rapidly when cells are inoculated into 10 % serum.

Solid media was also used to compare hypha production between *C. dubliniensis* Wü284, *Δmdp1/Δmdp1*, *C. albicans* BWP17 and *Δeed1/Δeed1*. Spider medium has previously been used in *C. albicans* for investigation into the contribution of genes to hypha formation (Liu *et al.*, 1994). In *C. dubliniensis* Wü284 and LOC4 (*Δmdp1/Δmdp1*) both produced smooth round colonies whereas the *C. albicans* control SC5314 produced rough filamentous colonies suggesting perhaps that on solid agar *MDP1* is not involved in filamentation. This result shows that *MDP1* does play a role in filamentation when grown in liquid media however on solid media *MDP1* does not appear to play a role in filamentation. In *C. albicans* BWP17 produced rough filamentous colonies whereas the mutant produced round smooth colonies, indicating that *EED1* is required for filamentation in both liquid and solid forms. On Lee's agar (rich in amino acids) again the *C. dubliniensis* Wü284 and LOC4 produced smooth

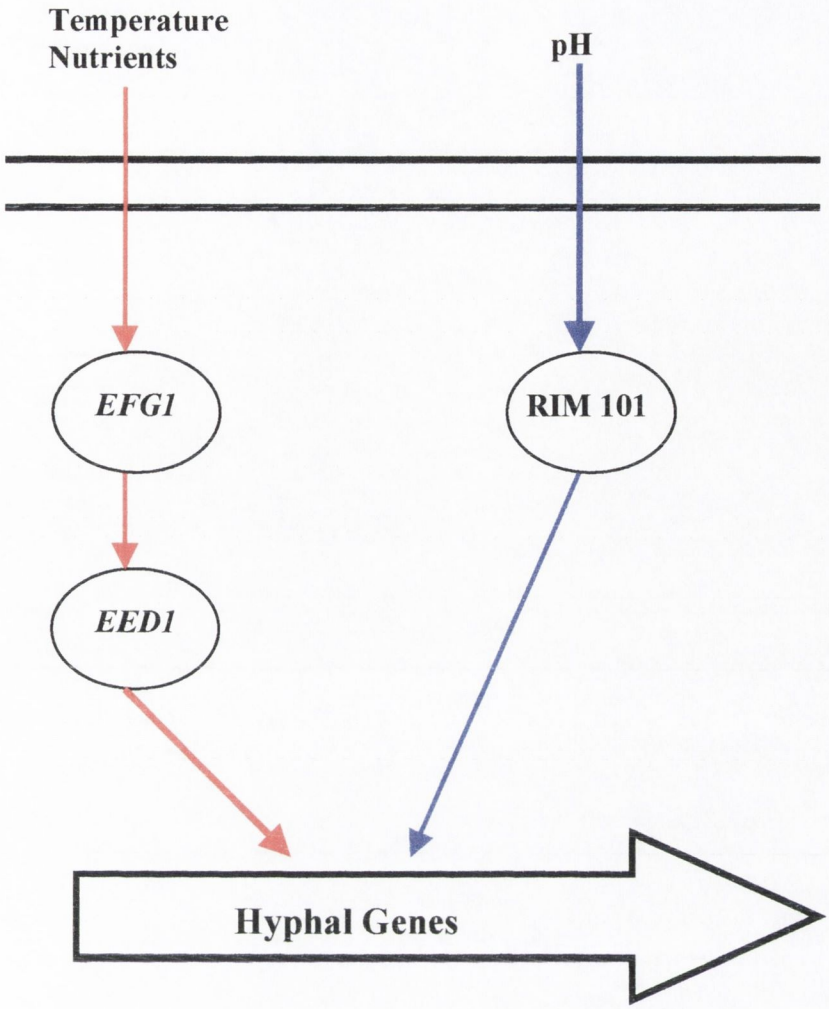


Figure 4.24. Proposed model of the different mechanisms that induce filamentation in *C. albicans*. Temperature and nutrients result (red arrows) in the activation of *EFG1* which in turn may activate *EED1*. Expression of *EED1* results in hyphal production. The alternative pH pathway (blue arrows) activates RIM 101 which itself results in hyphal production.

colonies whereas in *C. albicans* BWP17 again produced rough filamentous colonies, with the *Δeed1/Δeed1* mutant producing smooth round colonies.

Embedded conditions were also examined using a 'sandwich' of yeast extract peptone-sucrose (YPS) medium, where cells were plated between two solidified layers of YPS medium. When cells of wild type *C. albicans* SC5314 were embedded in YPS agar the majority of colonies showed filamentous growth within 48 h (Brown *et al.*, 1999). In the present study *C. dubliniensis* Wü284, *Δmdp1/Δmdp1*, *C. albicans* BWP17 and *Δeed1/Δeed1* were plated between two solidified layers of YPS. In *C. dubliniensis* neither Wü284 or LOC4 produced rough colonies. . In BWP17 hypha fringes are clearly visible on the embedded YPS, however, as has been observed on Lee's and Spider medium the *Δeed1/Δeed1* mutant failed to produce hyphae, indicating that in *C. albicans EED1* is an important regulator of filamentation.

Despite multiple attempts it was found to be impossible to complement the *Δmdp1/Δmdp1* mutant as it appears that gene is toxic to *E. coli*. Following these results, the virulence of *C. dubliniensis* Wü284, *Δmdp1/Δmdp1*, *C. albicans* BWP17 and *Δeed1/Δeed1* will be examined using two infection models, specifically the reconstituted human oral epithelial (RHE) cell model and the murine macrophage cell line RAW264.7.

Chapter 5

Analysis of the role of *MDP1* and *EED1* in virulence

5.0 Introduction

Candida dubliniensis which was first identified in 1995, is the most closely related species to *C. albicans* (Sullivan *et al.*, 1995). *C. dubliniensis* is less virulent than *C. albicans*, produces fewer hyphae and is less tolerant to environmental stress than *C. albicans.* Cllearly *C. albicans* is the most significant yeast pathogen. However, it is unclear why *C. albicans* is more virulent than *C. dubliniensis*. Moran *et al.* (2004) carried out comparative genomic hybridisation studies using DNA microarrays and found that only 4.4 % of *C. albicans* sequences analysed were likely to be absent or highly divergent in *C. dubliniensis*. In a previous study, Gilfillan *et al.* (1998) investigated both species with respect to potential virulence factors such as adhesion, hypha production (which is regarded as one of the most important virulence factors), and the possession of genes encoding secreted aspartyl proteinases (Saps). They found that *C. dubliniensis* produced hyphae more slowly than *C. albicans*, however the *C. dubliniensis* isolates tested formed true hyphae under most conditions tested but failed to produce true hyphae when induced using *N*-acetylglucosamine. Oral *C. dubliniensis* isolates tested were more adherent to human buccal epithelial cells than *C. albicans* when grown in glucose. The study also showed that all *C. dubliniensis* isolates examined possessed homologues to each of the seven *C. albicans SAP* genes tested. Another study by Stokes *et al.* (2007) found that *C. dubliniensis* produces fewer hyphae than *C. albicans* under most environmental conditions that promote filamentous growth such as serum and shifts in pH and temperature.

The aim of this study was to compare the roles of both *C. albicans EED1* and *C. dubliniensis MDP1*, in virulence using two infection models, specifically the reconstituted human oral epithelial (RHE) cell model and the murine macrophage cell line Raw 264.7. The purpose of using these two models of infection was to examine the filamentation and survival of both species during infection and to investigate the effects of the *MDP1* deletion and the *EED1* deletion. In addition, the effect of a pH shift prior to infection was examined in both models of infections to determine the effect on survival of wild-type *C. dubliniensis* and Δ*mdp1*/Δ*mdp1*, as a pH shift from 4.5 to 7.5 has been shown previously to enhance filamentation of *C. dubliniensis* to similar levels observed in wild-type *C. albicans*.

5.1 Results

5.1.1 Deletion of *EED1* effects invasion of *C. albicans* on RHE

Since this study began, *C. albicans* orf19.7561 has been described as *EED1*, a regulator of filamentation, which is essential for virulence during infection of Reconstituted Human Oral Epithelium (RHE) (Zakikhany *et al.*, 2007). RHE tissues were used to examine the virulence of both the *C. albicans* Δ*eed1*/Δ*eed1* and the *C. dubliniensis* Δ*mdp1*/Δ*mdp1* mutants. RHE tissues were purchased from Skinethic Laboratories (Nice, France) and were used as described previously (Stokes *et al.*, 2007; Zakikhany *et al.*, 2007) (Chapter 2, 2.9.3). In order to compare the ability of *C. albicans* BWP17, the Δ*eed1*/Δ*eed1* mutant, and *C. dubliniensis* Wü284 and LOC4 (Δ*mdp1*/Δ*mdp1*) to colonise and infect the RHE tissues, RHE tissues were inoculated with YPD-grown yeast cells from each strain. Tissue sections were examined for cell damage.

In RHE samples infected with *C. albicans* BWP17 extensive growth of both yeast and hyphae was evident by 12 h (Fig. 5.1). Between 12 h and 24 h post-infection the RHE tissue showed signs of tissue damage characterised by invading hyphae and vacuolisation in the cell layers. At 24 h, invasion of deeper parts of the epithelium was observed and hypha cells had penetrated throughout the tissue (Fig. 5.1). At 48 h post infection, very few epithelial cells were left attached to the membrane, and extensive damage had occurred to the tissue. Tissue damage was estimated by measuring the levels of the enzyme lactate dehydrogenase (LDH) released from the infected epithelial cells. In *C. albicans* BWP17, at 6 h little damage had occurred to the tissue and the levels of LDH released from the tissue were similar to those in uninfected controls (Fig. 5.2). High levels of LDH were released from the tissue at the later time points (12 h, 24 h and 48 h) when compared with the uninfected controls (Fig. 5.2) indicating that *C. albicans* BWP17 causes extensive damage to RHE tissue which correlated with the invasion of hyphae (Fig. 5.1). RHE samples were also inoculated with the *C. albicans* Δ*eed1*/Δ*eed1* mutant to confirm the results obtained by Zakikhany *et al* (2007), that the Δ*eed1*/Δ*eed1* mutant exhibited attenuated damage when inoculated onto RHE. At 6 h and 12 h post-infection, cells of the Δ*eed1*/Δ*eed1* mutant appeared to induce a ruffling of the membrane and induced endocytosis of the yeast cells (Fig. 5.1). At 24 h, Δeed1/Δ*eed1* cells remained predominantly in the yeast phase and the cells had been

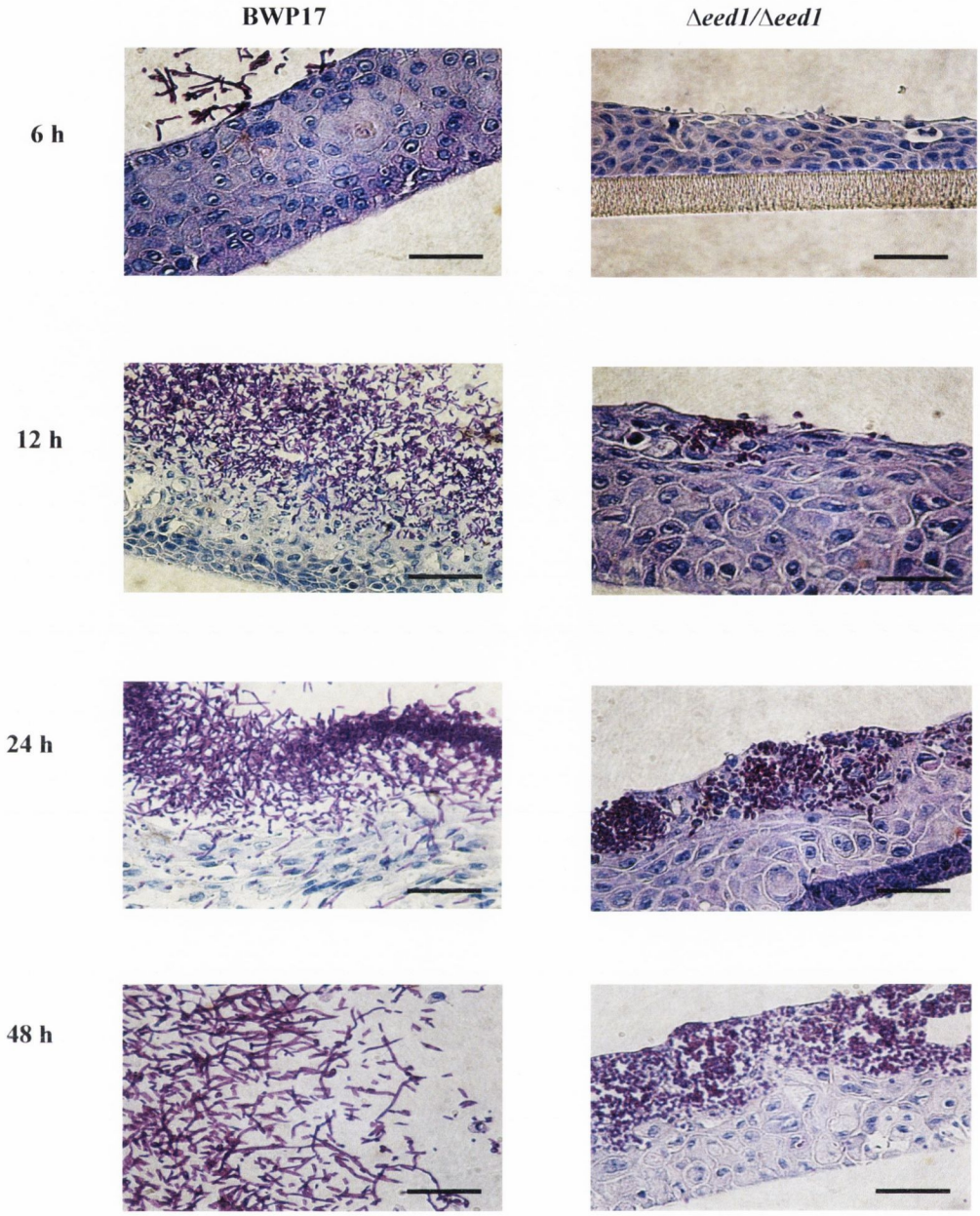


Figure 5.1. Light micrographs of reconstituted human oral epithelium (RHE; Skinethic, France) infected with *C. albicans* BWP17 and *Δeed1/Δeed1* following 6 h, 12 h, 24 h and 48 h of incubation. The black scale bar represents 25 μm

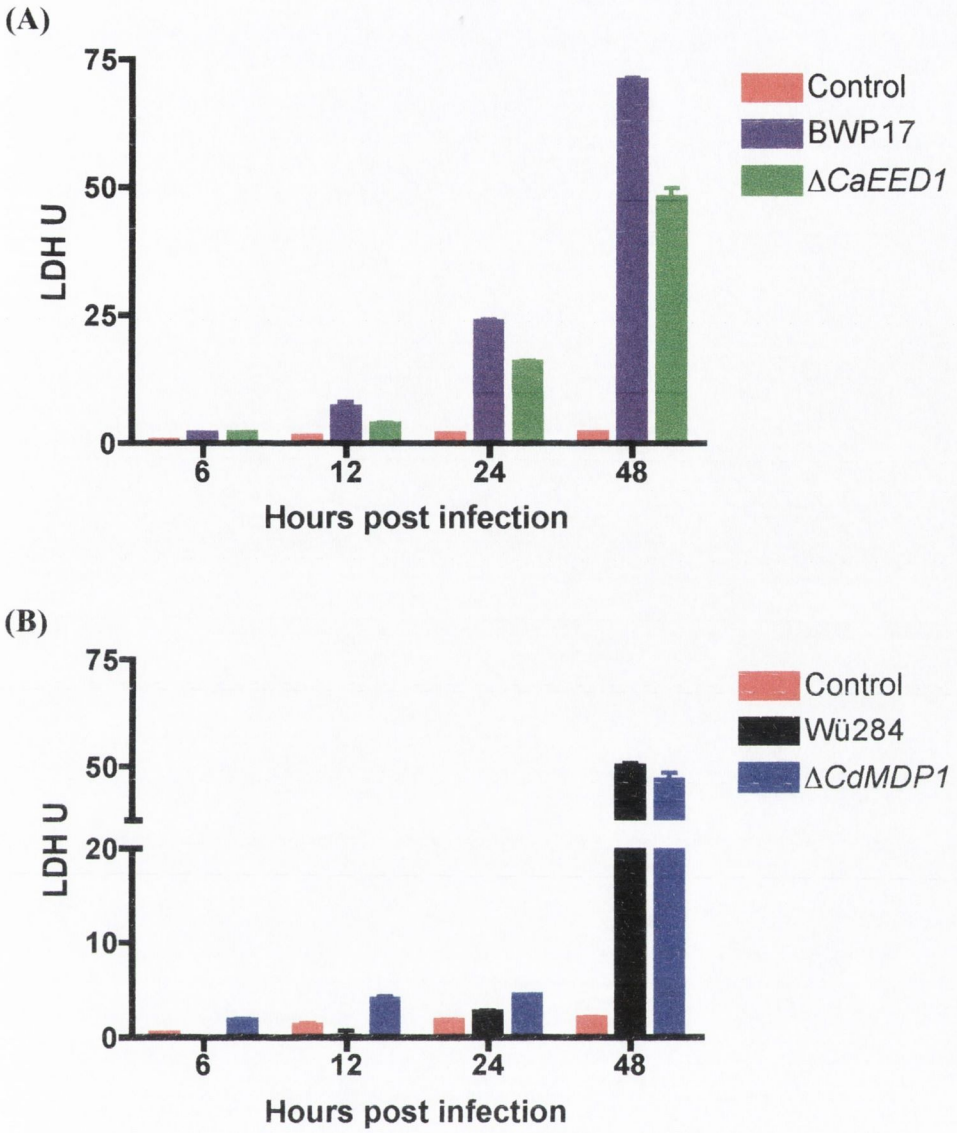


Figure 5.2. (A) Evaluation of tissue damage caused during infection of RHE by *C. albicans* BWP17 and Δ*eed1*/Δ*eed1* measured as LDH activity released into the culture medium over 48 h. **(B)** Evaluation of tissue damage caused during infection of RHE by *C. dubliniensis* Wü284 and LOC4 (Δ*mdp1*/Δ*mdp1*) measured as LDH activity released into the culture medium over 48 h. These results are the averages of two separate experiments. The control used in this experiment is uninfected RHE tissue.

endocytosed into the tissue. Some damage to the tissue had occurred but the tissue remained largely intact. At 48 h damage to the tissue had occurred and vacuolisation of the tissue was visible. Although damage had occurred to the tissue, the *Δeed1/Δeed1* cells remain trapped within the upper layer of the tissue unable to escape and disseminate throughout the tissue (Fig. 5.1). The levels of LDH released from the tissue post inoculation with *Δeed1/Δeed1* cells revealed a reduced ability to cause tissue damage (P <0.0001) (Fig. 5.2). These findings confirmed the results of Zakikhany *et al.* (2007) that *EED1* is not essential for the initial invasion into the epithelial cells but is required for epithelial escape and dissemination.

Inoculation of RHE tissue with *C. dubliniensis* Wü284 and LOC4 (*Δmdp1/Δmdp1*) revealed major differences in morphology and tissue damage. At all time points both Wü284 and LOC4 were found to grow exclusively in the yeast phase, although a small number of pseudohyphae were present in a small minority of tissue sections examined (Fig. 5.3). By 12 h, RHE that been inoculated with *C. dubliniensis* Wü284 and LOC4 had very few yeast cells attached directly to the epithelium. After 24 h a small amount of tissue damage was evident, as some epithelial cells in the top layer appeared to be detached. However, the lack of adherence and hypha invasion by both Wü284 and LOC4 meant that the tissue was largely intact. At 48 h damage had occurred to the epithelium, however, layers of the epithelial cells were still visible over the supporting membrane (Fig. 5.3). The LDH levels released from tissues at 6 h, 12 h and 24 h were similar to those in uninfected controls, indicating that up to and including the 24 h time point little damage had occurred to the tissue inoculated with the non-invasive *C. dubliniensis* Wü284 and LOC4. There was no significant difference observed between Wü284 and LOC4 following the LDH assay (P 0.0011) (Fig. 5.2). By 48 h however there were much higher levels of LDH released and the levels released was similar to *C. albicans* BWP17 at 48 h.

5.1.2 Can a pH shift rescue invasion defect in *C. dubliniensis?*

In chapter 4 it was demonstrated that a pH shift from 4.5 to 7.5 could increase hypha formation in both *C. dubliniensis* Wü284 and LOC4 (*Δmdp1/Δmdp1*). In order to assess whether a pH shift could enhance invasion of RHE, both strains were pre-cultured in Lee's medium pH 4.5 prior to infection. Tissue sections were examined at 6

h, 12 h and 24 h. *C. albicans* BWP17 and the *Δeed1/Δeed1* mutant were also pre-cultured in Lee's medium pH 4.5, and tissue sections were examined at 12 h and 24 h.

Inoculation of RHE tissue with *C. dubliniensis* Wü284 which had been pre-cultured in Lee's medium pH 4.5 enhanced filamentation on the RHE. In tissues infected with *C. dubliniensis* Wü284 there were both hyphae and yeast cells present at 6 h. Some of the hypha cells had embedded into the upper layer of the tissue (Fig. 5.4). There was a noticeable increase in the number of cells present and in contact with the epithelial surface compared to tissue infected with *C. dubliniensis* pre-cultured in YPD. At the 12 h time point, in some areas hypha cells had penetrated the upper layer of the tissue (Fig. 5.4). Both yeast and pseudohyphae were also present, adhering to the upper layer of the tissue and damage to the upper layer of the tissue was evident. By 24 h, extensive tissue damage had occurred, however, at this time point the cells were predominantly in the yeast phase (Fig. 5.4). Damage to the tissue was evident at this time point by the presence of what appeared to be vacuolisation within the tissue, scanning electron microscopy (SEM) would have to be carried out to confirm this. In tissue infected with *C. dubliniensis* LOC4 (*Δmdp1/Δmdp1*) by 6 h the presence of both yeast and pseudohyphae adhering to the upper layer of the epithelium was evident, with some hyphae also visible. By 12 h, the cells were predominantly in the yeast phase, although some pseudohyphae were visible above the upper layer of the tissue. Some damage had occurred to the upper layer of the epithelium, however the tissue remained largely intact (Fig. 5.5). At 24 h, extensive tissue damage had occurred and was clearly visible, where the yeast cells were surrounded by epithelial cell protrusions (Fig. 5.5), again SEM would have to be carried out to confirm this. By 24 h, almost all cells had reverted back to the yeast phase. At 12 h, some LDH release was detected indicating that damage to the tissue had occurred. This correlated with the presence of both yeast and hyphae within the epithelium in Wü284 and the large numbers of yeast cells of LOC4 adhering to the upper layer of the tissue. The LDH released into the culture medium at 24 h from tissue infected with both Wü284 and LOC4 was significantly higher than that detected from tissue infected with YPD grown cells and was similar to the levels obtained from *C. albicans* wild-type strains at 24 h ($P < 0.0001$) (Fig. 5.6). Interestingly, the LDH released at 24 h was far greater in LOC4 (Fig. 5.6). There was a significant difference observed following the LDH assay between Wü284 precultured in Lee's medium compared to YPD ($P < 0.0001$). There was also a significant difference observed between LOC4 precultured in Lee's medium compared to LOC4 precultured

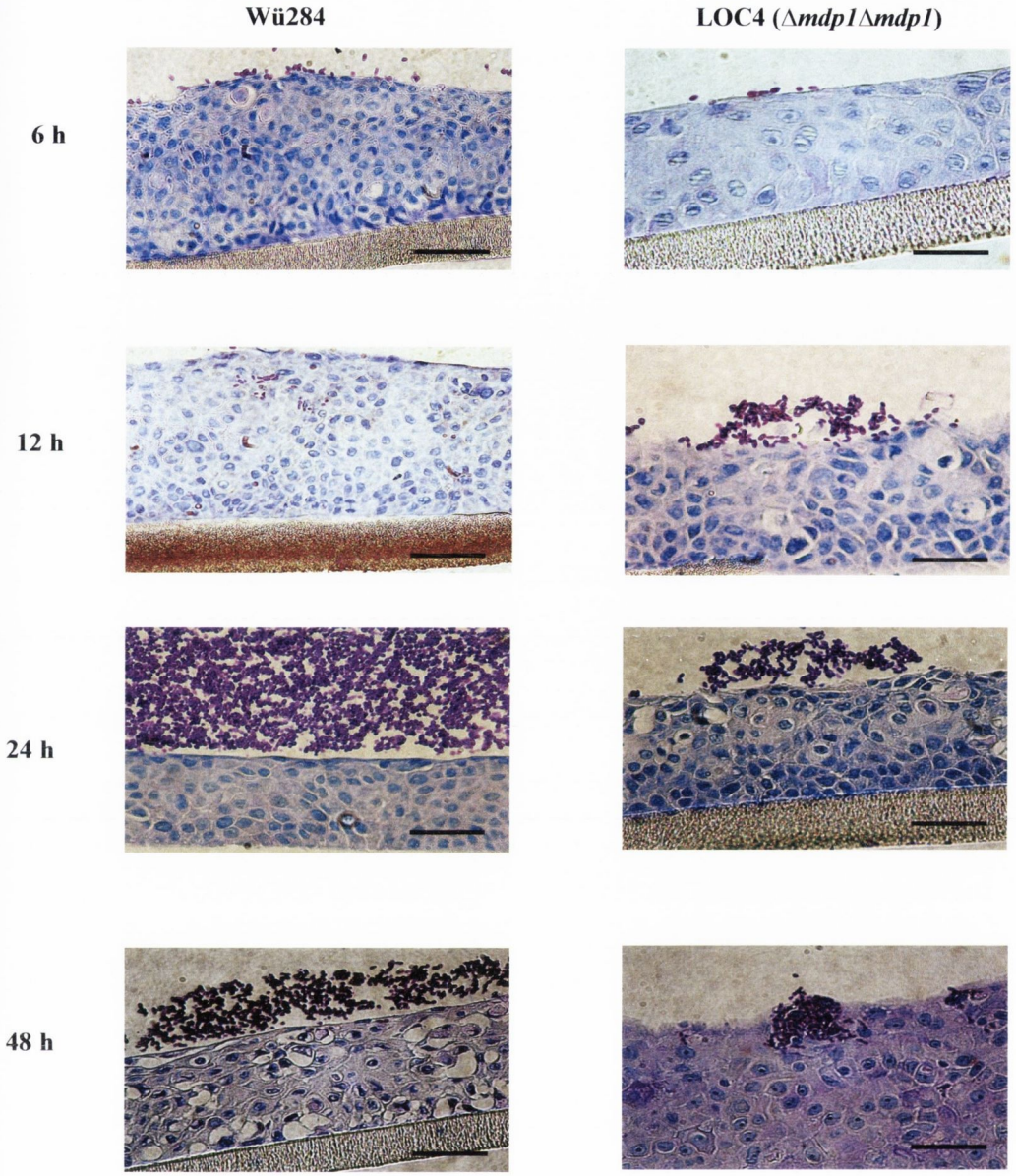


Figure 5.3. Light micrographs of reconstituted human oral epithelium (RHE; Skinethic, France) infected with *C. dubliniensis* Wü284 and LOC4 (*Δmdp1/Δmdp1)* following 6 h, 12 h, 24 h and 48 h of incubation. The black scale bar represents 25 μm

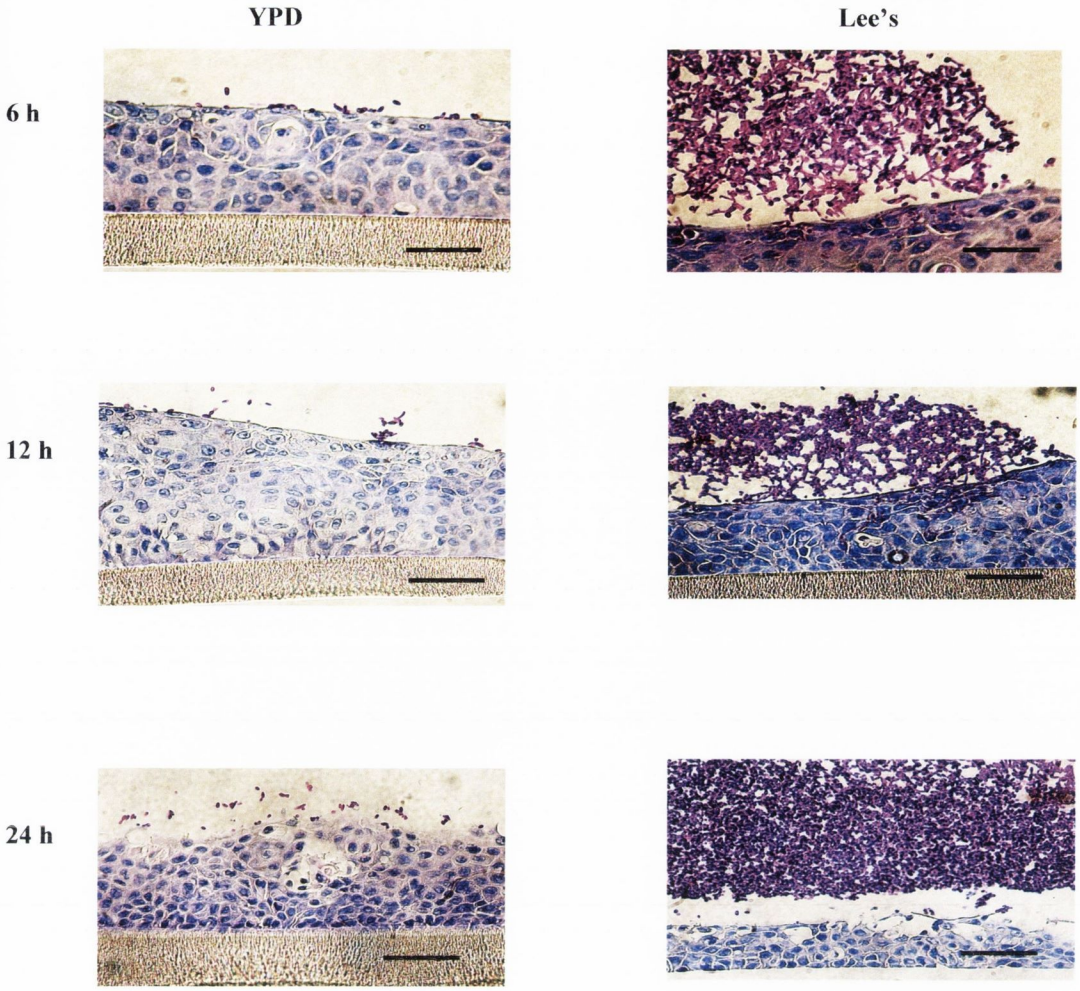


Figure 5. 4. Light micrographs of reconstituted human oral epithelium (RHE; Skinethic, France) infected with *C. dubliniensis* Wü284. Cells were pre-cultured in YPD or Lee's medium (pH 4.5) prior to inoculation onto RHE. Tissue samples were taken at 6 h, 12 h and 24 h. The black scale bare represents 25 μm.

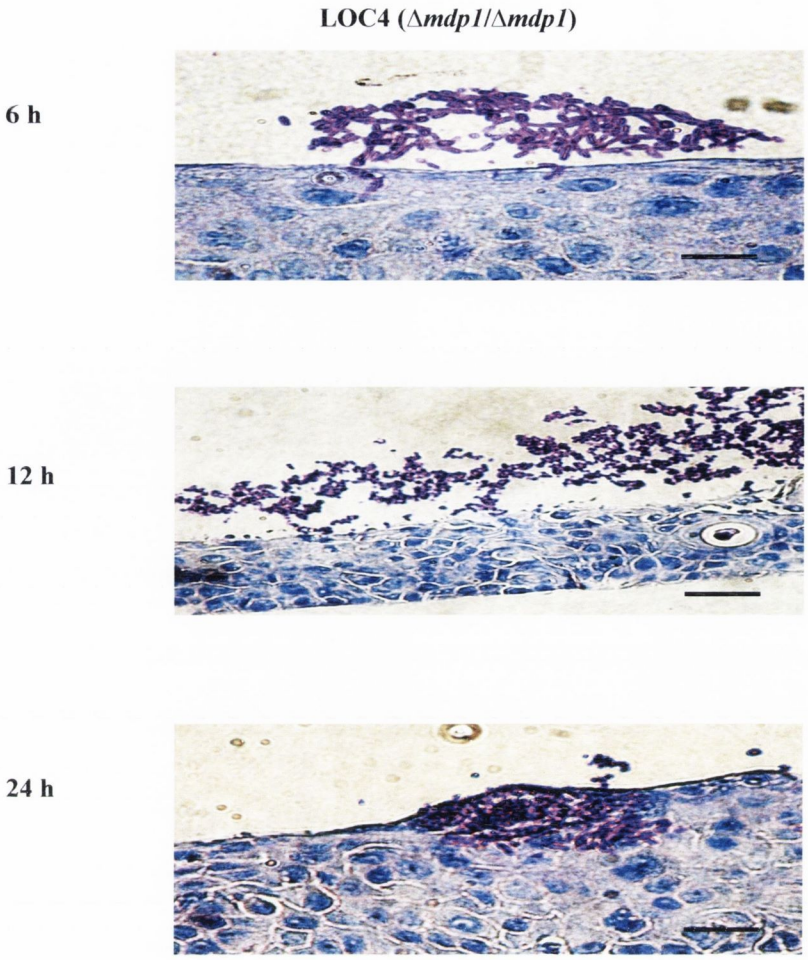
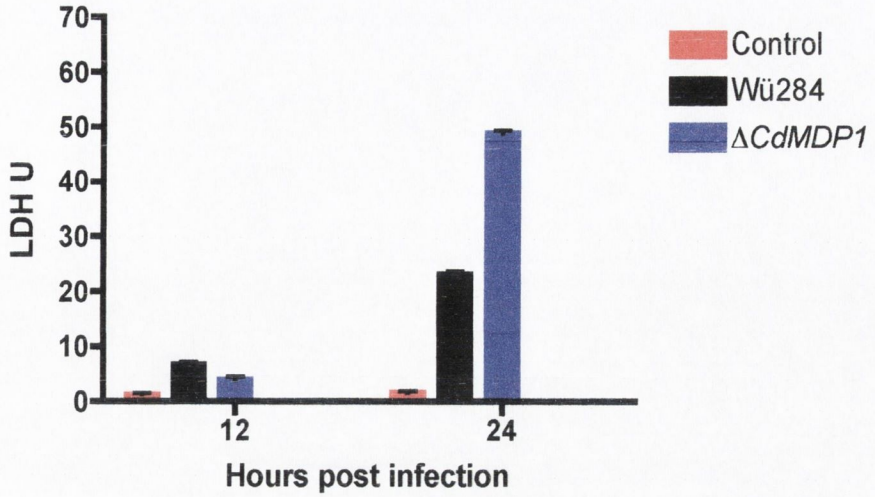


Figure 5. 5. Light micrographs of reconstituted human oral epithelium (RHE; Skinethic, France) infected with *C. dubliniensis* LOC4 (Δ*mdp1*/Δ*mdp1*). Cells were pre-cultured in Lee's medium pH 4.5 prior to inoculation onto RHE. Tissue samples were taken at 6 h, 12 h and 24 h. The black scale bar represents 25 μm.

(A)



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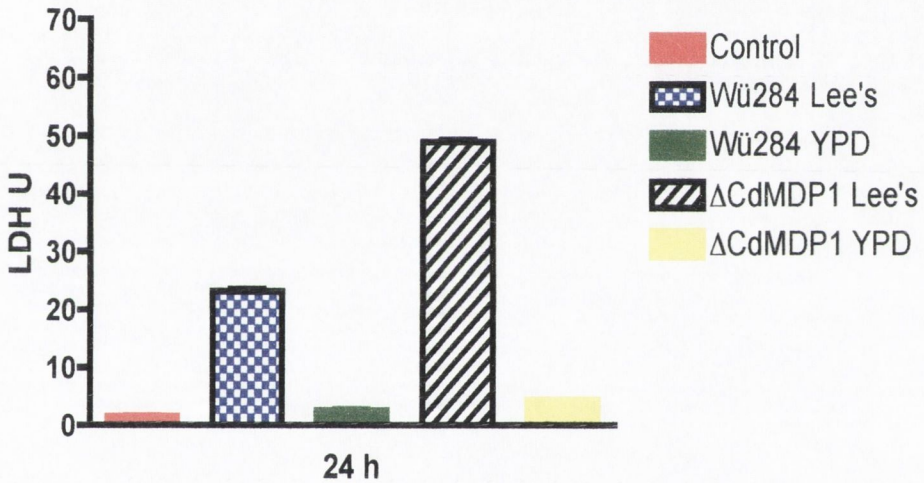


Figure 5.6. (A) Evaluation of tissue damage caused during infection of RHE by *C. dubliniensis* Wü284 and LOC4 (*Δmdp1/Δmdp1*) following pre-culture in Lee's medium pH 4.5, measured as LDH activity released into the culture medium over 24 h. **(B)** Tissue damage of *C. dubliniensis* Wü284 and LOC4 at 24 h post inoculation following preculture in both Lee's medium and YPD, measured as LDH activity released into the culture medium. This experiment was repeated on three separate occasions. The control used was uninfected RHE tissue.

in YPD (P <0.0001) (Fig. 5.6). This result shows that pre-culturing both Wü284 and LOC4 in Lee's medium enhanced the ability to damage RHE tissue.

Inoculation of RHE with *C. albicans* BWP17 and the Δ*eed1*/Δ*eed1* mutant which had been pre-cultured in Lee's medium pH 4.5 prior to inoculation did not enhance filamentation of the non-invasive Δ*eed1*/Δ*eed1* mutant. At 12 h the Δ*eed1*/Δ*eed1* mutant remained exclusively in the yeast phase, although the cells had adhered to the epithelium (Fig. 5.7). In *C. albicans* BWP17, as expected both yeast and hyphae were present and the hyphae had penetrated through the epithelium, vacuolisation was also visible within the cell layers (Fig. 5.7). At 24 h the Δ*eed1*/Δ*eed1* mutant remains largely on the surface of the epithelium and what appears to be induced endocytosis of the yeast cells was also visible within the epithelium (Fig. 5.7), again SEM would have to be carried out to confirm this. *C. albicans* BWP17 had caused extensive damage to the tissue at 24 h and very little epithelial tissue remains and hyphae are clearly visible throughout (Fig. 5.7) The levels of LDH released from tissues infected with *C. albicans* BWP17 showed that extensive tissue damage had occurred by 12 h (Fig. 5.8). There was a significant difference observed between BWP17 precultured in Lee's medium compared to preculture in YPD (P<0.0001) (Fig.5.8). The levels of LDH released in the Δ*eed1*/Δ*eed1* mutant at 12 h were similar to the uninfected controls (Fig. 5.8). By 24 h, the LDH level released had increased and were similar to those induced by YPD grown cells. However, there was a significant difference observed between *C. albicans* Δ*eed1*/Δ*eed1* precultured in Lee's medium to those precultured in YPD (P 0.0007) (Fig. 5.8). This result also correlates with the pH experiments in Chapter 4, as the pH shift from 4.5 to 7.5 as the Δ*eed1*/Δ*eed1* mutant was unable to produce hypha and penetrate the tissue.

5.1.3 Deletion of *MDP1* and *EED1* results in poor survival in a macrophage co-culture model

The growth and survival of *C. dubliniensis* Wü284 and LOC4 (Δ*mdp1*/Δ*mdp1*) was compared in co-culture with macrophages (Chapter 2, 2.8.2). During co-culture with Raw 264.7 macrophages Wü284 appears to be phagocytosed, as the cells is engulfed into the macrophage (Fig. 5.9) and at 1 h was found predominantly in the yeast phase. At 3 h and 5 h Wü284 still remained predominantly in the yeast phase however some hyphae and pseudohyphae were also visible within the macrophage (Fig. 5.9). Co-

culture of LOC4 with the Raw 264.7 macrophages resulted in the LOC4 cells appear to be phagocytosed by the macrophage, SEM would have to carried out to confirm this. At 1 h LOC4 remained exclusively in the yeast phase (Fig. 5.10). At both 3 h and 5 h LOC4 remained in the yeast phase and unlike Wü284, LOC4 failed to produce any hyphae or pseudohyphae and thus was unable to evade killing by the macrophage (Fig. 5.10).

Following 16 h incubation, the survival of both *C. dubliniensis* Wü284 and LOC4 (*Δmdp1/Δmdp1*) was assessed. Macrophages were lysed and the number of viable *C. dubliniensis* Wü284 and LOC4 cells was determined using an XTT dye reduction assay (Chapter 2, 2.8.2). Prior to co-culture with Raw 264.7 macrophages both Wü284 and LOC4 were grown overnight in YPD at 37°C. The XTT proliferation assay demonstrated that LOC4 had a poor survival rate when co-cultured with macrophages when compared to the wild-type Wü284. However, a significant difference was not observed ($P > 0.05$; Fig. 5.11). *C. dubliniensis* Wü284 also had a relatively poor survival rate following co-culture with macrophages (Fig. 5.11). At an MOI (multiplicities of infection) of 1:2 Wü284 had a 70 % survival rate, however at increased MOIs of 1:8 the survival rate of Wü284 was 46 %. A further drop in the survival rate was observed at MOIs of 1:32 and 1:128 to 22 % and 16 %, respectively.

The ability of *C. albicans* to form hyphae or pseudohyphae allows its escape from the macrophage, however as previously demonstrated the *Δeed1/Δeed1* mutant was unable to produce hyphae on RHE and in 10 % serum. Upon macrophage ingestion *C. albicans* BWP17 cells in the yeast phase form undergo a rapid switch to the filamentous form and hyphae are visible by 1 h (Fig. 5.12). At both 3 h and 5 h hypha can be seen protruding from the macrophage (Fig. 5.12). Thus as previously reported *C. albicans* has a much higher survival rate when compared to *C. dubliniensis* following co-culture with macrophages (Moran *et al.*, 2007). Following co-culture of the *Δeed1/Δeed1* mutant with RAW 264.7 macrophages, the mutant appears to be phagocytosed. At 1 h the *Δeed1/Δeed1* was found exclusively in the yeast phase (Fig. 5.13). However, at both 3 h and 5 h, both hyphae and pseudohyphae were visible within the macrophage and at 5 h some hyphae could be seen protruding out from the macrophage (Fig. 5.13). Following incubation the survival of both *C. albicans* BWP17 and the *Δeed1/Δeed1* mutant was measured by comparing their growth after 16 h. Macrophages were lysed and viable numbers of *C. albicans* BWP17 and *Δeed1/Δeed1* cells were determined using the XTT dye reduction assay. As with *C. dubliniensis*, the

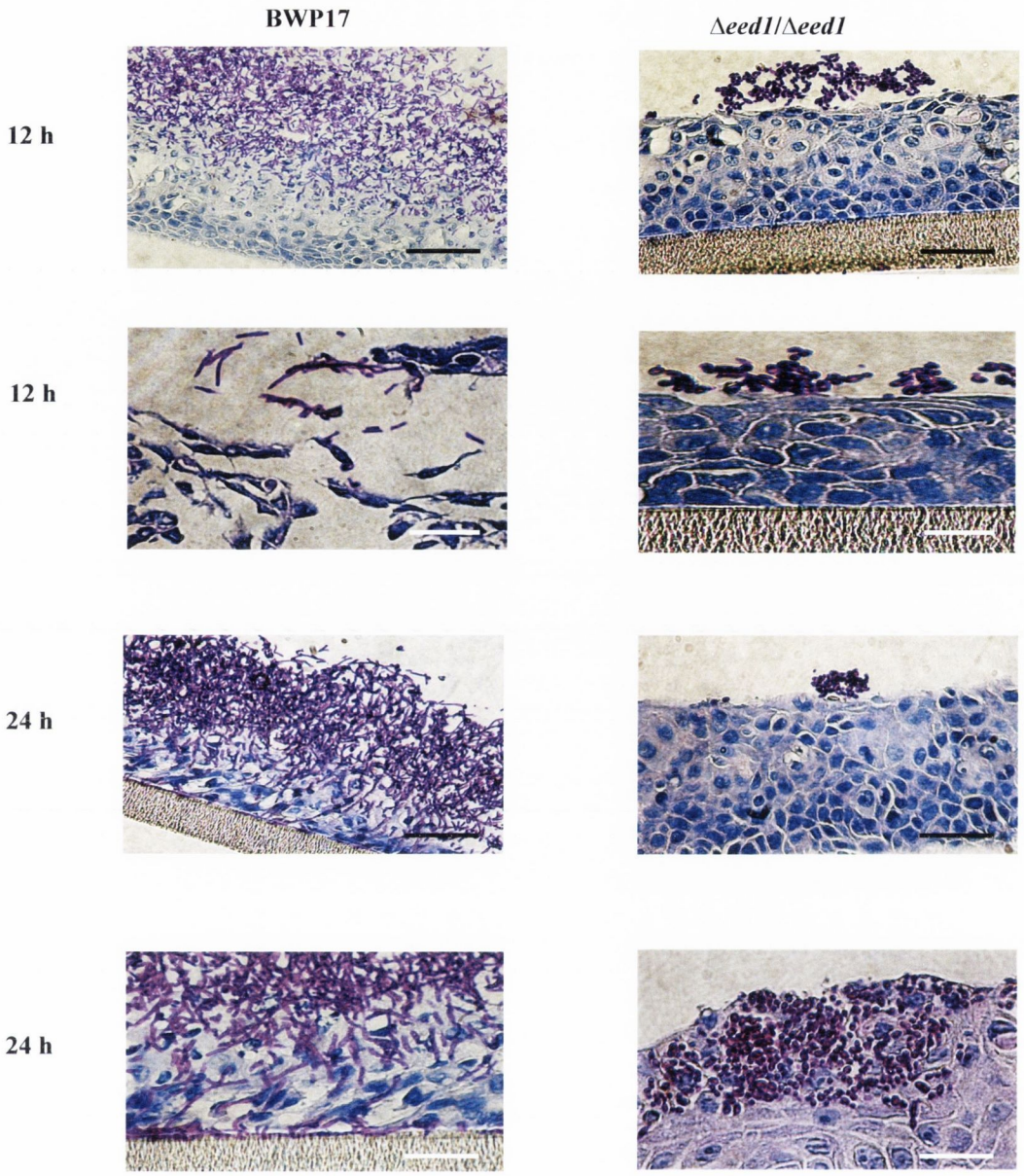
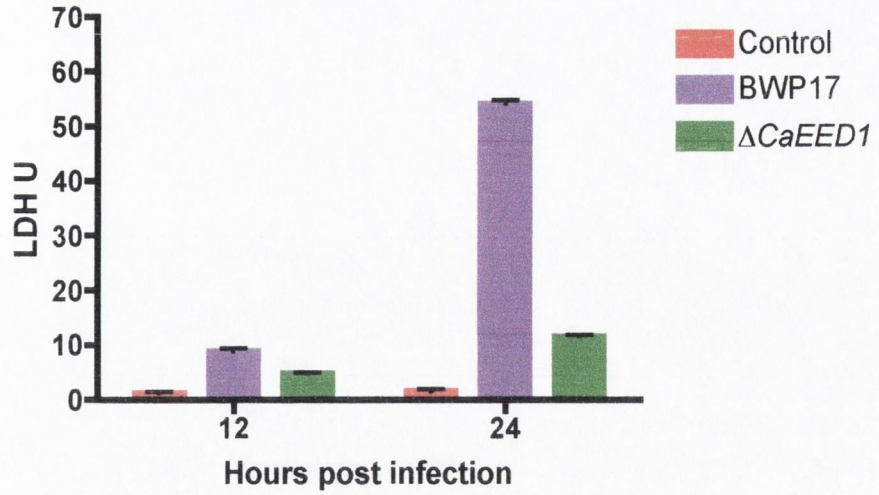


Figure 5.7. Light micrographs of reconstituted human oral epithelium (RHE; Skinethic, France) infected with *C. albicans* BWP17 and Δ*eed1*/Δ*eed1* mutant. Cells were pre-cultured in Lee's medium pH 4.5 prior to inoculation onto RHE. Tissue samples were taken at 12 h and 24 h. The black scale bar represents 25 μM and the white scale bar represents 10 μm.

(A)



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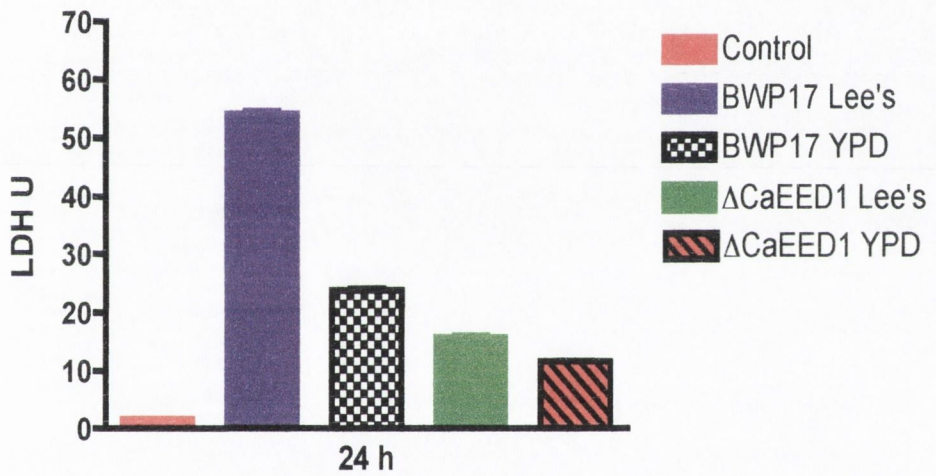


Figure 5.8. (A) Evaluation of tissue damage caused during infection of RHE by *C. albicans* BWP17 and *Δeed1/Δeed1* following pre-culture in Lee's medium pH 4.5, measured as LDH activity released into the culture medium over 24 h. **(B)** Tissue damage of *C. albicans* BWP17 and *Δeed1/Δeed1* at 24 h post inoculation following preculture in both Lee's medium and YPD, measured as LDH activity released into the culture medium.

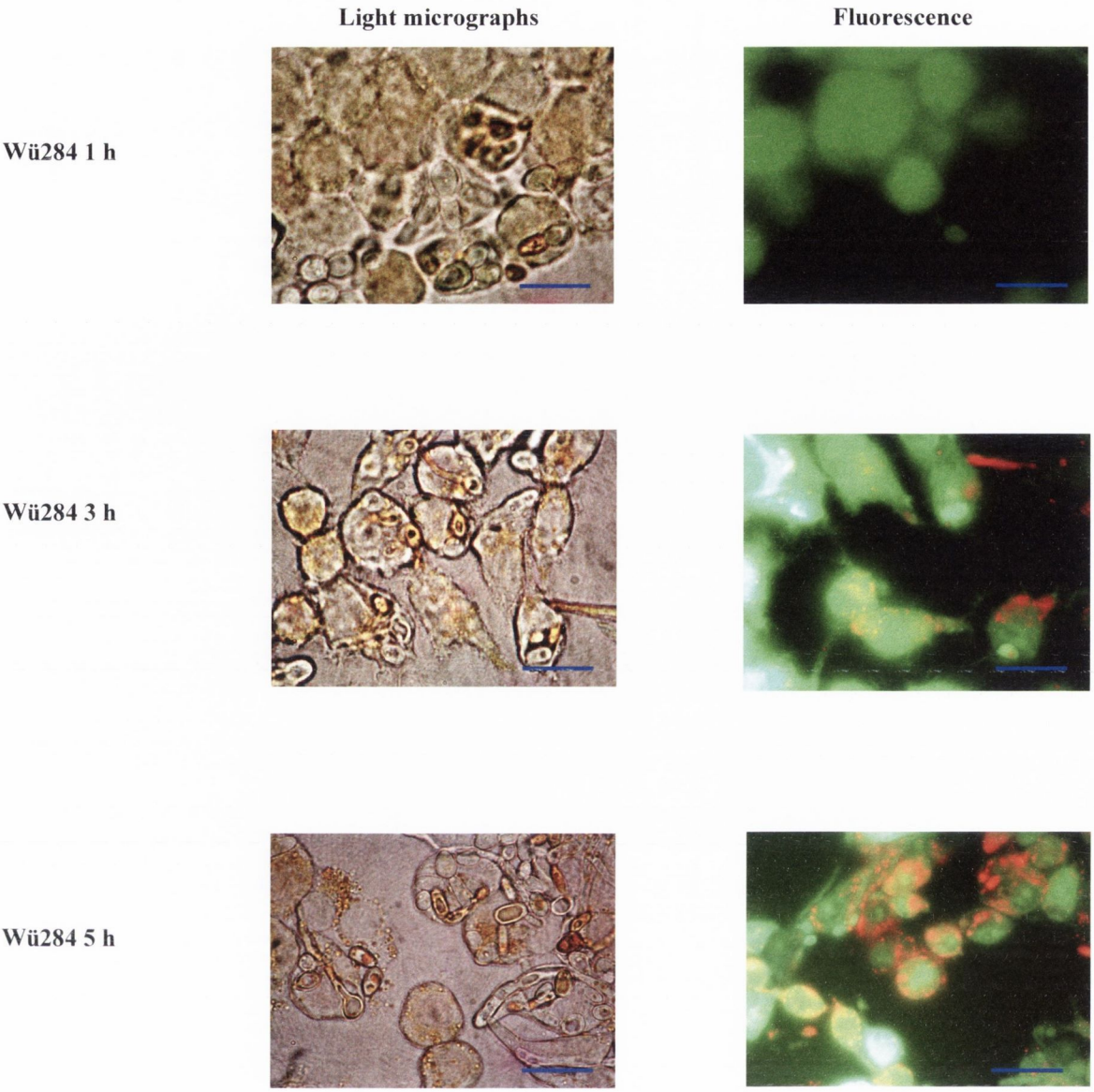


Figure 5.9. Analysis of *C. dubliniensis* Wü284 during co-culture with macrophages. Photomicrographs of Wü284 during co-culture with murine macrophages over 5 h following staining with acridine orange, this is a nucleic acid selective cationic dye useful for cell cycle determination, the dye fluoresces green to indicate live cell and red/orange to indicate dead cells. The blue scale bar represents 25 μm

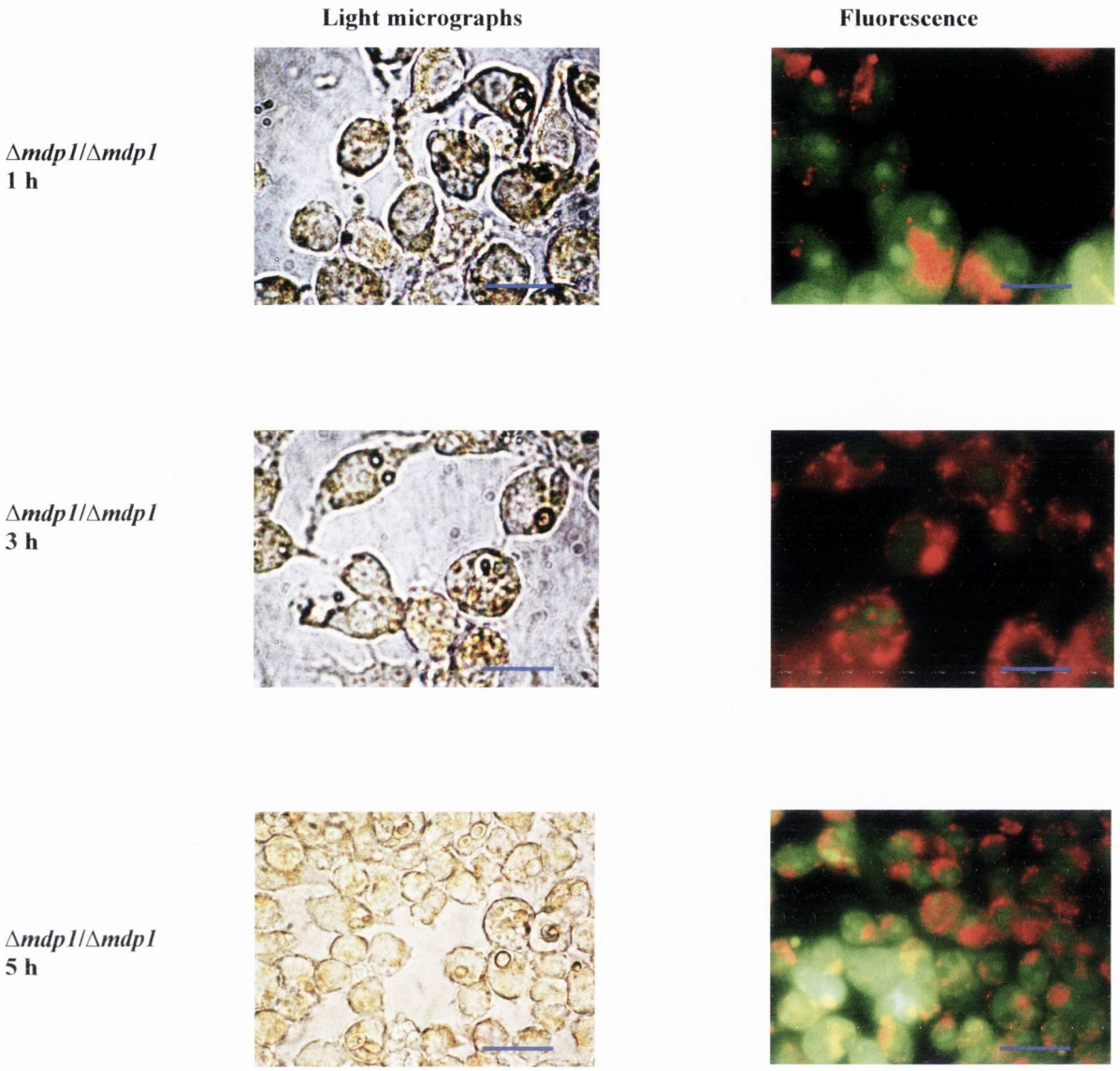
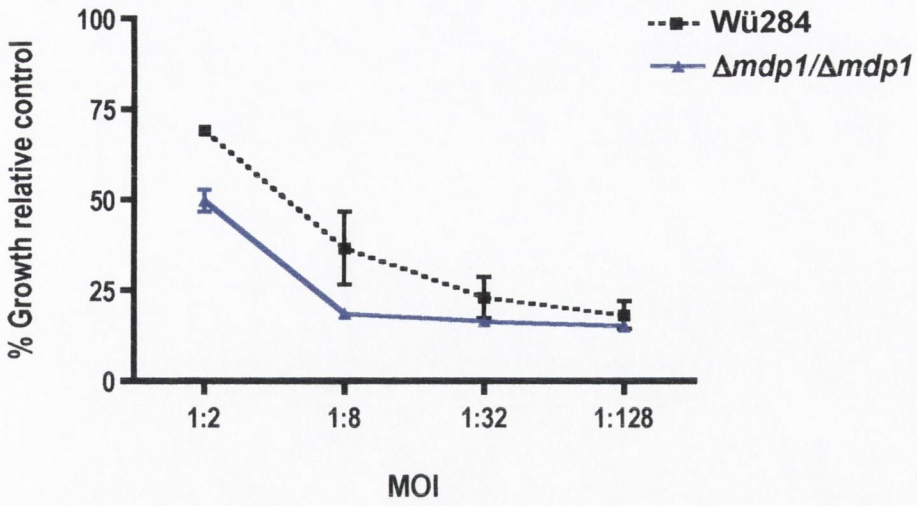


Figure 5.10. Analysis of *C. dubliniensis* LOC4 (Δ*mdp1*/Δ*mdp1*) during co-culture with macrophages. Photomicrographs of LOC4 (Δ*mdp1*/Δ*mdp1*) during co-culture with murine macrophages over 5 h following staining with acridine orange. The blue scale bars represents 25 μm.

(A)



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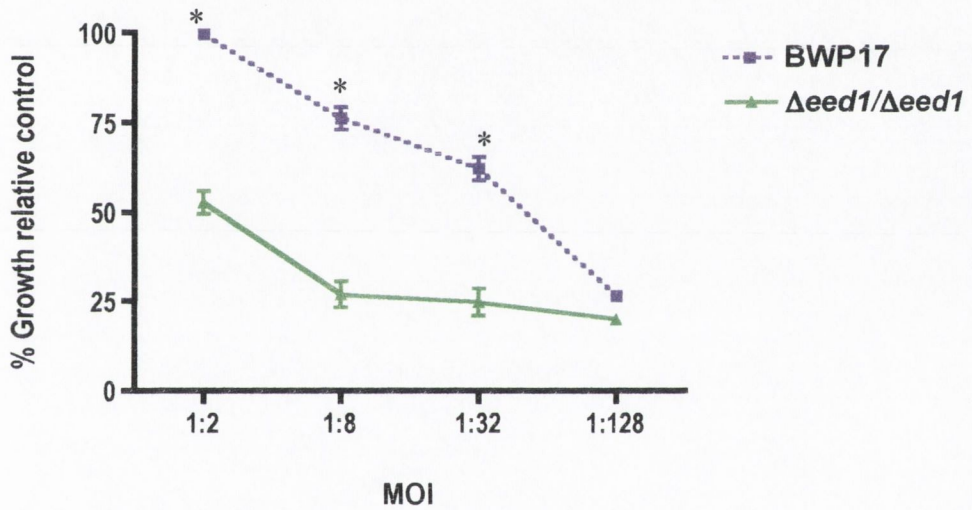


Figure 5.11. (A) Proliferation of *C. dubliniensis* Wü284 and LOC4 (*Δmdp1/Δmdp1*) in the presence of RAW 264.7 macrophages. **(B)** Proliferation of *C. albicans* BWP17 and the *Δeed1/Δeed1* mutant in the presence of RAW 264.7 macrophages. Proliferation of both *C. dubliniensis* and *C. albicans* was quantified in an XTT dye reduction assay. * Indicate where there was a significant difference observed (P <0.001) between the wild-type and the mutant. These experiments were repeated on three separate occasions.

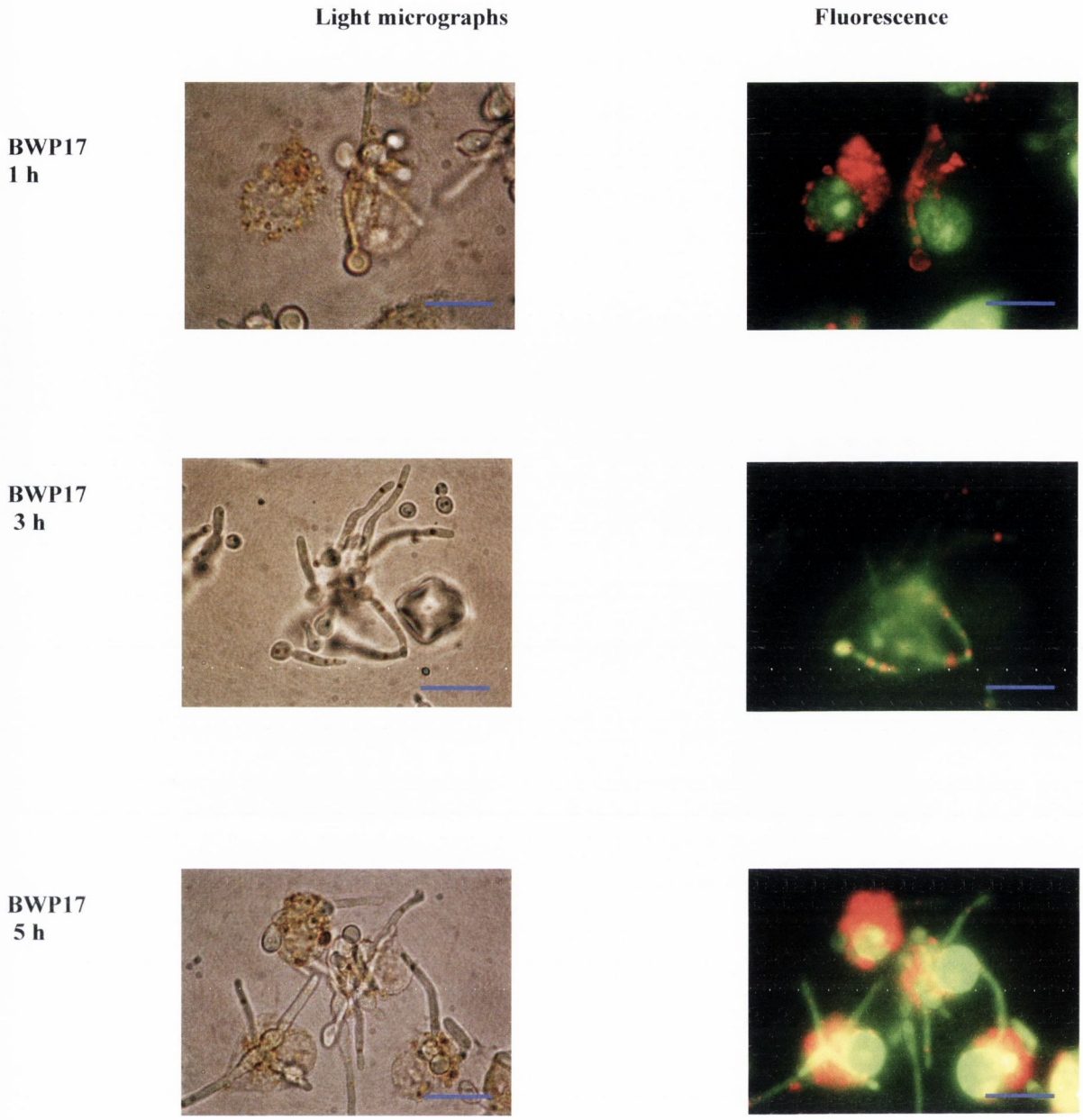


Figure 5.12. Analysis of *C. albicans* BWP17 during co-culture with macrophages. Photomicrographs of BWP17 during co-culture with murine macrophages over 5 h following staining with acridine orange. The blue scale bar represents 25 μm.

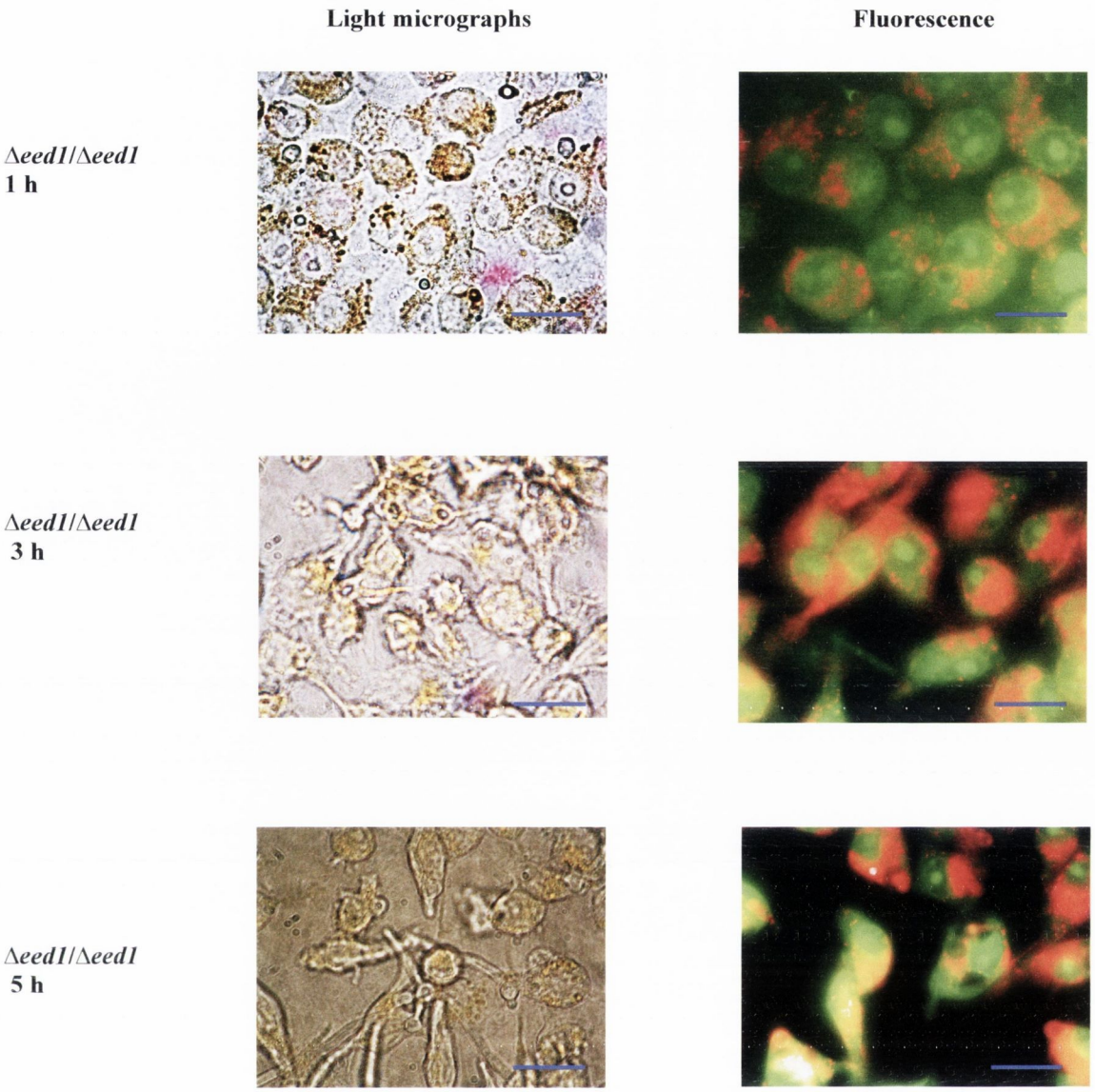


Figure 5.13. Analysis of *C. albicans* Δ*eed1*/Δ*eed1* mutant during co-culture with macrophages. Photomicrographs of Δ*eed1*/Δ*eed1* mutant during co-culture with murine macrophages over 5 h following staining with acridine orange. The blue scale bar represents 25 μm.

C. albicans BWP17 and *Δeed1/Δeed1* mutant were grown overnight in YPD at 37°C prior to co-culture with Raw 264.7 macrophages. BWP17 had a significantly higher survival rate when co-cultured with the macrophages at 1:2, 1:8 and 1:32 MOI compared to the *Δeed1/Δeed1* mutant and to *C. dubliniensis* with the exception of 1:128, there was a significant difference observed between BWP17 and *Δeed1/Δeed1* at MOIs of 1:2, 1:8 and 1:32 (P <0.001; Fig. 5.11). At MOIs of 1:2 the survival rate of BWP17 is 100 %, however at MOIs of 1:8 and 1:32 there was a drop in the survival rate to 77 % and 70 % respectively (Fig. 5.11). The increased survival of *C. albicans* BWP17 was associated with its ability to produce hyphae and to protrude from the macrophage and continue proliferation. The *Δeed1/Δeed1* mutant had a very poor survival rate when co-cultured with macrophages and had a survival rate very similar to *C. dubliniensis* LOC4. At MOIs of 1:2, the *Δeed1/Δeed1* mutant had a 53 % survival rate, however following an increase in MOIs the survival rate dropped. At MOIs of 1:8 and 1:32 the survival rate was 25 % and 20 % respectively. There were significant difference observed between BWP17 and *Δeed1/Δeed1* at MOIs of 1:2, 1:8 and 1:32 (P <0.001; Fig. 5.11). At MOIs of 1:128 the survival rate was just 17 % (Fig. 5.11).

5.1.4 A pH shift enhanced survival of *C. dubliniensis* Wü284 and LOC4 in a macrophage co-culture model

During co-culture with macrophages, the *C. dubliniensis* wild-type and LOC4 failed to filament and exhibited low virulence. However, by pre-culturing *C. dubliniensis* in Lee's medium pH 4.5, filamentation was enhanced and survival of both the *C. dubliniensis* wild-type and the *Δmdp1/Δmdp1* mutant was also enhanced (Figs. 5.14 and 5.15). At 1 h both the wild-type and the mutant were found predominantly in the yeast phase. However, by 3 h the wild-type and mutant cells had undergone the yeast hyphae transition and filaments were clearly visible within the macrophage and protruding from the macrophages (Figs. 5.14 and 5.15). In both the wild-type and the mutant it was noted that both pseudohyphae and hyphae were present. Following preculture in Lee's medium pH 4.5 it was evident that the pH shift had enhanced the level of hypha formation in both the wild-type and mutant. The XTT proliferation assay showed that survival of *C. dubliniensis* Wü284 and LOC4 was also greatly enhanced following preculture in Lee's medium pH 4.5 (Fig. 5.16). At MOIs of 1:2 Wü284 had a survival rate of 72 % whereas LOC4 had a survival rate of 85 %, at MOIs of 1:8 Wü284

had survival rate of 56 % and LOC4 had a survival rate of 56 %. When MOIs increased to 1:32 and 1:128 the survival rate did drop to 39 % and 28 % for both Wü284 and LOC4. However when survival rate is compared to preculture in YPD, it is clear that the pH shift resulted in significantly enhanced survival of both Wü284 and LOC4. There was a significant difference observed between Wü284 grown in Lee's medium compared to Wü284 grown in YPD at MOIs of 1:8 and 1:32 (P <0.01; Fig. 5.16). Also there was a significant difference observed between LOC4 grown in Lee's medium compared to LOC4 grown in YPD (P <0.001; Fig. 5.16).

C. albicans cells were also precultured in Lee's medium pH 4.5 to investigate if any the effect of a pH shift on survival of *C. albicans* cells. At 1 h *C. albicans* BWP17 cells had been phagocytosed and were found in both the yeast and hypha phase (Fig. 5.17). At both 3 h and 5 h almost all the cells were in the hypha phase and the hyphae were protruding from the macrophages and had continued to proliferate. However, this phenotype was also observed in *C. albicans* BWP17 following preculture in YPD. In the *C. albicans* Δ*eed1*/Δ*eed1* mutant cells were internalised by the macrophages and remained primarily in the yeast phase, however, some hyphae were visible from within the macrophages (Fig. 5.18). At both 3 h and 5 h hyphae were visible within the macrophage and some hyphae protruded out through the macrophage, allowing the *C. albicans* cells to continue proliferation (Fig. 5.17). The XTT proliferation assay demonstrated that a pH shift could not enhance the survival of the Δ*eed1*/Δ*eed1* mutant. At MOIs of 1:2 the survival rate of BWP17 was 93 %, and for the Δ*eed1*/Δ*eed1* mutant the survival rate was 52 % (Fig. 5.16). At an MOI of 1:8 the survival was 73 % and 34 % for BWP17 and the Δ*eed1*/Δ*eed1* mutant, respectively. Interestingly, there was no significant difference observed between BWP17 grown in Lee's medium or YPD (P > 0.05) (Fig. 5.16) As the MOIs increased no significant difference in survival of the mutant was observed compared to levels observed following preculture in YPD (P > 0.05) (Fig. 5.16). Thus pre-culturing of the Δ*eed1*/Δ*eed1* mutant in Lee's medium pH 4.5 did not enhance its survival following co-culture with macrophages. Therefore, a pH shift can enhance survival of both *C. dubliniensis* Wü284 and LOC4 (Δ*mdp1*/Δ*mdp1*) when co-cultured with macrophages, however this pH shift does not enhance the virulence of the Δ*eed1*/Δ*eed1* mutant.

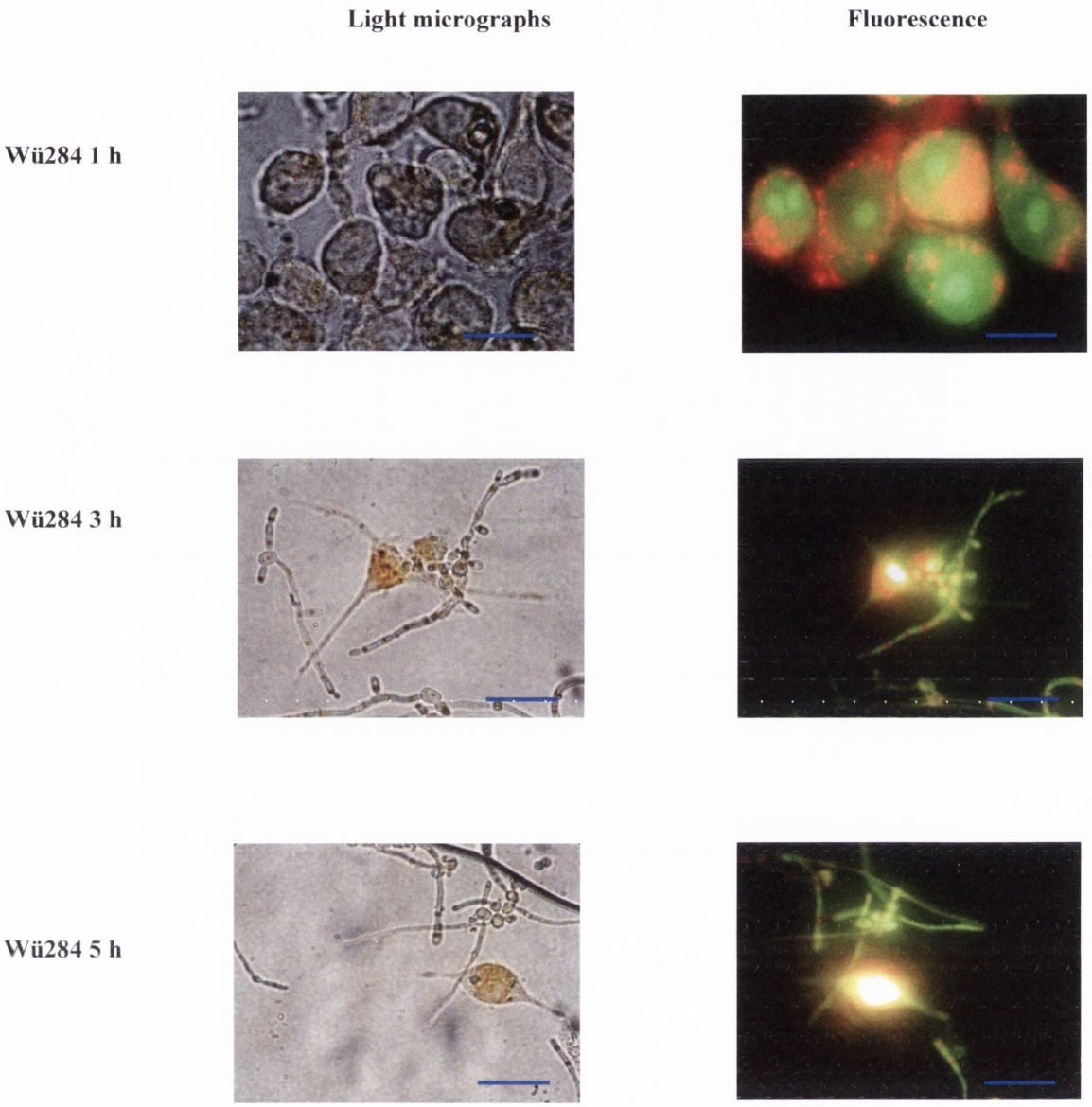


Figure 5.14. Analysis of *C. dubliniensis* Wü284 during co-culture with macrophages following preculture on Lee's medium (pH 4.5). Photomicrographs of Wü284 during co-culture with murine macrophages over 5 h following staining with acridine orange. The blue scale bars represents 25 µm.

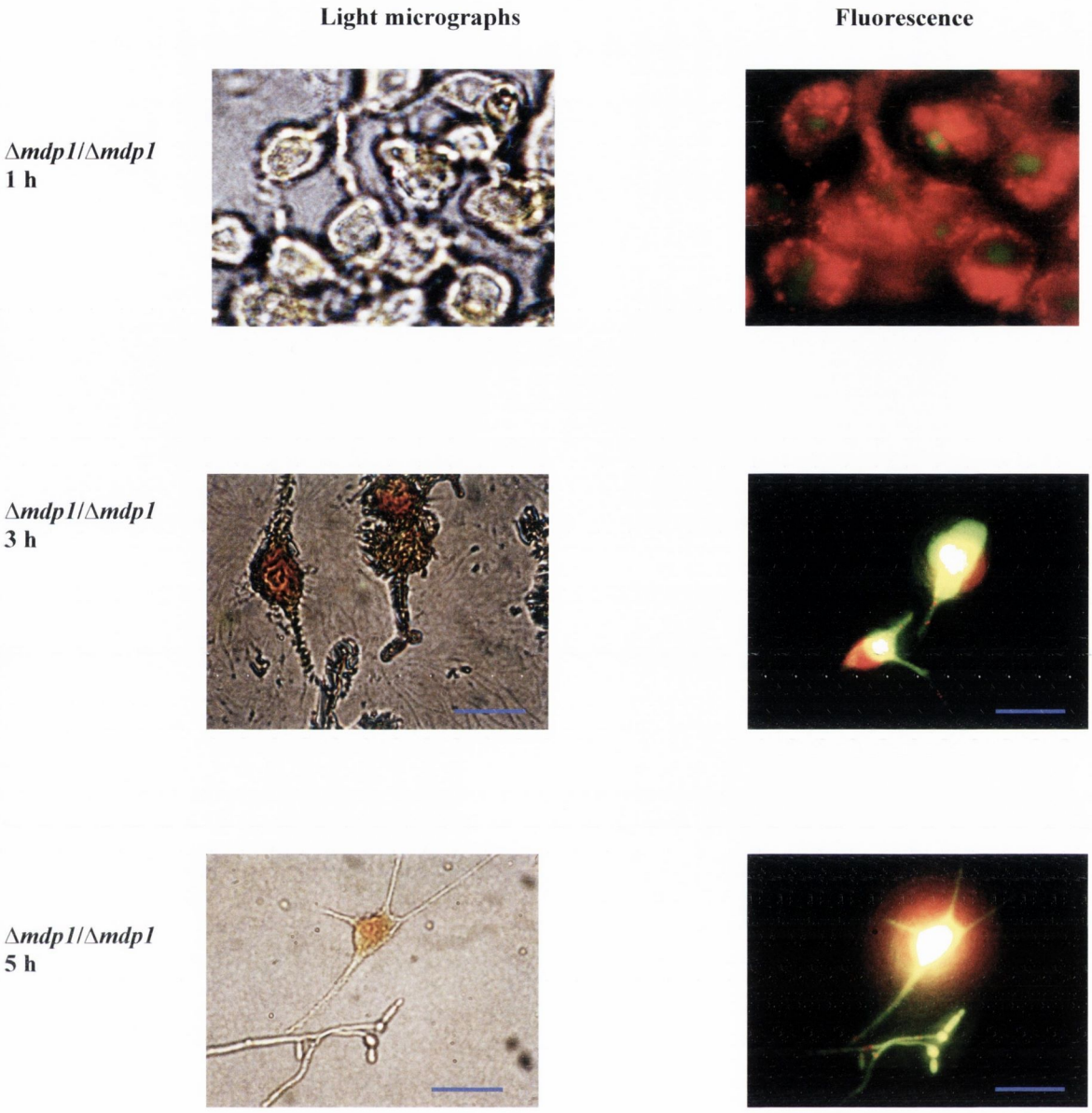
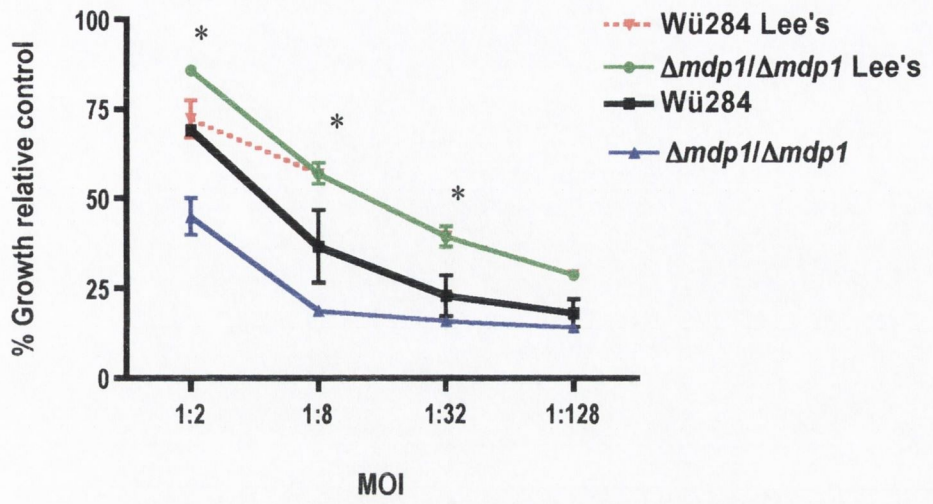


Figure 5.15. Analysis of *C. dubliniensis* LOC4 (*Δmdp1/Δmdp1*) during co-culture with macrophages following preculture in Lee's medium (pH 4.5). Photomicrographs of LOC4 (*Δmdp1/Δmdp1*) during co-culture with murine macrophages over 5 h following staining with acridine orange. The blue scale bars represents 25 μm.

(A)



(B)

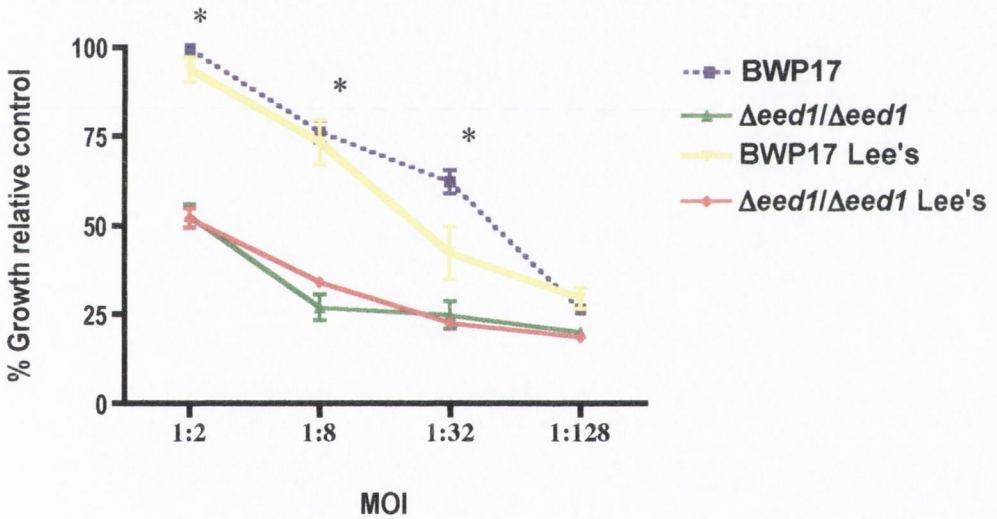


Figure 5.16 (A) Proliferation of *C. dubliniensis* Wü284 and LOC4 (*Δmdp1/Δmdp1*) in the presence of RAW 264.7 macrophages. *C. dubliniensis* cells were pre-cultured in either Lee's pH 4.5 or YPD prior to co-culture with murine macrophages. An * indicates where a significant difference between the strains following co-culture in Lee's medium and in YPD has occurred. There was significant difference between LOC4 grown in Lees and LOC4 grown in YPD (P <0.001; 2way ANOVA), There also was significant difference observed between Wü284 grown in Lee's medium and Wü284 grown in YPD at MOIs of 1:8 and 1:32 (P <0.01, P < 0.05). **(B)** Proliferation of *C. albicans* BWP17 and the *Δeed1/Δeed1* mutant in the presence of Raw 264.7 macrophages. *C. albicans* cells were pre-cultured either in Lee's pH 4.5 or YPD prior to co-culture with murine macrophages. There was no significant difference observed between BWP17 grown in Lee's medium and BWP17 grown in YPD (P > 0.05). Also there was no significant difference observed between *C. albicans Δeed1/Δeed1* grown in Lee's medium to *C. albicans Δeed1/Δeed1* grown in YPD (P > 0.05).

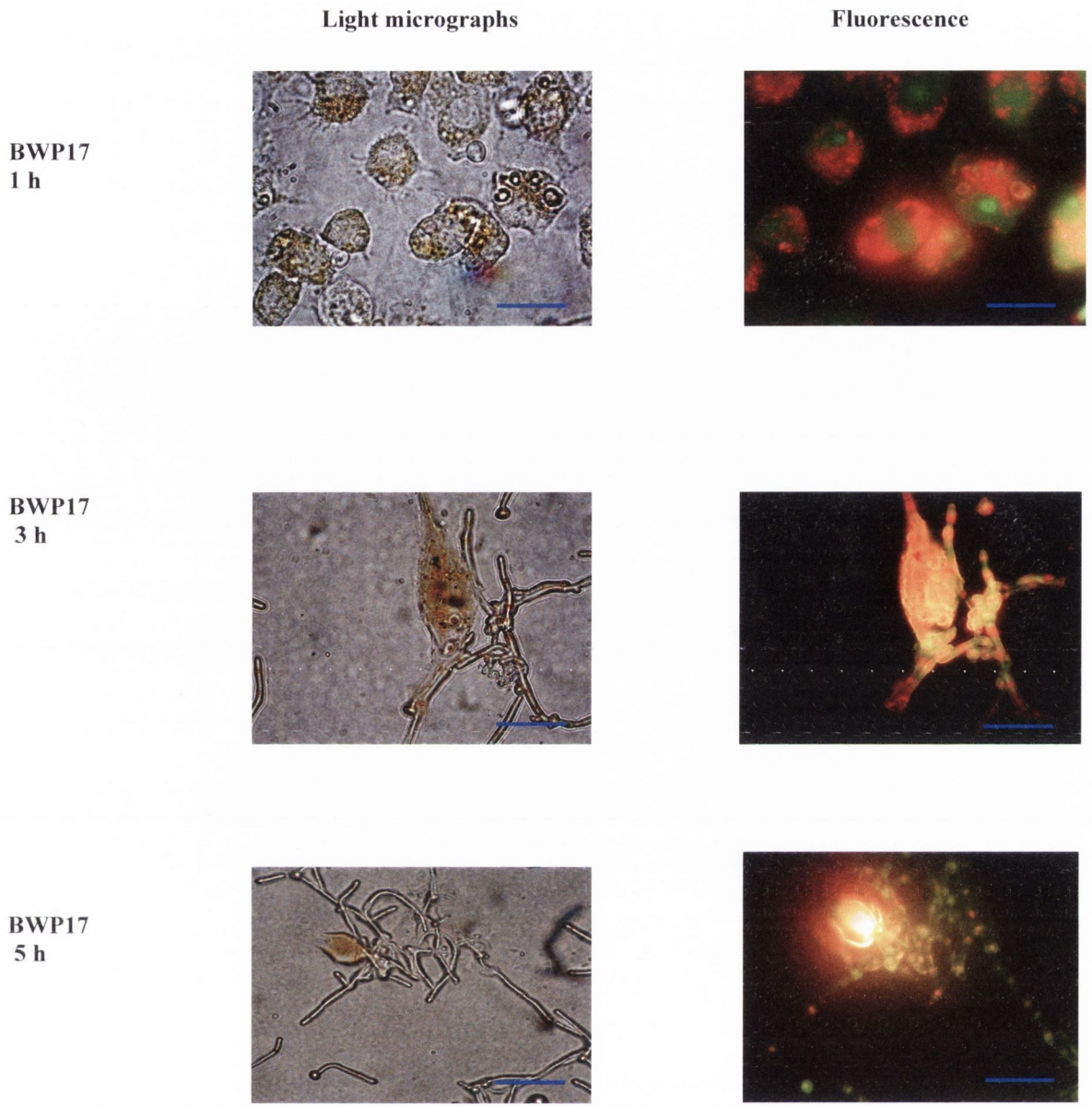


Figure 5.17. Analysis of *C. albicans* BWP17 during co-culture with macrophages following preculture in Lee's medium (pH 4.5). Photomicrographs of BWP17 during co-culture with murine macrophages over 5 h following staining with acridine orange. The blue scale bar represents 25 μm.

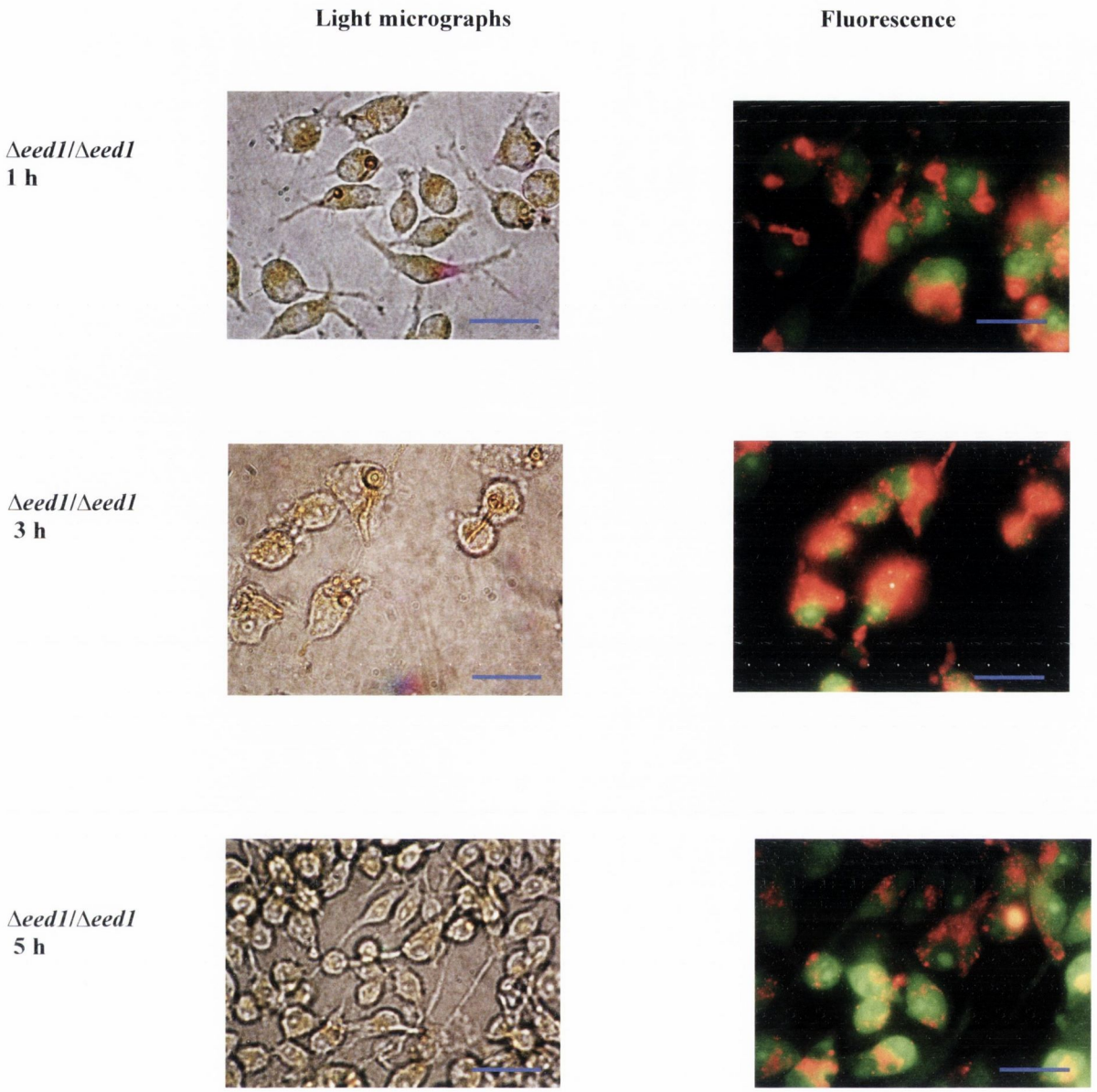


Figure 5.18. Analysis of *C. albicans* Δ*eed1*/Δ*eed1* mutant during co-culture with macrophages following preculture in Lee's medium (pH 4.5). Photomicrographs of Δ*eed1*/Δ*eed1* mutant during co-culture with murine macrophages over 5 h following staining with acridine orange. The blue scale bar represents 25 μm.

5.2 Discussion

5.2.1 *C. dubliniensis* Δ*mdp1*/Δ*mdp1* and *C. albicans* Δ*eed1*/Δ*eed1* exhibit attenuated virulence on RHE

The exact role of hypha growth in oral candidiasis is still unclear, although it has been demonstrated that hypha cells adhere better to oral epithelial and other cell types (Schaller *et al.*, 2002). Adhesion of *C. albicans* to host cells is one of the most important virulence traits of the organism and this could explain why the hypha form has a greater capacity to cause oral infections (Dieterich *et al.*, 2002). All morphogenetic forms of *C. albicans* are frequently encountered in the human oral mucosa. Oral epithelial cells constitute the first line of defence against oral *Candida* infections. Stokes *et al.* (2007), compared the growth of *C. albicans* and *C. dubliniensis* in the RHE infection model, and found that *C. dubliniensis* grew exclusively in the yeast form, forming a non-invasive layer along the surface of the epithelial tissue that appeared to be poorly adherent as it was easily dislodged from the RHE surface. This was in contrast to *C. albicans*, which attached to the epithelium and produced abundant hyphae at early time points and invaded deep into the tissue.

This study compared the virulence of *C. dubliniensis* Wü284 and LOC4 (Δ*mdp1*/Δ*mdp1*), *C. albicans* BWP17 and the Δ*eed1*/*eed1* mutant on the RHE infection model. The results from this study correlated with the results found by Stokes *et al.* (2007) as both *C. dubliniensis* Wü284 and LOC4 exhibited attenuated virulence following infection onto the RHE tissues. Both grew exclusively in the yeast phase and only a small number of cells adhered to the upper layer of the epithelial tissue. In tissues infected with *C. albicans* BWP17, a large number of cells adhered to the surface and at 6 h a lot of hyphae were visible and had become embedded within the tissue. In contrast, the *C. albicans* Δ*eed1*/Δ*eed1* mutant remained trapped within the tissues and grew exclusively in the yeast phase. However, the Δ*eed1*/Δ*eed1* mutant did cause what appeared to be induced endocytosis and membrane ruffling of the tissue as described by Zakikhany *et al.* (2007). Like *C. dubliniensis*, the Δ*eed1*/Δ*eed1* mutant exhibited attenuated virulence. This result correlated with the findings of Zakikhany *et al.* (2007) that *EED1* is not required for adhesion and initial invasion into epithelial cells but is required for escape and dissemination. *C. albicans* mutants with defects in filamentation have reduced virulence in animal models of infection (Lo *et al.*, 1997; Saville *et al.*,

2003). A study by Villar *et al.* (2004) compared the ability of yeast pseudohyphae and hyphae to adhere to and lyse oral epithelial cells. The study compared the ability of *C. albicans* SC5314 to adhere to oral epithelial cells with the filamentation deficient mutants *Δefg1/Δefg1*, *Δcph1/Δcph1* and *Δtup1/Δtup1*. The results showed that the ability of *C. albicans* to adhere to oral epithelial cells is compromised when the organism is unable to form true hyphae. Thus, the formation of true hyphae is necessary for the organisms to adhere to oral epithelial cells. This could perhaps explain why both *C. dubliniensis* Wü284 and LOC4 adhere poorly to the surface of the epithelial tissue as both remained predominantly in the yeast phase. The *C. albicans Δeed1/Δeed1* did adhere to the cell surface however, it was unable to penetrate down through the tissue. The LDH damage observed for the *C. albicans Δeed1/Δeed1* at 48 h would indicate that damage had occurred to the tissue, however, it is thought that by 48 h the tissue begins to degrade itself, therefore at the 48 h time point the LDH reading is inaccurate.

5.2.2 pH shift enhances virulence on RHE in *C. dubliniensis* Wü284 and LOC4

In Chapter 4, it was demonstrated that a pH shift from 4.5 to 7.5 could enhance filamentation of both *C. dubliniensis* Wü284 and LOC4 (*Δmdp1/Δmdp1*) in serum, when cells were pre-cultured in Lee's medium pH 4.5 prior to inoculation into 10 % serum. Therefore, it was investigated whether the pH shift could enhance virulence of *C. dubliniensis* Wü284 and LOC4 on RHE. Pre-culturing *C. dubliniensis* Wü284 in Lee's medium pH 4.5 prior to inoculation onto the RHE did enhance virulence and invasion. At the 6 h time point there was an increased number of yeast, hyphae and pseudohyphae visible adhering to the upper layer of the epithelium. At the 12 h time point both hyphae and yeast cells were visible throughout the epithelial tissue. However at 24 h Wü284 had reverted back almost exclusively to the yeast phase. Interestingly, in *C. dubliniensis* LOC4, the mutant remained almost predominantly in the yeast phase, however there were some hyphae and pseudohyphae present at earlier time points (6 h and 12 h), whereas at 24 h cells were almost exclusively in the yeast phase. However, although LOC4 did not produce as many hyphae as the wild-type Wü284, there was a large increase in the number of cells adhering to the upper epithelial tissue and at 24 h a significant amount of damage had occurred to the epithelial tissue. The increased damage that has occurred to the epithelial tissue following pre-culture of *C. dubliniensis* Wü284 and LOC4 in Lee's medium pH 4.5 may be due to an increased adherence of the

cells to the tissue. This could be due to the fact that the pH shift from 4.5 to 7.5 resulted in an increased number of hyphae (Wü284), and pseudohyphae (LOC4) which facilitated an increased adherence of the cells to the epithelium. Compared to YPD grown cells, adherent cells were visible at 6 h. Kimura & Pearsall (1980), found that germination of *C. albicans* enhanced adherence to human buccal epithelial cells. The increased germination of *C. dubliniensis* in response to pH may involve Rim101p, which is essential for the regulation of genes in response to external pH in *C. albicans*. The pH-dependent transcription factor Rim101p is known to regulate *PHR1* and *PHR2* in *C. albicans*, two glycosidases involved in cell wall biosynthesis. Lotz *et al.* (2004) performed transcriptional profiling to identify *RIM101*-regulated cell wall genes with a cell wall-specific DNA microarray. The experiments were carried out comparing *RIM101* wild-type cells and a Δ*rim*101 mutant. Transcriptional profiling revealed that Rim101p induced a set of cell wall genes in response to changes in the environmental pH. There was significant activation of hypha-specific genes such as *HWP1, RBT1, ALS1* and *ALS5*. The results correlated with the Δ*rim101*/Δ*rim101* phenotype, which is characterised by filamentous growth at acidic pH and an excess adhesion phenotype at neutral pH and 37°C. At acidic pH (pH 4.0) activation of *RBT1* and *HWP1* by *RIM101* was significant but not as strong as at pH 8.0 (Lotz *et al.*, 2004). This data suggest that perhaps in *C. dubliniensis* Wü284 and LOC4 when a pH shift occurs from 4.5 to 7.5, cell wall genes such as *HWP1*, *ALS1* and *ALS5* could be expressed resulting in increased adherence to the epithelial tissue (Figs. 5.4 and 5.5) and thus increased damage to the tissue. The pH shift did not affect virulence of the Δ*eed1*/Δ*eed1* mutant. There was no increase in adherence to the upper layer of the tissue, and what appears to be induced endocytosis was visible, however SEM anaylsis would have to be carried out to confirm this. However, the Δ*eed1*/Δ*eed1* mutant was unable to escape and disseminate through the tissue and perhaps this suggests that *EED1* is required for activation of the pH response in *C. albicans*. *C. albicans* BWP17 produced numerous hypha and these embedded down through the tissue following pre-culture in Lee's medium pH 4.5 (Fig. 5.7). Similarly perhaps the pH shift resulted in the expression of cell wall genes which allowed for the adherence and dissemination of the hypha cells down through the tissue.

5.2.3 Survival of *C. dubliniensis* and *C. albicans* following co-culture with Raw 264.7 murine macrophages.

C. albicans can cause life-threatening infections in immunocompromised patients but causes superficial mucosal infections in immunocompetent individuals. The mammalian immune system is a powerful barrier to *Candida* infections (Soll, 2002). Analysis of the response of *Candida* to macrophages provides a glimpse into the necessary processes for the organism to survive its first encounter with the immune system. Once inside a macrophage, the yeast form of *C. albicans* differentiates into the filamentous from which can break through the macrophage allowing it to resume proliferation. The survival of both *C. dubliniensis* Wü284 and LOC4 (*Δmdp1/Δmdp1*), *C. albicans* BWP17 and the *Δeed1/Δeed1* mutant was examined in co-culture with Raw 264.7 macrophages. This model examines the proliferation of *Candida* cells at a range of yeast: macrophage ratios (MOIs). This provides an interesting insight into the functions necessary for virulence (Marcil *et al.*, 2002). This could perhaps resemble the *in vivo* activity of the macrophages in patients with a compromised immune system (Moran *et al.*, 2007). *C. dubliniensis* LOC4 had a poorer survival rate compared to wild-type Wü284. LOC4 remained in the yeast phase and failed to filament following phagocytosis, whereas Wü284 did produce filaments. However, when compared to wild-type *C. albicans* both the *C. dubliniensis* wild-type and LOC4 were significantly less able to proliferate in co-culture with macrophages and this can be attributed to the low levels of filamentation. Moran *et al* (2007) also found that the survival of *C. albicans* during co-culture with Raw 264.7 murine macrophages was significantly greater than *C. dubliniensis*. *C.albicans* BWP17 produced hyphae rapidly following co-culture with Raw 264.7 macrophages, and was able to proliferate out of the macrophage. The *Δeed1/Δeed1* mutant did produce pseudohyphae and hyphae, however hyphae were only seen protruding from the macrophage at 5 h. One of the main reasons for the dramatic differences in survival rates between BWP17 and the *Δeed1/Δeed1* mutant is the poor ability of *Δeed1/Δeed1* mutant to produce filaments Lo *et al.* (1997) demonstrated that both the *C. albicans Δcph1/Δcph1* and the *Δefg1/Δefg1* mutants were ingested by macrophages. However, following ingestion of the filamentous form, the *Δcph1/Δcph1* and the *Δefg1/Δefg1* mutants failed to form filaments and had a very poor survival rate. These data show how vital the ability to switch from yeast to the filamentous form is for virulence.

5.2.4 A pH shift enhanced survival of *C. dubliniensis* following co-culture with Raw 264.7

It was also decided to investigate whether a pH shift from 4.5 to 7.5 could enhance survival of *C. dubliniensis* Wü284 and LOC4 and *C. albicans* BWP17 Δ*eed1*/Δ*eed1*. As with the RHE experiments both *C. dubliniensis* and *C. albicans* were pre-cultured in Lee's medium pH 4.5 prior to co-culture with Raw 264.7 macrophages. In *C. dubliniensis*, both Wü284 and LOC4 displayed increased in survival. Both produced hyphae, which allowed the cells to protrude from the macrophage and continue proliferation. The pH shift from 4.5 to 7.5 increased survival rates of both strains measured by XTT reduction levels. This increase in filamentation enabled both *C. dubliniensis* Wü284 and LOC4 to escape from the macrophage at a similar level to *C. albicans* (Lo *et al.*, 1997; Marcil *et al.*, 2002). As mentioned previously, it is likely that the pH shift enhances the expression of hypha specific genes in *C. dubliniensis* (Lotz *et al.*, 2004). Interestingly, as with inoculation on to the RHE tissues the pH shift following preculture in Lee's medium did not enhance the virulence of the Δ*eed1*/Δ*eed1* mutant, supporting the previous conclusion that perhaps *EED1* is required for activation of the pH response in *C. albicans*.

Chapter 6

Tetracycline induced expression of *C. dubliniensis MDP1*

6.0 Introduction

Systems that allow researchers to experimentally control the expression of specific genes in an organism under study are highly valuable tools for investigating gene function and also for manipulating the behaviour of the organism. Several regulating promoters have been utilised by investigators to induce or repress gene expression. In *C. albicans* for example, the *PCK1* promoter, the *MAL2* promoter and the *MET3* promoter, which are repressed by glucose, methionine and cysteine respectively, have been used previously (Backen *et al.*, 2000; Care *et al.*, 1999; Leuker *et al.*, 1997). When a target gene is placed under the control of a tightly regulated promoter, its expression can be turned on or off by incubating *C. albicans* in an appropriate inducing or repressing media. Under many circumstances it is desirable to control gene expression without the need to change the growth medium but by adding an inducing or repressing substance that does not affect metabolism (Park & Morschhauser, 2005). The tetracycline (Tet) system allows such a growth medium independent control of gene expression by a small molecule that easily diffuses into the cell. It is based on the tetracycline repressor protein (TetR) from *E. coli*, which binds to its target sequence, the *tet* operator (*tetO*), in the promoter region of the tetracycline resistance genes to repress their expression in the absence of tetracycline. When present in the media, tetracycline binds with high affinity to TetR, resulting in dissociation of the repressor from the promoter and the expression of the *tet* genes (Gossen & Bujard, 1992). Park and Morschhauser (2005), developed a Tet-inducible gene expression system for *C. albicans*, using the tetracycline derivative doxycycline (Dox) and used it to investigate the expression of specific genes on the behaviour of *C. albicans* yeast and hypha cells. They demonstrated that the *C. albicans*-adapted tetracycline-dependent gene expression system is a multipurpose tool with which to study gene function and manipulate cellular behaviour in *C. albicans.*

As direct complementation was unsuccessful following multiple attempts at cloning (Chapter 4, 4.1.10), it was decided to utilise the tetracycline inducible promoter in order to further assess the function of *MDP1*. *MDP1* was placed under control of the tetracycline promoter, thus overexpression of the gene was achieved by the addition of doxcycline to the medium.

6.1 Results

6.1.1 Construction of a *MDP1* and *EED1* tetracycline-inducible gene expression cassette

We were kindly given a gift of the pNIM1 plasmid from J. Morschäuser (University of Würzburg, Germany). The pNIM1 plasmid contains the dominant selection marker *caSAT1*, which confers resistance to nourseothricin (Fig. 6.1). In pNIM1 the *caGFP* ORF was fused to the Tet-inducible promoter and this ORF is flanked by unique *Sal*I and *Bgl*II restriction sites. To replace the *GFP* ORF, a 881 bp fragment from the 5' end of *C. dubliniensis MDP1* was amplified using the primer pair CdTetF/CdTetR (primers contained the unique restriction sites *Sal*I and *Bgl*II, Table 2.2). Purified DNA fragments were ligated using the pGEM-T Easy Vector. Following this, ligations were transformed into *E. coli* DH5α (Chapter 2, 2.3.4). Transformants were screened by digesting plasmid DNA with the restriction enzymes *Sal*I and *Bgl*II to identify inserts. Inserts from positive transformants were gel purified along with the *Sal*I/*Bgl*II digested pNIM1 plasmid and these were then ligated together and transformed into *E. coli* DH5α, as before. Following the identification of positive transformants, the pNIM1 derivative containing the *MDP1* fragment was linearised within the cloned *MDP1* region with *Eco*RI and transformed into the *C. dubliniensis* heterozygous mutant (Fig. 6.1).

Positive transformants were grown overnight in YPD at 37°C. Cells were washed twice in (PBS) and inoculated to a cell density of 2 x 10^6 CFU/ml in 5 ml of the appropriate inducing medium (Table 6.1) in a 6 well tissue culture plate. The concentration of doxycycline (Dox) used in all experiments was 50 μg/ml. Cells were examined every hour for 5 h using a Nikon TMS-F inverted microscope (Nikon, Japan).

6.1.2 Construction of tetracycline inducible allele of *MDP1* in *C. dubliniensis*

The *C. dubliniensis MDP1* heterozygous mutant was transformed with the *Eco*RI linearised *MDP1*-pNIM1 vector (6731 bp) (Chapter 2, 2.3.4). It was predicted that this fragment would insert into the 5' end of the intact chromosomal copy of the *MDP1* gene in the heterozygous mutant, thus placing this copy of the gene under the control of the tetracycline inducible promoter (Fig. 6.1 c). In order to screen for correct transformants, nourseothricin resistant transformants were screened using the primer

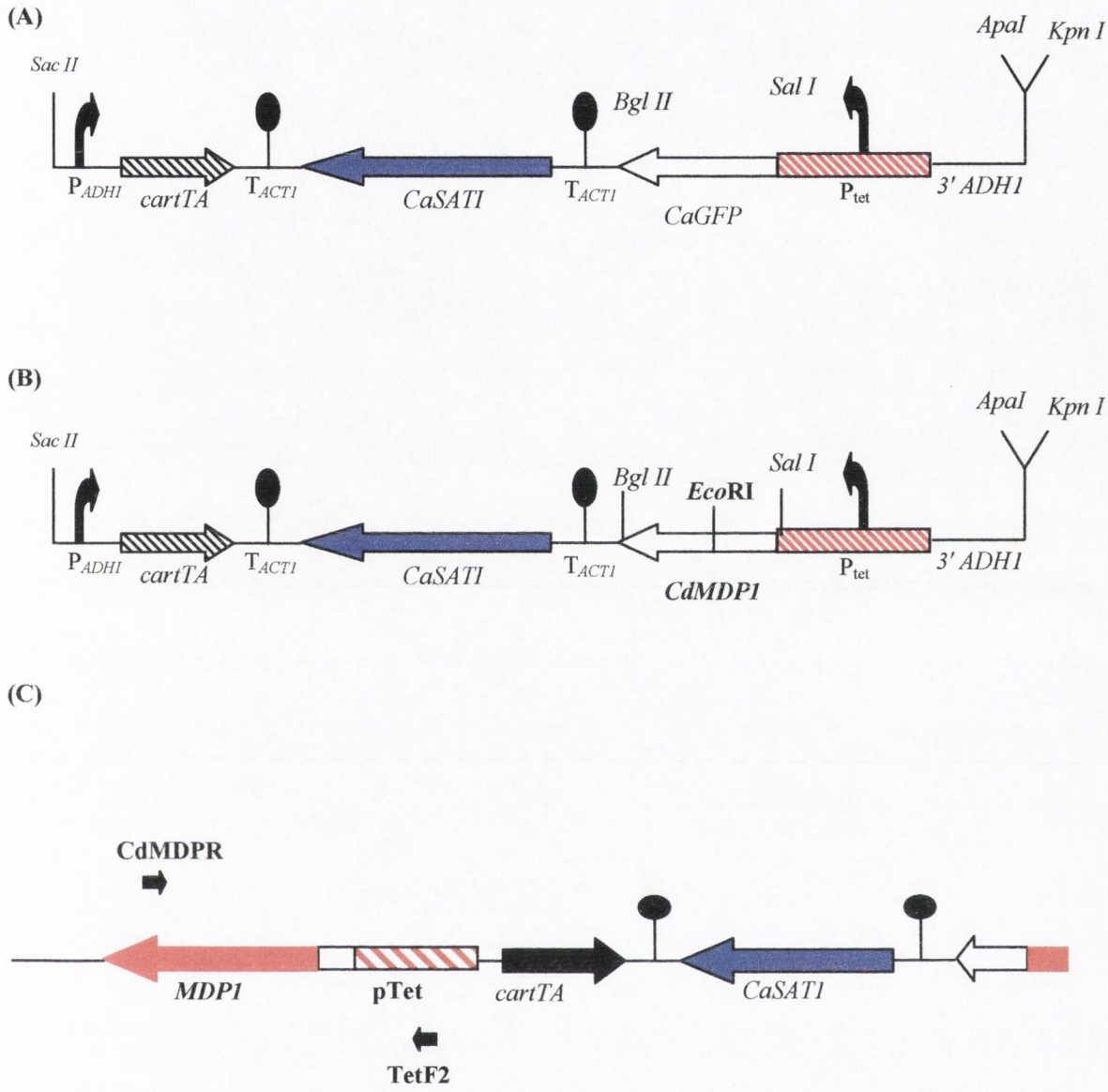


Figure 6.1. (A) Structure of the Tet-inducible gene cassette contained in plasmid pNIM1. Unique restriction sites (*Bgl*II/*Sal*I) can be used to substitute other ORFs for *caGFP* and to excise the whole cassette from the vector. **(B)** The *Bgl*II/*Sal*I restriction sites were utilised to insert a 881bp fragment from *C. dubliniensis MDP1* for *caGFP*. **(C)** Structure of the *MDP1* locus in the *C. dubliniensis* heterozygous *MDP1* mutant containing the *MDP1*-pNIM1 fragment. Positive transformants were identified using the primer pair TETF2/CdMDPR. The chromosomal copy of *MDP1* is shaded red.

Bent arrows symbolize promoters (P), and the filled circles indicate the transcription termination sequence of the *ACT1* gene (T*ACT1*). The cartTA is represented by ⇒. The prefix *ca* indicates *C. albicans*-adapted versions of heterologous genes. . Only relevant restriction sites used to construct the plasmids or to obtain the fragments used for transformation are shown. *Apa*I, *Bgl*II;,*Eco*RI *Kpn*I, *Sac*I, *Sac*II. Unique restriction sites that can be used to substitute other ORFs for *caGFP* and to excise the whole cassette from the vector backbone are indicated.

This figure is adapted from a paper by Park, Y. N. & Morschhauser, J. (2005). Tetracycline-inducible gene expression and gene deletion in *Candida albicans*. *Eukaryot Cell* **4**, 1328-1342.

Table 6.1 Experimental conditions for doxycycline expression

Pre-culture Media	Pre-culture Temperature	Experimental Media	Experimental Temperature
YPD	37°C	YPD	37°C
YPD	37°C	YPD + doxycycline	37°C
YPD	37°C	10 % serum	37°C
YPD	37°C	10 % serum + doxycycline	37°C
YPD	37 °C	PBS + CO_2	37°C
YPD	37 °C	PBS + CO_2 + doxycycline	37°C

pair TETF2/CdMDPR (Table 2.2). The TETF2 annealed to the tetracycline promoter region and the CdMDPR annealed to chromosomal DNA for *MDP1* in *C. dubliniensis* (Fig 6.1). The latter primer was specific for the full length (intact) copy of *MDP1* in the heterozygous mutant, and would therefore only detect integration into that allele. Four positive transformants were identified. Following this, phenotypic tests were carried out.

6.1.3 Doxycycline induced expression of *MDP1* in *C. dubliniensis* transformants

In order to further assess the function of the *C. dubliniensis MDP1* gene under the regulation of Dox, filamentous growth was examined under different conditions. When grown in YPD overnight and inoculated into YPD with and without Dox the transformants tested remained in the yeast phase during the course of the experiment. However when the pTet-*MDP1* transformants were grown overnight and inoculated into 10 % serum in the presence and absence of Dox, a difference was observed. In the parental strain (heterozygous mutant) at 2 h, cells grown in the presence and absence of Dox were found predominantly in the yeast phase with some pseudohyphae. At both 3 h and 4 h the heterozygous mutant was found as both yeast and pseudohyphae with some true hyphae present. At 2 h the pTet-*MDP1* transformants grown in the absence of Dox resembled the parental strain and remained largely in the yeast phase with some pseudohyphae visible (Fig. 6.2). The transformants grown in the presence of Dox were also predominantly in the yeast phase, however, germ tubes and short hyphae could be observed (Fig. 6.2). At 3 h the effect of Dox was more pronounced. Transformants grown in the presence of Dox were predominantly hypha with the majority of cells producing true hyphae (Fig. 6.2). In the absence of Dox, cells were predominantly pseudohypha, with a small number of true hyphae present. Thus the tetracycline-induced expression of *C. dubliniensis MDP1* resulted in increased true-hypha production compared to both non-induced cultures and the parental strain. At 4 h the transformants grown in the absence of Dox were found as both yeast and pseudohyphae with some true hyphae present (Fig. 6.2). pTet-*MDP1* transformants grown in the presence of Dox were both pseudohypha and true hyphae were also present in the medium (Fig. 6.2).

Following the increased filamentation observed in pTet-*MDP1* transformants grown in the presence of Dox in 10 % serum, it was decided to grow the transformants

in PBS in the presence of CO_2. These conditions are similar to those used in RHE infection experiments and were performed in order to determine if Dox-induced *MDP1* expression could enhance filamentation and possibly increased epithelial invasion and dissemination. Cells were grown overnight in YPD, and were washed with PBS and inoculated into fresh PBS with and without Dox. The cells were then incubated at 37°C and 5 % CO_2. Under these conditions all cells, whether grown in the presence or absence of Dox, remained predominantly in the yeast phase with some pseudohyphae produced (Fig. 6.3).

Following the analysis of pTet-*MDP1* strains in liquid medium it was decided to investigate their growth on known hypha inducing agar (Lee's agar) to determine if a difference could be observed between those grown in the presence and absence of Dox. These were compared to the *C. dubliniensis* wild type Wü284 and the heterozygous mutant (*Δmdp1/MDP1*). *C. dubliniensis* Wü284 produced rough filamentous colonies on Lee's agar with and without Dox (Fig. 6.4). The *C. dubliniensis* heterozygous mutant (*Δmdp1/MDP1*) produced predominantly smooth colonies on Lee's agar without Dox, however some rough colonies were visible. In the presence of Dox the *Δmdp1/MDP1* mutant also produced mainly smooth colonies. The tetracycline induced pTet-*MDP1* transformants produced colonies which were predominantly smooth when grown on Lee's agar without Dox. In the presence of Dox the *Δmdp1/MDP1* mutant produced almost completely smooth colonies (Fig. 6.4).

6.1.4 Expression analysis of the tetracycline induced *C. dubliniensis MDP1* gene

In order to examine tetracycline induction of *MDP1*, cells were grown overnight in YPD, and diluted 1 in 500 into fresh YPD with and without Dox and grown to $O.D._{600\ nm}$ 1.0. Following this, cells were collected by centrifugation and subsequently RNA isolation and cDNA synthesis was carried out for real-time PCR analysis. As previously shown (Fig 3.16) expression of *MDP1* in both Wü284 and the heterozygous mutant (*Δmdp1/MDP1*) was extremely low relative to *ACT1* (Fig. 6.5). However, there was a 7.5-fold increase in expression of the pTet-regulated allele of *MDP1* compared to the heterozygous mutant (*Δmdp1/MDP1*). In the presence of Dox (50 $\mu g/ml^{-1}$) a 13.5-fold increase in tetracycline induced *MDP1* expression was detected compared to the heterozygous mutant (Fig. 6.5). The levels of expression observed in the pTet-*MDP1*

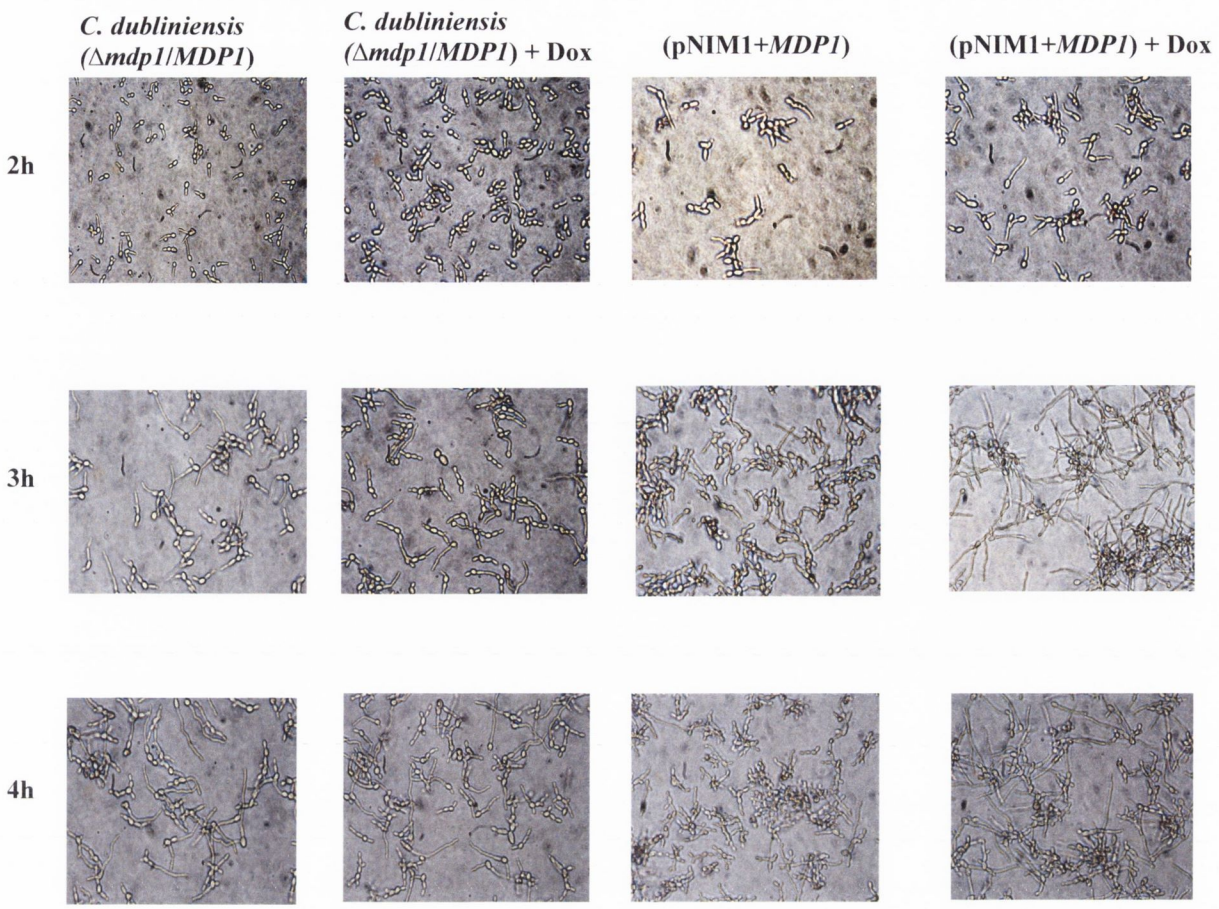


Figure 6.2. Light micrographs of the *C. dubliniensis* heterozygous mutant and the tetracycline induced expression of *C. dubliniensis MDP1* in 10 % serum. Strains were grown overnight in YPD at 37°C, the following day strains were washed with PBS and inoculated into 10 % serum in the absence or presence of 50 μg ml^{-1} doxycycline.

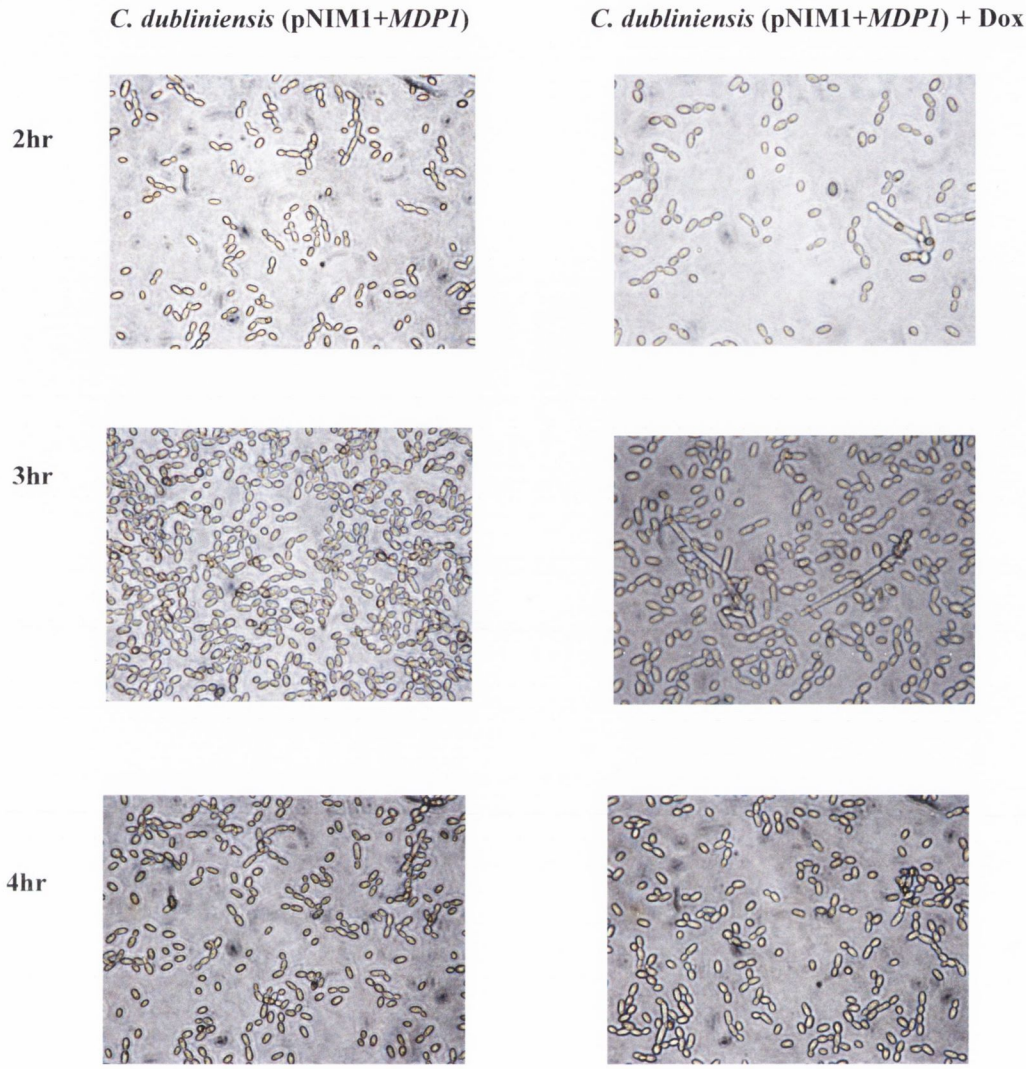


Figure 6.3. Light micrographs of tetracycline induced expression of *C. dubliniensis MDP1* in PBS + CO_2. Strains were grown overnight in YPD at 37°C the following day strains were washed with PBS and inoculated into fresh PBS and incubated at 37°C with 5 % CO_2. in the absence or presence of 50 μg ml^{-1} doxycycline.

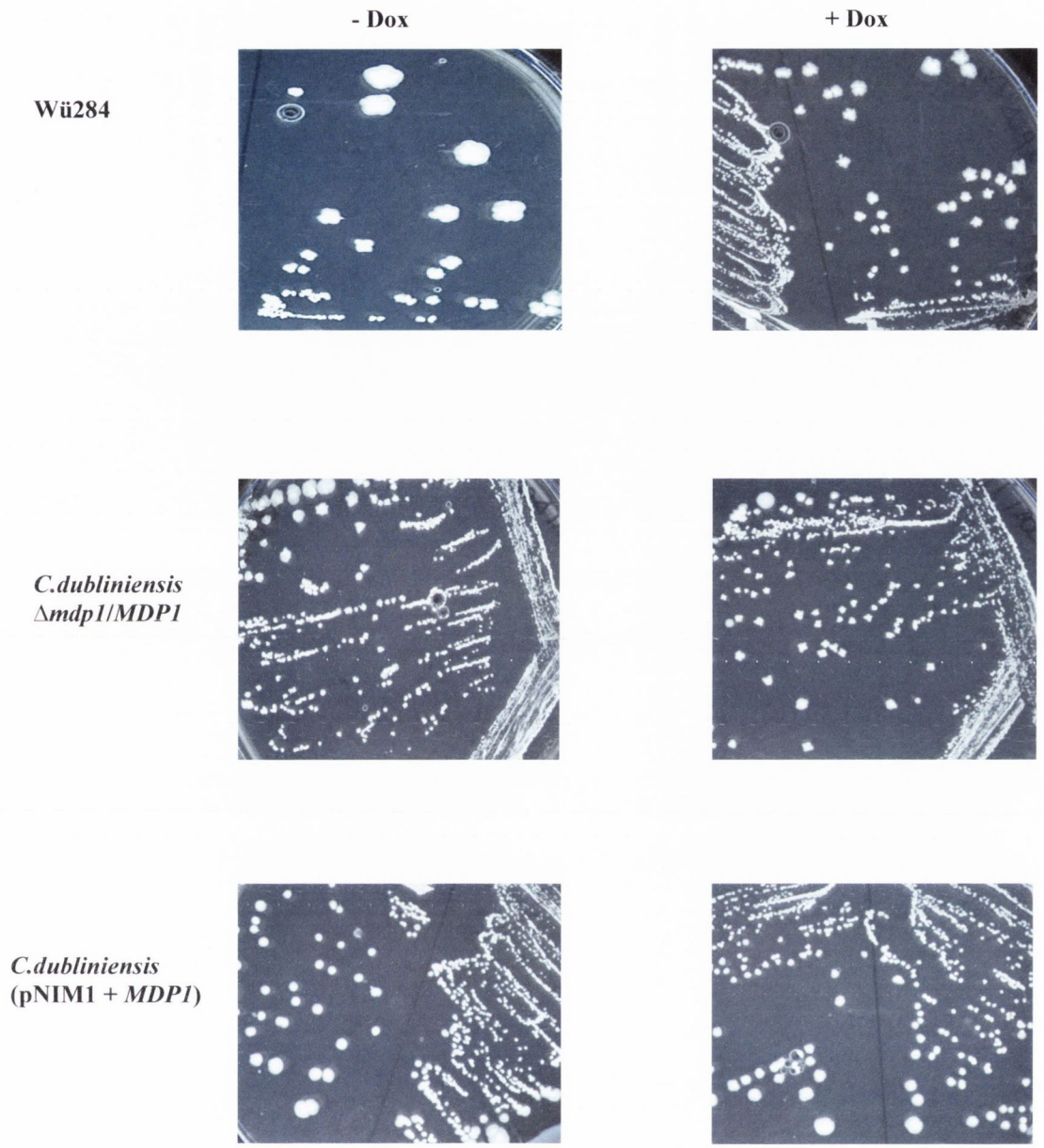


Figure 6.4. Photographs of *C. dubliniensis* wild-type Wü284, *Δmdp1/MDP1*, and the pTet-*MDP1* strain following growth on Lee's agar. *C. dubliniensis* Wü284 produced rough colonies in the presence and absence of Dox, the heterozygous mutant produced both rough and smooth colonies in the presence and absence of Dox. The tetracycline induced *C. dubliniensis MDP1* produced smooth colonies, and some colonies slightly pinched in appearance when grown in both the presence and absence of Dox

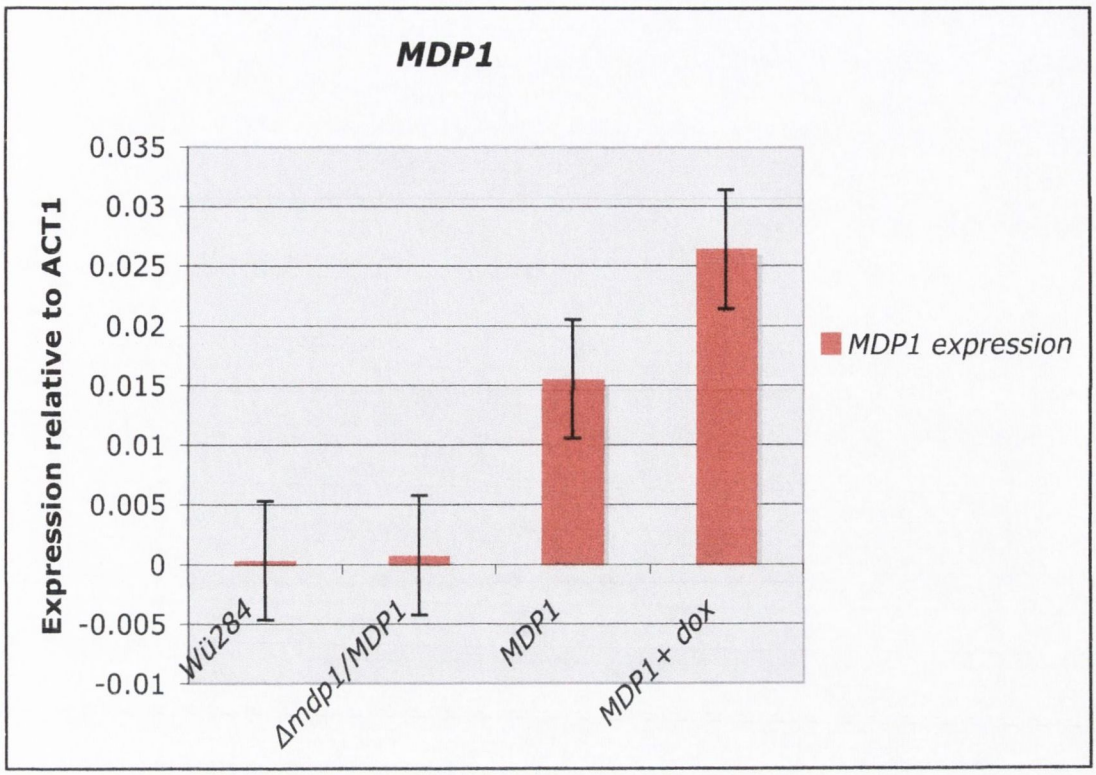


Figure 6.5. Expression of *MDP1* relative to *ACT1* in *C. dubliniensis* wild type Wü284, the heterozygous mutant *MDP1*/Δ*mdp1*, the pTet-*MDP1* strain in the absence of Dox and the pTet-*MDP1* strain in the presence of Dox following growth in YPD to an O.D. $_{600nm}$ 1.0. This experiment was repeated on three separate occassions.

strain grown in the absence of Dox indicate that perhaps the tetracycline promoter was leaky in exponential YPD cultures.

6.1.5 PCR amplification of pTet-*MDP1* cassette

As cloning of the *MDP1* gene proved impossible due to possible toxicity to *E. coli*, we could not insert the gene into a cassette which would allow us to re-insert the gene into the heterozygous mutant (i.e. complementation). However in this chapter we have inserted the *SAT1* marker and a regulatable promoter upstream of *MDP1*. This was achieved by use of the pNIM1 plasmid, *MDP1* is replaces in the *caGFP* ORF and hence is expressed from the Tet-inducible promoter. Thus, a cassette has been instructed in vivo on the *C. dubliniensis* chromosome. In this part of the study, we attempted to PCR amplify the entire region containing the *SAT1* marker, the tetracycline inducible promoter and the entire *MDP1* gene in order to use this fragment to transform and complement the *MDP1* double mutant. Firstly the structure of the cassette was confirmed using the primer pairs CdTetF/CdCEDTR (Table 2.2) and SATTetF/SATTetR (Table 6.2). In order to ensure the presence of the *MDP1* cassette PCRs were run using the TETF2/CdCEDTR, which confirmed the presence of the tetracycline promoter fused to the *MDP1* gene. Following this it was attempted to amplify this whole region using the primer pair SATTetF/CdCEDTR and this was done using the Expand long template PCR system (Roche). However, this was unsuccessful. Subsequently, it was decided to amplify this cassette in two portions. These two over-lapping fragments could be transformed together into *C. dubliniensis* and could potentially recombine to form the full cassette. This "split-marker" approach has been used previously (Schaub *et al.*, 2006; Walther & Wendland, 2008). The *SAT1* cassette and the pBluescript ampicillin resistance gene were first amplified with SATTetF/PbAmpR and subsequently we attempted to amplify the other half of the pNIM1 cassette from the ampicillin resistance gene to the end of the *MDP1* gene. The *SAT1*-Ampicillin fragment amplified successfully using the primer pair SATTetF/PbAmppR giving a 4.2 Kb fragment (Table 6.2). Following this, amplification of the pBluescript ampicillin resistant gene and the *MDP1* fragment of the pNIM1 cassette was attempted. The primer pair PbAmpf/CdCEDTR, did not yield the correct products despite optimisation of the annealing temperature and the $MgCl_2$ concentration. Following this result it was decided to design two further primers within

the ampicillin resistance gene in pBluescript (Table 6.2). The new primers were used in conjunction with CdEDTR (e.g. AMPF1/CdCEDTR and AMPF2/CdCEDTR) in an attempt to amplify this 5.5 Kb fragment from this cassette. Unfortunately neither of these primer pairs yielded a fragment. Primer optimisation was carried out where various annealing temperatures from 48°C to 60°C were tested, extension times were also increased and finally $MgCl_2$ titrations were carried out, however, none of these optimisation processes yielded any products.

Following unsuccessful attempts to amplify the tetracycline induced *MDP1* allele, it was decided to design a *SAT1-ACT1* cassette. This cassette consisted of the *ACT1* promoter fused to the *SAT1* cassette. To specifically target the *ACT1* promoter, the *MDP1* gene and sequences from the porter of the *MDP1* gene were incorporated into the primers by tagging the primers with 80 bp of *MDP1* promoter sequence (Table 6.2). This primer pair would then amplify the *SAT1-ACT1* cassette to yield a product flanked with 80 bp of *MDP1* promoter sequence at the 5' and 3'ends. This would then allow for direct transformation of the *ACT1-SAT1* cassette into the *C. dubliniensis* heterozygous mutants. The PCR was set up using the Expand high fidelity PCR system (Roche) (Chapter 2, 2.4.2). However, following multiple attempts no PCR product was obtained.

Table 6.2 Primers used to contsruct the pTet-*MDP1* cassette

Primer	Nucleotide Sequence (5'-3')	Nucleotide coordinates
MDP1SATF	CCAATCTAAATGACTCATATACTTTTCCCG GTTCTGGTTCAAAACAATTACTGTCTTTT TTTTTTTATTTAGTCAATCTGAAAGTATA GGAACTTC	-1304 to -1226[a] +1 to +21
MDP1ACTR	CAGAAATAGTGACACTAGTATTTCTTTCT GGTGTAGAAGCAGGACTGGTTGGATATTT AGAATTGGGTGTTTTCATTGCTTGTTGAA TGATTATTATTTTTTAATATTAATATC	-5 to +75[a] +3473 to +3507
TETF2[b]	TCCAGTTACCACTCCTATC	+23 to +43
SATTetF[c]	TCAAAATGTCGAGCGTCAAAACTAGAG	-501 to -529[b]
SATTetR[c]	GGCATTTCTATCTTGTTGTTGTTG	+1446 to + 1471[b]
PbAmpF[d]	TCGCCCCGAAGAACGTTTTCCAATG	+390 to + 414[c]
PbAmpR[d]	TGATCCCCCATGTTGTGCAAAAA	+660 to +683[c]
AMPF1[d]	GCAATAAACCAGCCAGCCGGAA	+160 to + 190[c]
AMPF2[d]	GTTAATAGTTTGCGCAACGTT	+280 to +301[c]

[a] The nucleotide coordinates of the *MDP1* gene (where position +1 corresponds to the first base of the ATG translational start codon)

[b] Nucleotide coordinates of TETF2 from PNIM1 plasmid, accession no DQ090840

[c] Nucleotide coordinates of the SAT1 cassette in the pNIM1 accession number DQ090840

[d] Nucleotide coordinates of the ampcillin resistant gene in pBluescript accession number X5329

6.2 Discussion

6.2.1 Tetracycline induction of *MDP1* increases filamentation in 10 % serum

Park & Morschhauser (2005) designed a Tet-inducible gene expression system for *C. albicans* and used this system to further investigate the expression of a specific set of genes. Under the control of the inducible promoter, the *C. dubliniensis MDP1* gene should be minimally expressed in the absence of the inducer (Dox). Addition of doxycycline to the cells would result in overexpression of the gene under the required conditions. Induction of *MDP1* expression in the tetracycline promoter increased the rate of filamentation in *C. dubliniensis* compared to non-induced cells or cells of the parental strain (heterozygous mutant) following incubation in 10 % serum. The conditions used here (10 % serum) are normally permissive for filamentation in *C. dubliniensis*, however the rate of filamentation is generally slower under these conditions relative to *C. albicans* strains. Tetracycline induction of *MDP1* expression increased the rate of filamentation in *C. dubliniensis* Therefore, the low levels of filamentation observed in *C. dubliniensis* under these conditions may be due to the low levels of *MDP1* expression observed in wild-type strains. However, increasing *MDP1* expression could not increase filamentation under conditions that are normally non-permissive for hypha formation in *C. dubliniensis*, indicating that other regulatory factors may repress filamentation under these conditions, for example in YPD or in PBS in the presence of 5 % CO_2. The increased filamentation observed could be attributed to a stress response mechanism in *C. dubliniensis*. The latter results would also suggest that overexpression of *MDP1* would not lead to increased virulence in the RHE infection model, as CO_2 is one of the main inducers of filamentation in this model.

In YPD cultures expression of tetracycline induced *MDP1* was detected. However, basal levels of expression of pTet-*MDP1* grown in the absence of Dox in exponential YPD cultures were higher than expected. This result would indicate a leaky promoter. Further real-time analysis is needed to determine the basal levels of expression of pTet-*MDP1* in 10 % serum. On solid Lee's medium, the wild-type produced rough filamentous colonies whereas the pTet-*MDP1* produced smooth colonies, suggesting that perhaps overexpression of *MDP1* on solid agar results in repression of hypha formation.

6.2.2 Complementation of *MDP1* through PCR Amplification

As previous attempts to complement the *MDP1* mutant by cloning had failed, it was decided to use a rapid method for homologous integration of genes using PCR products that contain short regions of homology. The main asset of these methods is that they do not require cloning of the gene of interest. Several attempts were made to clone the *MDP1* gene in order to carry out complementation, which was unsuccessful (Chapter 4, 4.2.8). As a result it was decided to utilise the genome integrated tetracycline induced cassette. Amplification of the complete cassette was attempted using primers at either end. Despite optimisation of the PCR running conditions no product was obtained. Subsequently it was decided to amplify the cassette in parts. Firstly the *SAT1* cassette and part of the pBluescript ampicillin resistance gene were amplified successfully. Amplification of the other section of the cassette containing part of the pBluescript ampicillin resistance gene and the *MDP1* gene was attempted however, despite optimisation of the annealing temperatures and extension times this did not yield a product. Various primers were applied and these also failed to yield a product. Thus this PCR amplification method was unsuccessful in allowing further insight into the function of this gene. The use of the *SAT1-ACT1* cassette also failed to yield any products. If complementation had been successful this would have allowed us to further the study of *MDP1* by inserting this gene into the *C. albicans EED1* mutant. This could have allowed us to assess whether *MDP1* could restore the ability to disseminate in epithelial tissue. In *C. dubliniensis* it would be expected that once complementation of the mutant had occurred that the wild-type phenotype would be restored.

Chapter 7

General Discussion

7.0 General Discussion

Comparative genomic hybridisation studies carried out by Moran *et al.* (2004) led to the screening of thousands of genes which were highly divergent or even absent in *C. dubliniensis*. One such ORF deemed absent or highly divergent in *C. dubliniensis* was IPF946/orf19.7561. When our studies began, no function had yet been assigned to the *C. albicans* IPF946/orf19.7561 gene. During the course of our studies Chen *et al.*(2004) identified a potential role for this gene as an *EFG1* dependent transcript (*EDT1*). Subsequently, Zakikhany *et al.* (2007) described this gene as an essential regulator of filamentation (*EED1*).

Prior to these studies our goal was to determine whether a *C. dubliniensis* orthologue of IPF946/orf19.7561 existed. Following extensive database searches and protein alignments, we identified a syntenic *C. dubliniensis* gene (Cd36_34980). The genes either side of this orf (*EED1*) on chromosome R were conserved between the two species, however, the intervening region encoding the corresponding *C. dubliniensis* gene was highly divergent. Prior to this study, it was found that the *C. albicans* Eed1 protein was expressed at high cell densities and acts as a regulator of filamentation and is also required for epithelial escape and dissemination in an RHE model of infection(Zakikhany *et al.*, 2007). Due to the high level of divergence between the *C. albicans* and *C. dubliniensis* orthologues this raised the possibility that these two genes may differ in the regulation and or function. Our initial hypothesis was that the *C. dubliniensis* homologue may not have an essential role in regulating filamentation and may not play a role in virulence as the protein identified only shares 22 % homology with Eed1p, with no significant or highly conserved domains. The *C. dubliniensis* protein could act as a functional homologue that does not contain sequence homology or perhaps these two proteins may not be functionally related at all. Following comparative genome analysis using the CGOB (*Candida* gene order browser) of *C. albicans*, *C. dubliniensis* and several other yeast species were identified to contain a gene at the same locus as *EED1* (Fig 3.5). Pfam searches of these genes identified a Myb-like DNA binding domain in all these proteins with the exception of *C. albicans EED1*. Myb-like DNA binding domains play an important role in the regulation of gene expression. Baranowskij *et al.* (1994) found that Myb-like DNA binding proteins can be divided into three classes depending on their amino acid composition, these are either rich in acidic amino acids, proline or glutamines. Although a Myb-like DNA binding

domain was not found in Eed1p, a partially conserved domain was found. In addition, Eed1p was also found to be rich in proline and glutamine like these other Myb domain proteins. Thus following the identification of a Myb-like DNA binding protein in *C. dubliniensis*, it was decided to refer to Cd36_34980 as *MDP1* (Myb domain protein).

Interestingly, another protein identified as having similarity to the Cd36_34980 protein was *S. cerevisiae DOT6*. The ends of chromosomes in *S. cerevisiae* initiate a repressive chromatin structure that spreads internally and inhibits the transcription of nearby genes, a phenomenon called telomeric silencing. Singer *et al.* (1998) used genetic screens to identify genes whose overexpression disrupts telomeric silencing and the study isolated ten *DOT* (Disrupter of telomere silencing) genes. The *DOT6* gene sequence encodes a predicted 670 amino-acid protein with a Myb-related motif between residues 78 and 116. A study by Bilaud *et al.,* (1996) found that various telomere sequence-binding proteins, including the telomere repeat binding factors from human cells and *Schizosachromyces pombe* contain a single repeat of a Myb-related sequence. Lorenz & Heitman (1998) overexpressed *DOT6* among 17 other genes and found that these genes suppressed growth or pseudohyphal defects of the *Δmep1/Δmep1* and *Δmep2/Δmep2* mutants on low ammonium. Until this report *DOT6* had not been known to affect filamentous growth. The *EED1*-related proteins identified in this study (Fig 3.5) may also play a role in regulating telomeric gene expression. Interestingly, *C. albicans* contains 16 *TLO* (TeLOmere-associated) genes located close to the telomeres of the *C. albicans* chromosomes and these genes are thought to encode transcription factors (Kaiser *et al.*, 1999; van het Hoog *et al.*, 2007). Recent data from our laboratory suggests that the *TLO* genes play a role in the control of filamentation and switching (Dr. Derek Sullivan, personal communication).

In order to investigate the function of *EED1* and *MDP1*, phenotypic analysis of both *EED1* and *MDP1* following gene disruption studies revealed that both the *Δeed1/Δeed1* and the *Δmdp1/Δmdp1* mutants were unable to form hyphae in 10 % serum which is a potent inducer of hyphae. Hyphal induction experiments on the *C. dubliniensis* wild-type and *MDP1* mutant correlated with results from Stokes *et al.*, (2007) and Gilfillan *et al.*,(1998) that *C. dubliniensis* does produce hyphae but at a much slower rate then *C. albicans*. The *Δmdp1/Δmdp1* mutant produced ~ 5 % hyphae in 10 % serum, whereas the *C. dubliniensis* wild-type produced ~ 50 % hypha indicating that *MDP1* is required for efficient filamentation in *C. dubliniensis*. This demonstrates that both *EED1* and *MDP1* play a role in regulating filamentation and that both *EED1* and *MDP1* may play

a role in the cAMP pathway. Further evidence for this was revealed following real-time PCR expression analysis of *EED1*. As shown in figure 3.18 there was a 12.5-fold drop in expression of *EED1* in an Δ*efg1* mutant when compared to the wild-type, indicating that when transcription of *EFG1* is switched off in *C. albicans* so is the expression of *EED1*; indicating that *EED1* may play a role in *EFG1*-dependent filamentation (Fig. 7.1). In the absence of a pH shift the Δ*eed1*/Δ*eed1* mutant failed to form true hyphae, therefore indicating that the *EED1* gene is required for filamentation under these conditions (e.g. serum). Temperature shift experiments revealed an effect on the morphology of the *EED1* wild-type, which produced predominantly pseudohyphae whereas the Δ*eed1*/Δ*eed1* mutant remained in the yeast phase. The greatest effect was observed following nutrient starvation experiments, where the *EED1* wild-type produced true hyphae whereas the Δ*eed1*/Δ*eed1* mutant remained exclusively in the yeast phase, suggesting that *EED1* may play a role in regulation of the morphological shift under nutrient poor conditions. These results suggest that *EED1* is required for temperature and nutrient induced filamentation. This evidence suggests that in the presence of 10 % serum, a temperature shift and low nutrients, *EED1* potentially acts in conjunction with or downstream of both *CPH1* and *EFG1* (Fig 7.1). These results suggest that *EED1* may be a transcriptional activator, acting alone or alongside *EFG1* to activate transcription. In order to test this hypothesis a yeast two hybrid screen could be carried out to determine whether CaEed1p has protein-protien interactions with Cph1p and Efg1p, thus confirming its role as a transcriptional activator. Microarray technology could also be used to identify genes regulated by *EED1* by comparing the different gene expression patterns of the *C. alibcans* wild-type with the Δ*eed1*/Δ*eed1* mutant. A more direct microarray technique to identify *EED1* binding sites would be chIP-on-chip. This is a technique that combines chromatin immunoprecipitation with microarray technology. It is used to investigate the interaction between proteins and DNA in vivo. This technique allows for the identification of binding sites of DNA binding proteins on a genome wide basis, including the promoters of the *TLO* gene family.

On solid agar, the *C. albicans* wild-type produced rough filamentous colonies on agar media known to induce hyphal formation (Spider and Lee's), whereas the Δ*eed1*/Δ*eed1* mutant produced smooth colonies. This would indicate that *EED1* is required for filamentation in solid media as well as liquid media. Hypha development in *C. albicans* is also controlled by the MAPK pathway, and in this pathway Cph1 plays a role in the activation of hypha development. However, this pathway is required for

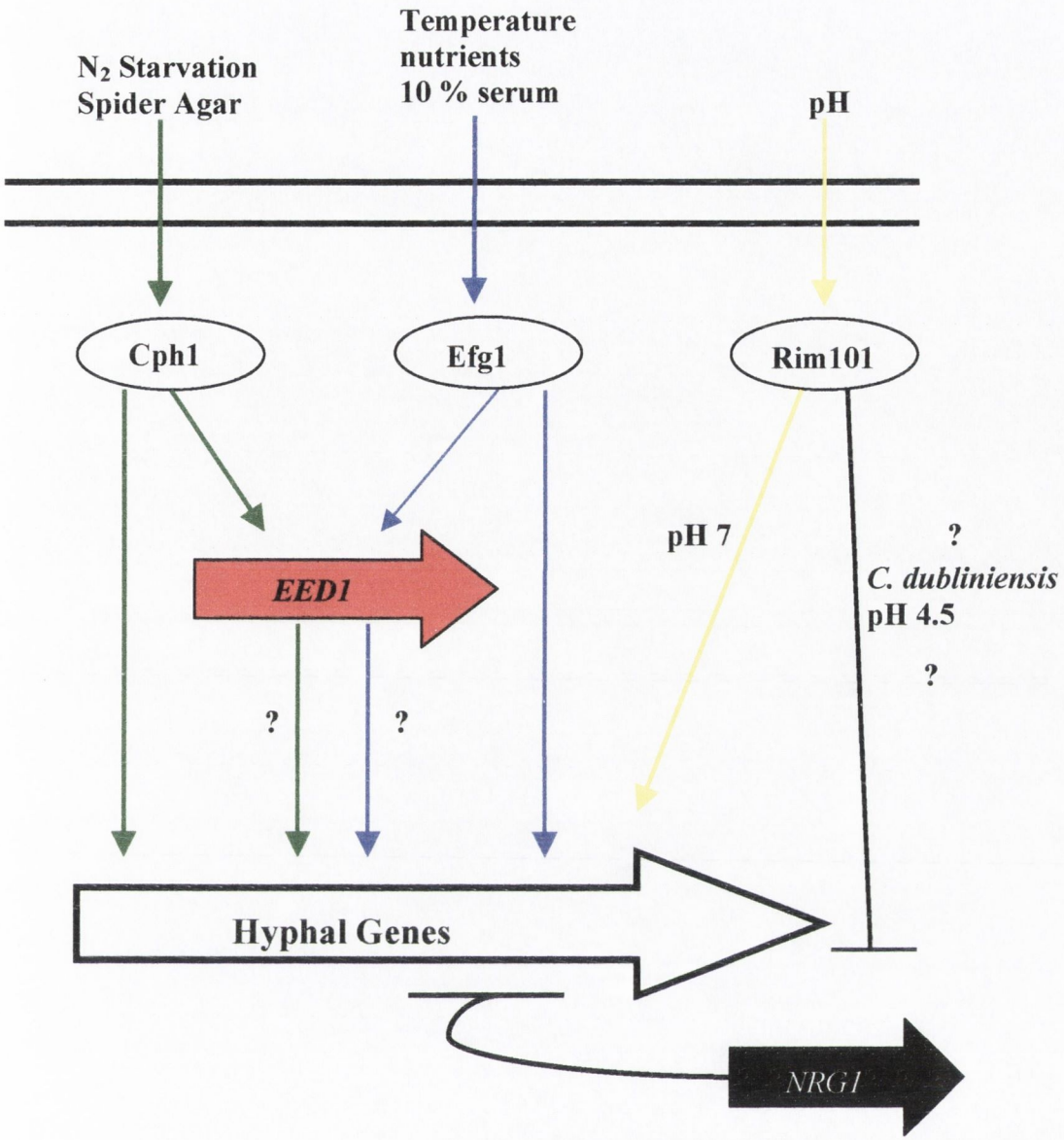


Figure 7.1. Proposed model of the different mechanisms that induce filamentation in *C. albicans*. Nitrogen starvation and spider agar (green arrows) result in the acitvation of Cph1p, which in turn may activate Eed1p and hence transcription of Eed1p could result in hypha production. It is also possible that Cph1p activates hypha specific genes in the absence of *EED1*.Temperature, nutrients and serum (blue arrows) result in the acitivation of *EFG1* which in turn may acitivate *EED1* and result in transcription of hypha specific genes. Though it is also probable that Efg1 itself could transcribe hypha specific genes in the absence of *EED1*.
The alternative pH pathway (yellow arrows) activates *RIM101* which itself results in hyphal production. As demonstrated in this study it is thought that in *C. dublinienisis* the *RIM101* pathway represses *NRG1* (Black) which results in an increase in hypha production. Expression of *NRG1* results in repression of hypha specific genes.

hypha formation under a specific set of conditions such as nitrogen starvation (Ernst, 2000; Leberer *et al.*, 1996). Real-time PCR expression analysis of *EED1* revealed that this gene is not expressed in the Δ*cph1* mutant indicating that perhaps *EED1* is dependent on the MAPK pathway or could be implicated in this pathway (Fig 7.1). However analysis of both the *C. dubliniensis* wild-type and the *MDP1* mutant on solid agars known to induce filamentous growth in *C. albicans* (e.g. Spider and Lee's media) both strains produced smooth round colonies which could suggest that *MDP1* may not play a role in the MAPK pathway. However, a *C. dubliniensis* Δ*nrg1*/Δ*nrg1* mutant produces rough filamentous colonies on these agars, indicating that the MAPK pathway is intact in this species (Moran *et al.*, 2007). The phenotype of the *C. dubliniensis* wild-type and the Δ*nrg1*/Δ*nrg1* mutant strain on these media suggest that on solid media, *NRG1* is expressed at high levels and hence represses hyphal formation. Therefore, the high levels of expression previously observed for *NRG1,* could possibly repress hyphal formation in both the wild-type and the *MDP1*mutant on solid agar.

Real-time PCR expression analysis of *C. dubliniensis MDP1* expression revealed that transcription of *MDP1* was very low compared to *C. albicans EED1*. Real-time PCR expression analysis also found that expression of *MDP1* in *C. dubliniensis* decreased with increasing cell densities. Further analysis following gene disruption experiments gave a clearer insight into a possible function for *C. dubliniensis MDP1*. Overexpression of *MDP1* was carried out using the Tet-inducible gene expression system (Park & Morschhauser, 2005), in order to determine if the low level of filamentation in *C. dubliniensis* was due to the low level of *MDP1* expression. Induction of *MDP1* expression from the tetracycline promoter did result in an increased rate of filamentation in *C. dubliniensis* when compared to non-induced cells following incubation in 10 % serum. Therefore, the low level of hypha formation observed in wild-type strains may be due to low levels of expression of *MDP1* (Spider medium). However, overexpression of *MDP1* in *C. dubliniensis* did not increase filamentation under conditions that do not normally result in hypha formation. However, under conditions usually non-permissive of hypha formation in *C. dubliniensis* (e.g. Spider medium) other regulatory pathways may be repressing filamentation. Perhaps under these conditions in *C. dubliniensis*, *NRG1* is expressed at a high level, and may possibly directly repress hypha specific genes or even *MDP1* expression. To further investigate the function of *MDP1*, one could create a Δ*mdp1*/Δ*mdp1* deletion in the Δ*nrg1*/Δ*nrg1* mutant background. As the Δ*nrg1*/Δ*nrg1* deletion allows filamentation under these

conditions we could determine whether the *Δmdp1/Δmdp1/Δnrg1/Δnrg1* double mutant could still filament. On solid agar one could speculate that this double mutant would produce smooth round colonies as the deletion of *MDP1* in the *Δnrg1/Δnrg1* background would possibly result in loss of function downstream of *CPH1* through the deletion of *MDP1*.

The virulence models of infection clearly demonstrated that *EED1* is required for escape and dissemination in the RHE model as described by Zakikhany *et al.*, (2007). In this study we have also shown that *EED1* is required for escape and survival in the macrophage model of infection. Following inoculation onto the RHE the *Δeed1/Δeed1* mutant exhibited attenuated virulence. The mutant cells were able to adhere to the surface of the epithelium, however, the cells remained in the yeast phase and failed to disseminate down through the tissue. In *C. dubliniensis*, both the wild-type and the *Δmdp1/Δmdp1* mutant exhibited attenuated virulence, both grew exclusively in the yeast phase and only a small number of cells adhered to the epithelium. In the macrophage model of infection the wild-type *C. albicans* was phagocytosed by the macrophage, however, this resulted in the wild-type producing hypha which enabled it to protrude from the macrophage and continue proliferation. The *Δeed1/Δeed1* mutant did produce pseudohyphae and some true hypha following co-culture with the macrophages however, only a small number were visible protruding from the macrophage at 5 h when compared to the wild-type, however these protruding cells did appear increase survival. In *C. dubliniensis* the *Δmdp1/Δmdp1* mutant had a poorer survival rate compared with the wild-type. The poor survival of the *Δmdp1/Δmdp1* mutant demonstrates that although *C. dubliniensis* is less filamentous than *C. albicans*, *MDP1* is still essential for filamentation to occur and hence survival.

The ability of *C. albicans* to exist and survive in diverse environments of different pH and temperature is crucial for its pathogenicity. In *C. albicans* gene expression is known to be regulated in response to pH, including the pH regulated genes *PHR1* and *PHR2*. The *PHR* homologous genes of *C. dubliniensis* exhibit a pH-dependent pattern of expression similar to that of *PHR1* and *PHR2*, therefore it is likely that the pH response system of *C. albicans* is conserved in *C. dubliniensis* (Kurzai *et al.*, 1999). In the current study the role of *MDP1* in the pH response was investigated in *C. dubliniensis*. Lee's medium is a known inducer of the yeast to hypha transition in *C. albicans* (Lee *et al.*, 1975) and this medium was used to investigate the effect of a pH shift from pH 4.5 to pH 7.5 in both *C. albicans* and *C. dubliniensis*. The pH shift

increased hyphal formation in the *C. albicans* wild-type BWP17, however, the pH shift only partially restored the ability of the *Δeed1/Δeed1* mutant to produce hypha. In *C. dubliniensis* a more significant effect was observed. Hypha formation in both the wild-type and LOC4 (*Δmdp1/Δmdp1*) increased to levels similar to a *C. albicans* wild-type. This evidence would indicate that the increase in filamentation observed following a pH shift from 4.5 to 7.5 occurred independently of *MDP1*. We investigated the basis for this by investigating the expression *NRG1* in *C. dubliniensis* following a pH shift. As discussed previously, deletion of both copies of *NRG1* in *C. dubliniensis* resulted in an increase in filamentation (Moran *et al.*, 2007). Therefore, we hypothesized that the increased hypha formation observed following a pH shift in the *C. dubliniensis* wild-type and mutant could be a direct result of changes in *NRG1* expression. Real-time PCR expression analysis demonstrated that *NRG1* levels in *C. dubliniensis* following preculture in Lee's medium pH 4.5 were 4- fold lower than that observed at pH 5.6. Thus, the very low levels of expression of *NRG1* observed could be responsible for the increased filamentation observed in both Wü284 and LOC4 when cells are precultured in a low pH medium, followed by inoculation into 10 % serum. The increased hypha production observed in *C. dubliniensis* following a pH shift may involve the *RIM101* pathway, which is essential for the regulation of genes in response to external pH. A study by Lotz *et al.*,(2004) identified *RIM101*-regulated cell wall genes using DNA microarrays. The study correlated with the *RIM101* phenotype, which is characterised by filamentous growth at acidic pH and increased adhesion at neutral pH (Fig 7.1). From this current study, the data obtained would possibly implies that in the *C. dubliniensis* wild-type, under low pH environments Rim101p represses *NRG1* thus resulting in increased filamentation. This increase in filamentation occurs independently of *MDP1* (Fig 7.1).

Our diets have become extremely acidic with the large increase of snack foods, meat, grains and coffees but in particular highly processed foods. The resting pH of plaque in the mouth is usually around 6.5 however following ingestion of such foods it has been shown that pH levels can drop to as low a pH 4.5. A study by Aamdal-Scheie *et al.*(1996) identified several microbial species in plaque including *Candida* species. This pH variation could potentially favour *C. dubliniensis* colonization in the oral cavity, as in this current study; a similar pH shift resulted in an increase in hyphal formation. As results obtained in this study that the pH shift is required for increased hyphal formation in *C. dubliniensis*. This increase in hyphal formation resulted in

increased adherence, as was demonstrated in the RHE model of infection in both *C. dubliniensis* Wü284 and LOC4. Therefore, oral *C. dubliniensis* isolates could potentially adhere to acidic dental plaque, and following an increase in pH to 6.5 (resting pH of the oral cavity), hypha production would possibly occur at similar levels to those observed in *C. albicans,* thus promoting invasion into the epithelial cells, possibly leading to a persistent *Candida* infection.

This study has demonstrated that a change in pH can increase the adherence and hence survival of *C. dubliniensis* and has also demonstrated that at low pH *NRG1* is transcribed at very low levels. Staib & Morschhauser (2005) also demonstrated that *C. dubliniensis* but not *C. albicans* specifically down-regulates *NRG1* expression on Staib medium to allow for chlamydospore production in *C. dubliniensis*. Also recent experimental data from our laboratory has demonstrated that a synthetic chlamydospore inducing media requires a low pH (3.7 to 4.5) for the production of chlamydospores. This study has found that the low pH is crucial for chlamydospores production along with specific carbohydrates and amino acids. At a higher pH (pH 6) *C. dubliniensis* fails to form chlamydospores (Citiulo *et al.*, unpublished data).

In summary this study has demonstrated that the *C. albicans EED1* gene is important for filamentation and possibly plays a role in the *EFG1* pathway. However real-time PCR expression analysis also showed that *EED1* may also play a role in the MAPK pathway downstream of *CPH1*. This study demonstrated that the syntenic gene in *C. dubliniensis MDP1, c*is required for filamentation and that a pH shift could greatly enhance survival in macrophages, however this appears to be occurring independently of *MDP1*. *NRG1* may mask the effect of *MDP1* under most hypha inducing conditions. Real-time PCR expression analysis suggested that perhaps repression of *NRG1* could be responsible for the increased filamentation observed following a pH shift from 4.5 to 7.5. The low levels of filamentation observed prior to overexpression of *MDP1* could be due to the low levels of expression of *MDP1* observed in wild-type strains. Overexpression of *MDP1* did not induce hyphae under conditions where hyphae are not normally produced indicating that other regulatory pathways may be involved in repressing filamentation under these conditions. Therefore from these results it may be possible that divergent evolution has occurred between *MDP1* in *C. dubliniensis* and *EED1* in *C. albicans* and this could explain the low homology between these two proteins. This could also suggest a reason as to why *C. albicans* is far more virulent on the RHE model of infection and in the macrophage model of infection.

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